A GENETIC VARIANT OF ANGIOTENSIN RS699 ASSOCIATED WITH DIABETIC NEPHROPATHY IN PATIENTS OLDER THAN 50 YEARS OLD

Anggelia Puspasari[1], Elfiani[2], Citra Maharani[1], Susan Tarawifa[3]

[1]Departement of Medical Biology and Biochemistry, Faculty of Medicine and Health Sciences, Universitas Jambi,

[2]Departement of Internal Medicine, Faculty of Medicine and Health Sciences, Universitas Jambi, Jambi, Indonesia.

[3]Departement of Anatomy, Faculty of Medicine and Health Sciences, Universitas Jambi, Jambi, Indonesia.

email: anggelia.puspasari@unja.ac.id

ABSTRACT

Background: *Beyond controlling blood pressure and blood glucose, a genetic factor may play role in diabetic nephropathy (DN) progression in Type II Diabetes Mellitus (T2DM). Previous studies reported a genetic variant of Angiotensin rs699 (M235T) associated with diabetic nephropathy, with conflicting results across populations worldwide. To the best of our knowledge association of this genetic variant has never been conducted in the Jambi Malay population.* ***Aims*** *The Aims of this study is to the reveal association of the genetic variant of Angiotensin rs699 with diabetic nephropathy in the Jambi Malay population.*

Methods: *This study design was cross-sectional. Totally 70 T2DM patients aged 22-67 years old were participated, as many 41 patients have aged older than 50 years old. Genotyping was performed using tetra ARMS PCR specific for rs699.*

Result: *Patients aged older than 50 who have CTTT genotype have lower risk for suffering DN than CC genotype (p-value 0.029; OR (95% CI) 0.233 (0.06-0.88)), the statistic significant persist in multivariate analysis (p-value 0.030; OR (95% CI) 0.19 (0.04-0.86)).*

Conclusion: *CTTT genotype was protective for DN in patients older than 50 years old. Further study with larger sample size, multi-centre and measuring confounding factors was needed.*

Keyword: *Genetic variant of Angiotensin, rs699, diabetic nephropathy, Type II Diabetes Mellitus, Malay population*

ABSTRAK

Latar belakang: Kendali Tekanan darah dan glukosa darah saja tidak cukup untuk mencegah kejadian nefropati diabetik (DN) pada Diabetes Mellitus Tipe II (T2DM), faktor genetik juga berperan penting. Studi sebelumnya melaporkan varian genetik Angiotensin rs699 (M235T) yang terkait dengan DN, dengan hasil yang berbeda antar populasi. Sejauh studi literatur yang dilakukan, varian genetik ini belum pernah diteliti pada populasi Melayu Jambi. **Tujuan** dari penelitian ini adalah untuk mengetahui hubungan varian genetik Angiotensin rs699 dengan nefropati diabetik pada populasi Melayu Jambi.

Metode: penelitian ini merupakan penelitian potong lintang. Sebanyak 70 pasien T2DM berusia 22-67 tahun berpartisipasi, 41 diantaranya berusia lebih dari 50 tahun. Genotyping dilakukan dengan menggunakan *tetra arms* PCR spesifik untuk rs699.

Hasil: Pasien berusia lebih dari 50 tahun yang memiliki genotipe CTTT memiliki risiko lebih rendah untuk menderita DN daripada genotipe CC (nilai p 0,029; OR (95% CI) 0,233 (0,06-0,88)), analisis multivariat juga menunjukkan hasil serupa (nilai p 0,030; ATAU (95% CI) 0,19 (0,04-0,86)).

Kesimpulan: CTTT genotipe adalah pelindung untuk DN pada pasien yang lebih tua dari 50 tahun. Studi lebih lanjut dengan ukuran sampel yang lebih besar, berbagai pusat studi dan mengukur faktor risiko genetik dan non-genetik lain perlu dilakukan.

Kata kunci: Varian genetik angiotensin, rs699, nefropati diabetik, Diabetes Mellitus Tipe II, populasi Melayu

INTRODUCTION

T2DM is a major degenerative health problem worldwide including in Jambi Province. It is a concern due to the increasing prevalence of long term macrovascular dan microvascular complications. The most common microvascular complication of T2DM is diabetes nephropathy. Diabetes nephropathy leads to end-stage renal diseases which cause burden to health costs and patient quality of life (1–3).

Controlling blood pressure and plasma glucose as a clinical modified risk factor cannot always prevent DN progression (4,5). The DN genetic factors have played role in DN progression and are reported in many populations. Assessing genetic factors may enhance personalized medicine which promises better treatment for the diseases (6–8).

The genetic variant of Angiotensin rs699 T/C is located at the exon of the angiotensin gene. This variant cause missense mutation which results in the exchange of methionine to threonine at codon 268. This genetic variant cause variability of AGT level, a protein that is coded by the gene and affects DN progression due to hemodynamic and metabolic pathway (9–11). Genotype-phenotype association studies reported genetic variant of rs699 associated with risk of DN with the conflicting result beyond population (9–14).

Epidemiology study in Jambi Malay ethnic which resides in Province reported the DN as a major complication of T2DM. In addition, controlling plasma glucose is also not associated with DN progression in this population. A previous study reported genetic variation of TGF- ß associated with decreased renal function in T2DM (15). The progression of DN is multifactorial and multi genetic associated diseases. It promises that many other genetic variants may influence DN progression in this population (16).

To the best of our knowledge, the study phenotype-genotype association study of DN-genetic variant of Angiotensin rs699 T/C has never been published with subjects from Jambi Malay ethnic. Due to the concern describe before this study aims to reveal of association of genetic variant Angiotensin rs699 with DN in the Jambi Malay population.

METHOD

Research design and subject recruitment

This study was a cross-sectional study, as many 70 subjects suffering T2DM participated in this study. The inclusion criteria were duration of T2DM at least five years, age range 35-67 years old, Jambi Malay ethnic. The inclusion criteria were suffering urinary tract infection based on clinical examination and urine laboratory measurement, having a history of other renal diseases, pregnant and breastfeeding women and immunocompromised patients. The T2DM diagnosis was based on fasting plasma glucose >126 mg/dL and or 2 hours postprandial plasma glucose > 200 mg/dL as listed in medical records.

All the subjects signed informed consent after receiving a detailed explanation about the study objectives and design. The research protocol was arranged based on the declaration of Helsinki. This study obtained ethical permission from the medicine and health sciences faculty, Universitas Jambi ethical research committee number 2136/UN21.8/PT.01.04/2021.

Blood pressure measurement and blood metabolic measurement

Systolic and diastolic blood pressure was measured using a calibrated sphygmomanometer. The measurement was taken twice in a sitting position after the patients rested for 5 minutes. Subjects with systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg were classified as uncontrolled hypertension.20

Blood peripheral samples (5 ml) were obtained from the antecubital vein after eight to ten hours of fasting. This blood sample was used to measure serum creatinine levels and fasting blood glucose plasma levels. The 2 hours postprandial plasma glucose was measured two hours after glucose loading (75 g glucose loading). The Plasma glucose was measured with Glucose oxidase peroxidase amino-antipyrin (GOD-PAP) methods performed by PRODIA laboratories. Subjects with plasma fasting glucose >130 mg/dL were classified as uncontrolled plasma fasting glucose. Subjects with two hours postprandial plasma fasting glucose ≥ 180 mg/dL were classified as uncontrolled plasma 2 hours postprandial plasma fasting glucose based on PERKENI guideline.

Random spot urine samples of as much as 30 mL were collected from the patients. Quantitative measurements of urine albumin were made with immunoturbidimetric and urine creatinine was measured with enzymatic colourimetric. ACR was calculated based on the ratio of quantitative creatinine to albumin in random spot urine sampling. The DN was diagnosed

according to ACR, where DN was defined as ACR ≥ 30 mg/g.

Genotyping

Deoxyribonucleic Acid (DNA) was extracted from peripheral vein buffy coat using a commercial DNA blood extraction kit from Macrogen^{R}. Quality and quantity of DNA were measured using nanodrop with a nanodrop index of at least ~1.8.

Genotyping was performed by two-step tetra Amplification refractory mutation system polymerase chain reaction (ARMS-PCR), which showed general and allele-specific products. Primer design was adapted from El-Garawani et al (17). Primer sequences and its product showed in table 1.

The PCR mixture was 5uM primer for each specific allele (Genetica SciencesR); 10 mL PCR master mix (Go taq green, Promega^{R}); nuclease-free water 9, mL; DNA template 2 uL. The thermocycler (BioradR) condition was 95 °C for 7 minutes as initial denaturation and 1 minute as denaturation; 60 °C for 1 minute as annealing; 72 °C as an extension for 1 minute and 7 minutes as a final extension. The PCR product was then visualized with 1.5% agarose gel for 35 minutes with 100 mV

Table 1. Primer and the product size

Primer		*Fragmen*
Outer Forward	*5'TGCGCACAAGGTCCTGTCTG3'*	
Inner Forward / T allele	*5'ATGGAAGACTGGCTGCTCCCTTAT3'*	*197 bp*
Outer Reverse	*5'GTCACCAGGTATGTCCGCAGG3'*	
Inner Reverse / C allele	*5'GCTGTCCACACTGGCTCACG3'*	*295 bp*

In-silico analysis was performed to measure primer sequences and the size of their product. The genotyping method was optimized based on our laboratory resources in which optimal fragment visualization. Quality measurement of PCR reaction was determined based on the specific fragment size of the PCR product for the allele. As much as 10% of the sample was performed twice for genotyping to ensure the result, all the second genotyping processes showed consistency with the first one.

A bivariate analysis test was performed for baseline subject characteristics and association between genotype and decreased renal function. Moreover, Multivariate analysis was performed to analyzed blood pressure and blood glucose as covariable of genotype.

RESULT AND DISCUSSION

Baseline subject characteristic

Totally 70 patients who suffering T2DM and fulfilled research criteria participated in this study. As many 40 patients suffering nephropathy based on random spot ACR measurement. The baseline characteristic of them showed in Table 1.

Table 1. Baseline characteristic

Characteristic	*Diabetic nephropathy (n=40)*	*T2DM without nephropathy (n=30)*	*p-value*
Age (years)	*51.7±685*	*49.87±8.41*	*0.476*
Albumin/Creatinine Ratio	*277.36 (32.16-1440.18)*	*12.82 (3.84-28.21)*	*<0.001*
Gender			
Male, n	*17*	*11*	*0.622*
Female, n	*23*	*19*	
Blood pressure (mmHg)			
Uncontrolled blood pressure	*13*	*3*	*0.027*
Controlled blood pressure	*27*	*27*	
Fasting plasma glucose (mg/dL)			
Uncontrolled FPG	*14*	*17*	*0.071*
Controlled FPG	*26*	*13*	
2 hours PP plasma glucose (mg/dL)			
Uncontrolled 2 hours PP plasma glucose	*4*	*6*	*0.201*
Controlled 2 hours PP plasma glucose	*36*	*24*	

*Data were analyzed using a t-test for numeric scale, and all the numeric data were normally distributed. A Chi-square test was performed for the categoric scale. *statistically significant, p-value < 0.05. DM refers to diabetes mellitus; FPG refers to fasting plasma glucose; PP refers to postprandial.*

The patients who suffer nephropathy have older age than those without nephropathy, but the difference is not statistically significant. The proportion between gender not different significantly between nephropathy and without nephropathy. Previous epidemiology studies reported older age and longer duration of T2DM associated with increased risk of DN(2,4,18,19).

The proportion of subjects who have uncontrolled blood pressure is higher in the nephropathy group than without nephropathy and is statistically significant. Uncontrolled blood pressure in T2DM enhanced increasing glomeruli capillary pressure and activating RAAS. This is cause

proinflammatory and profibrotic cytokines released which fastens deterioration of kidney function leads to DN progression (4).

The proportion of subjects who have uncontrolled plasma glucose does not differ significantly between the two groups (Table 1). Previous epidemiology studies also reported controlling blood glucose cannot always prevent DN. The genetic variants may influence the DN progression, as reported in a genotype-phenotype associated study in this population performed earlier (5,15).

Genotype distribution rs699

Based on genotyping had performed, the CC genotype was wild type genotype in this population. The T allele was a minor allele, the frequency of the T allele was 20.7 %. The distribution of the genotype of rs699 is presented in Table 2. Based on the NCBI database, this minor allele frequency was higher than other Asian populations but lower than the Caucasian population.

Table 2. Frequency of genotype rs699

Genotype	*Observed value*	*Expected value*	*Chi-square*	*p-value*	*MAF*
CC	*43*	*44*			
CT	*25*	*23*	*0.533*	*0.465*	*0.207*
TT	*2*	*3*			

The genotype frequency of rs699 did not deviate from Hardy Weinberg equilibrium. MAF was minor allele frequency.

Based on the calculation of Hardy-Weinberg equilibrium, the frequency of genotype in this population not deviated from HWE equilibrium although limited sample size. This is may represent the genotyping method has a minimal bias other than the assumption of HWE for the population.

This research used one step ARMS PCR as the genotyping method. This technique allows genotyping performed faster, reliable enough, and relatively low budget than PCR-RFLP, Taq man assay or HRM-PCR. In the setting of limited laboratory resources, this technique was proper to adopt (20,21). Analysis in silicon for primer sequences, twice genotyping for 10% sample used negative control for contamination exclusion was performed to minimize genotyping error.

Association of a genetic variant of angiotensin rs699 with diabetic nephropathy

Bivariate analysis of this research reported proportion of T2DM with CT genotype and CT+TT genotype was lower in diabetic nephropathy and age group older

than 50 years old. The difference was statistically significant. Odds ratio calculation showed CT and CT+TT genotype as a protective factor for DN in older than 50 years old. The difference of genotype is not statistically significant as the risk for DN in age younger than 50 years old (Table. 3).

Continue to bivariate analysis, binary logistic model in multivariate analysis was performed. Blood pressure and plasma glucose were added in models as the covariate. This model shows CT and CT+TT associated with DN and statistically significant. Adjusted odds ratio calculation of that genotype showed as a protective factor for DN in T2DM older than 50 years old (Table 4).

Table 3. Bivariate Analysis Association of a genetic variant of angiotensin rs699 with diabetic nephropathy

Genotype	*Diabetic nephropathy*	*T2DM without nephropathy*	*p-value*	*OR (95% CI)*
Age older than 50 years old				
CC	*18*	*6*	*ref*	
CT	*6*	*10*	*0.018[b]*	*0.20 (0.05-0.79)**
TT	*1*	*0*	*0.760[a]*	*1.33 (1.06-1.68)*
CTTT	*7*	*10*	*0.029[b]*	*0.23 (0.06-0.89)**
Age younger than 50 years old				
CC	*10*	*9*	*ref*	
CT	*5*	*4*	*0.604[a]*	*1.12 (0.23-5.54)*
TT	*0*	*1*	*0.500[a]*	*2.11 (1.31-3.40)*
CTTT	*5*	*5*	*0.600[a]*	*0.90 (0.19-4.16)*

[b]for chi-square test; [a] for fisher exact test; *statistically significant; CI for confident interval; OR for odds ratio; T2DM for type 2 diabetes mellitus.

Concordant to our study, the previous study in the Turkish and Tunisian population also reported the rs699 of angiotensin gene associated with risk of DN. In both populations the T allele of rs699 as the risk for DN, difference risk allele with this research population. Among Caucasoid and Mexican American this genetic variant lack association with the risk of DN (9,11,14,17). This difference may be influenced by ethnicity and the difference in subject criteria for recruitment.

Table 4. Multivariate Analysis Association of a genetic variant of angiotensin rs699 with diabetic nephropathy in patients older than 50 years old

Variable	*B*	*SE*	*Adjusted p-value*	*Adjusted OR*	*95% CI*
Additive model					
CC		*ref*			
CT	***-1.721***	***0.764***	***0.024***	***0.18***	***0.04-0.80***
TT	***Statistically insignificant***				
Uncontrolled blood pressure	*1.573*	*0.966*	*0.104*	*4.82*	*0.73-32.05*
Uncontrolled fasting plasma glucose	*0.664*	*0.755*	*0.380*	*1.94*	*0.44-8.54*
Uncontrolled 2 hours post prandial plasma glucose	*1.307*	*1.382*	*0.344*	*3.69*	*0.25-55.41*
Recessive model					
CC		*ref*			
CTTT	***-1.636***	***0.755***	***0.030***	***0.19***	***0.04-0.86***
Uncontrolled blood pressure	*1.689*	*0.969*	*0.081*	*5.41*	*0.81-36.18*
Uncontrolled fasting plasma glucose	*0.566*	*0.746*	*0.448*	*1.76*	*0.41-7.60*
Uncontrolled 2 hours post prandial plasma glucose	*0.657*	*1.225*	*0.592*	*1.93*	*0.17-21.27*

Hosmer and *Lemeshow test was performed to analyze the good fitness in both models, p-value for all models was > 0.05. B refers to logistic regression model coefficient; SE refers to standard error; OR refers to odds ratio with 95% Confident Interval (CI); ref refers to reference genotype.*

The angiotensin genetic variant rs699 is also known as M235T. This missense mutation in single nucleotide polymorphism causes the substitution of methionine to threonine amino acid in coding sequences. A previous study reported this variant responsible for the variability of angiotensin levels (9). Hyperglycemia which occur in T2DM enhanced Angiotensin expression in circulation and kidney. Angiotensin in circulation caused vasoconstriction, sodium retention, water retention which leads to increased blood pressure. Activation of angiotensin in the kidney enhanced proliferation of the mesangial cell, podocytes apoptosis, excess extracellular matrix formation and activated inflammation cascade. All those concerts cause deteriorated functional and structure of the kidney leading to decreasing

glomerular filtration rate and increased proteinuria (22–24).

The limitation of this study was a single centre pilot study and single genetic variant measurement. Further research with a larger sample, multicenter, more comprehensive genetic and non-genetic analysis is needed to determine this genetic variant as genetic screening for DN. Genetic screening serves as a promising step that can promote better treatment and prognosis.

CONCLUSION

The genetic variant of Angiotensin gene rs699 is associated with DN in the Jambi Malay population. The CT and CT+TT were reported as a protective factor for DN in the Jambi Malay population. Further study with a larger sample size was needed to confirm this result.

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