



The Role of Angiotensinogen rs699 in Diabetic Nephropathy Among Type 2 Diabetes Mellitus Patients with Uncontrolled Postprandial Glucose Levels

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A B S T R A C T

Diabetic Nephropathy (DN) is the most common complication of Type 2 Diabetes Mellitus (T2DM), leading to the highest mortality rate of DM complications. However, its etiology is still questionable. Hyperglycemia, hypertension, and particular genetic susceptibility are associated with DN. Not all patients with uncontrolled hyperglycemia suffer DN. Thus, genetic susceptibility may be a risk factor for DN. The genetic variant of angiotensinogen rs699 is known to be associated with the risk of DN with inconsistent results between ethnicities. This study aims to reveal the correlation between the AGT rs699 with the incidence of diabetic nephropathy among type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels in the Jambi Malay ethnicity. This study was observational analytic research with a cross-sectional design. It used 48 DNA samples from type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels. The authors took 24 DNA samples from patients with DN and 24 without DN (as a control group). The genotyping method used ARMS-PCR specific for AGT rs699. Subjects with the CT genotype had a lower risk for diabetic nephropathy than the CC genotype, but it was not statistically significant ($p=0.247$; $OR=0.508$; $95\%CI=0.160-1.607$). In addition, subjects with the T allele ($p=0.331$; $OR=0.621$; $95\%CI=0.237-1.630$) had a lower risk for diabetic nephropathy than the C allele, but it was not statistically significant. In conclusion, Angiotensinogen rs699 is not a risk factor for diabetic nephropathy among type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels in the Jambi Malay ethnicity.

INTRODUCTION

According to the International Diabetes Federation (IDF), the prevalence of Type 2 Diabetes Mellitus (T2DM) has increased from the previous year, and the estimation will continue to grow (International Diabetes Federation, 2019). This phenomenon also occurs in Indonesia, especially Jambi Province (Kementerian Kesehatan Republik Indonesia, 2018). Increased incidence of T2DM can lead to complications, one of which is Diabetic Nephropathy (DN). In Jambi Province, DN is the most common complication in individuals with diabetes. The prevalence is approximately 35-45% of individuals with T2DM. In addition, DN has the highest mortality rate compared to other diabetic complications (Bistara and Rusdianingseh, 2019).

The causes of diabetic nephropathy in T2DM are multifactorial and occur due to interaction between genetic and environmental factors. Hyperglycemia, hypertension and genetics are risk factors for DN (Xue *et al.*, 2017). However, previous studies found that uncontrolled blood glucose levels or hyperglycemia were not significantly correlated with an increased risk of DN in individuals with T2DM

(Elfiani *et al.*, 2020). It certainly indicates the possibility of other risk factors more involved in developing DN, one of which is genetic factors.

Genetic variations in the renin-angiotensin-aldosterone System (RAAS) play a role in developing diabetic nephropathy in T2DM patients. Angiotensinogen (AGT) rs699 is one of them. T allele of this genetic variation is associated with an increased risk of DN through increased plasma angiotensinogen levels, blood pressure, and insulin resistance in various tissues (Rahimi, 2016; Ramalingam *et al.*, 2017; Yako *et al.*, 2018). AGT rs699 gene variant is located on the long arm of chromosome 1 (1q41-q45) in the form of a T to C substitution in exon 2, resulting in a functional exchange of methionine (M) to threonine (T) at codon 268 (M268T). It is related to the variability of plasma and tissue levels of the encoded protein (Yako *et al.*, 2018).

Studies reported that the AGT rs699 was significantly associated with DN in T2DM patients in Turkish, Tunisian, and Indian populations (Rahimi, 2016; El-garawani *et al.*, 2021). However, the AGT rs699 did not correlate with diabetic nephropathy among people in Caucasians, Mexican Americans, subgroups of Asians, and Indians (Ahluwalia *et al.*, 2009; Rahimi, 2016; Tziastoudi, Stefanidis and Zintzaras, 2020). Controversial results regarding the association between genetic variation of AGT rs699 and diabetic nephropathy among the population of Jambi Malay ethnic are undoubtedly intriguing to investigate. This study aims to reveal the correlation between the AGT rs699 with the incidence of diabetic nephropathy among type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels in the Jambi Malay ethnicity.

METHOD

Study design

This study was observational analytic research with a cross-sectional design. It used 48 DNA samples from type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels. The authors took 24 DNA samples from patients with DN and 24 without DN (as a control group). The inclusion criteria were DNA samples that fulfilled standard quality and quantity based on a nanodrop index of at least ~ 1.8 for genotyping. The exclusion criteria were DNA samples that were not successfully genotyping using the ARMS-PCR method specific for AGT rs699 and DNA samples that did not have complete demographic data. All subjects signed informed consent forms after receiving a detailed explanation of the study objectives and design. The Faculty of Medicine and Health Sciences, Universitas Jambi Ethics Commission approved this study with certificate number: 2136/UN21.8/PT.01.04/2021.

Blood pressure and laboratory measurements

We measured respondents' blood pressure twice in a seated position after 5 minutes of rest using a calibrated sphygmomanometer. Then, we recorded the mean value of those measurements. In addition, we drew 5 milliliters of blood from respondents' antecubital veins after eight to ten hours of fasting to evaluate the serum creatinine and DNA extracted. The Prodia laboratory measured serum creatinine levels using enzymatic colorimetric Jaffe methods. Serum creatinine levels measure the estimated glomerular filtration rate (eGFR) based on Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.

In addition, we took 3 milliliters of blood from a peripheral vein after respondents ate to measure 2-hour postprandial glucose levels. *Perkumpulan Endrokrinologi Indonesia* (PERKENI) guideline 2015 categorizes 2-hour postprandial glucose levels ≥ 180 mg/dL as uncontrolled postprandial glucose levels. Furthermore, we calculated the albumin creatinine ratio (ACR) based on the ratio of quantitative creatinine to albumin from a random spot urine sample. The DN is diagnosed when $ACR \geq 30$ mg/g. Moreover, enzymatic colorimetric methods measure quantitative urine creatinine, and immunoturbidimetric evaluate quantitative urine albumin.

Genotyping

The Deoxyribonucleic Acid (DNA) was extracted from peripheral blood leucocytes using a commercial DNA extraction kit (Macrogen^R). Nanodrop was used to measure the quality and quantity of DNA yielded in the extraction process. We genotyped using the Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method for angiotensinogen rs699. The primary design in this paper was adapted from El Garawani et al (El-garawani *et al.*, 2021). Table 1 shows primary sequences and their product sizes.

Table 1. PCR primers and the product sizes

Primers		Product sizes or Fragment
Outer Forward	5'TGCGCACAAGGTCCTGTCTG3'	
Inner Forward / T allele	5'ATGGAAGACTGGCTGCTCCCTTAT3'	197 bp
Outer Reverse	5'GTCACCAGGTATGTCCGCAGG3'	
Inner Reverse / C allele	5'GCTGTCCCACTGGCTCACG3'	295 bp

The PCR mixture was 10 μ L of PCR master mix (GoTaq Green), 9 μ L of Nuclease Free Water (NFW), 1 μ L for each specific primer AGT rs699 and DNA template 2 μ L. The thermocycler condition was 95°C for 7 minutes as initial denaturation and 1 minute as denaturation. Then, 60 °C for 1 minute as annealing. Next, 72 °C for 1 minute as an extension and 7 minutes as a final extension. The PCR product was then visualized with 1.5% agarose gel for 35 minutes with 100 mV. The PCR product results obtained 197 bp DNA fragments for the T allele and 295 bp DNA fragments for the C allele.

Data Analysis

Data analysis used the Shapiro-Wilk test to test the normality of data with a continuous scale. Normally distributed data were analyzed using an independent t-test and presented as mean (\pm SD). Data not normally distributed were analyzed using Mann-Whitney and presented as median (min-max). The Chi-square test analyzed the correlation between angiotensinogen rs699 and the incidence of diabetic nephropathy. Then, the authors calculated the Hardy Weinberg equilibrium. P-value $>$ 0.05 indicated that allele frequency was concordant with the Hardy Weinberg equation.

RESULT

Baseline Subject Characteristics

This study used a matching method in age and gender between case and control groups. In addition, subject grouping was based on urine ACR value, with urine ACR value \geq 30 mg/gr Cr for the case group. Table 2 indicates the baseline subject characteristics in this paper.

Table 2. Baseline subject characteristics

Characteristics	DN (n=24)	Non DN (n=24)	<i>p</i>
Age (Years)	51 (29-60)	50.5 (22-60)	0.869 ^b
Gender			
Male	10 (41.7%)	10 (41.7%)	1.000
Female	14 (58.3%)	14 (58.3%)	
Systolic Blood Pressure (mmHg)	140.83 \pm 17.17	121.38 \pm 11.04	$<$ 0.001 ^a
Diastolic Blood Pressure (mmHg)	80 (60-100)	80 (70-90)	0.361 ^b
Postprandial glucose (mg/dL)	249 (180-358)	222 (180-456)	0.375 ^b
Urine ACR (mg/g Cr)	377.30 (32.16-5237.69)	12.03 (3.84-26.26)	$<$ 0.001 ^b
eGFR (mL/minute/1.73 m ²)	66.73 \pm 31.58	94.03 \pm 22.83	0.001 ^a

Statistically significant, $p <$ 0.05, ^aThe Independent t-test, ^bThe Mann-Whitney test

Due to the matching method, there were no significant differences between the mean value of age and gender between the case and control groups. The mean value of systolic blood pressure in the case group was statistically higher than in the control group. However, both groups had no statistically significant difference in the mean of diastolic blood pressure and postprandial glucose. The urine ACR mean in the case group was significantly higher than the control group. Still, the eGFR mean was statistically more elevated in the case group.

Genotyping Results of Angiotensinogen rs699

Figure 1 shows the polymerase chain reaction (PCR) product visualization. The T allele was a 197 bp DNA fragment, and the C allele was a 295bp DNA fragment, with the main band being a 448 bp DNA fragment.

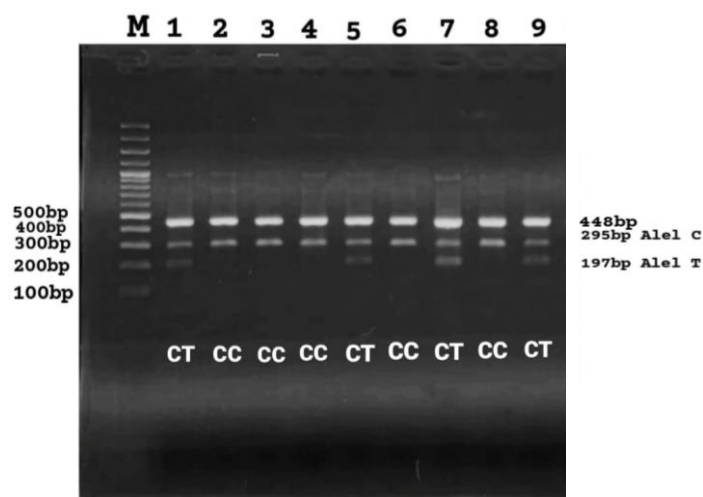


Figure 1. ARMS-PCR results of Angiotensinogen rs699. The CC genotype was marked by the appearance of the C allele and main band in 295 bp and 448 bp DNA fragments. The CT genotype was characterized by the C and T alleles in 295 bp and 197 bp fragments and the main band in the form of 448 bp DNA fragments. M as a marker.

Table 3. Genotype distribution and Hardy Weinberg equation

Genotype	Observed value	Expected value	X ² (Df)	<i>p</i>
TT	0	3		
CT	22	17	4.2425	0.00001
CC	26	29		

MAF=0.34; DF=1; statistically significant, $p>0.05$

The genotype distribution found that the CC genotype was reported as wild type in the respondents, and the T allele was the minor allele. The MAF value of this SNP was around 0.34 in the SNPedia database. Our studies' proportion of genotype frequency deviated from the Hardy Weinberg equation (Table 3).

The Correlation between Angiotensinogen rs699 and the Incidence of Diabetic Nephropathy

The bivariate analysis revealed that Angiotensinogen rs699 showed a higher frequency distribution in the CC genotype than the CT genotype in the diabetic nephropathy group. Thus, subjects with CT genotype had a lower risk for DN than CC genotype. The CT genotype acted as a protective factor against the incidence of diabetic nephropathy, but it was not statistically significant (OR=0.508; 95% CI= 0.160 – 1.607; $p= 0.247$) (Table 4).

Table 4. The bivariate analysis between Angiotensinogen rs699 and the incidence of diabetic nephropathy

Genotype	DN (n=24)	Non DN (n=24)	P value ^a	OR (95% CI)
Additive model				
CT	9 (40.9%)	13 (59.1%)	0.247	0.508 (0.160-1.607)
CC	15 (57.7%)	11 (42.3%)	ref	
Allele				
T	9 (40.9%)	13 (59.1%)	0.331	0.621 (0.237-1.630)
C	39 (52.7%)	35 (47.3%)	ref	

^aChi-Square test; DN refers to diabetic nephropathy; ref refers to reference genotype/allele.

In addition, the frequency distribution of the C allele was higher than the T allele in the diabetic nephropathy group. Thus, subjects with the T allele ($p=0.331$; $OR=0.621$; $95\%CI=0.237-1.630$) had a lower risk for diabetic nephropathy than the C allele. The T allele was a protective factor against diabetic nephropathy but was not statistically significant ($OR=0.621$; $95\% CI=0.237 - 1.630$; $p= 0.331$) (Table 4).

DISCUSSION

Baseline Subject Characteristics

This paper showed no significant differences between the mean value of age and gender between the case and control groups (Table 2). It may be due to the matching method between both groups (DN and non-DN) to minimize confounding factors contributing to diabetic nephropathy. Based on Basic Health Research in 2013, previous epidemiology reported that older age and male T2DM patients contributed increased risk of DN (Mihardja *et al.*, 2018).

In addition, our findings found that the DN group (case group) had statistically higher mean systolic blood pressure than the non-DN (control group) (Table 2). Meanwhile, the diastolic blood pressure was not statistically different between the two groups. A prior study in Slovenia also reported similar results (Makuc *et al.*, 2017). Increased blood pressure in diabetic nephropathy patients is associated with increased activity in the sympathetic nervous system due to insulin resistance in patients with diabetes mellitus. High renal sympathetic nerve tone stimulates renin release and increases sodium reabsorption in renal tubules. In addition, increased renin release will increase cardiac output and peripheral vascular resistance, leading to increased blood pressure (Maqbool, Cooper and Jandeleit-Dahm, 2018; Ohishi, 2018).

Furthermore, this research reported that postprandial glucose levels in the DN group did not have a statistically significant difference compared to the non-DN group (Table 2). However, theory explains a contradiction in the association between blood glucose and DN. Hyperglycemia in T2DM patients leads to insulin resistance and impaired β cell function. The failure of the feedback function between insulin action and insulin secretion causes abnormally increased blood glucose levels. Dysfunction of β cells and reduced insulin secretion leads to impaired blood sugar regulation. As a result, insulin does not work optimally, leading to the pancreas compensates by producing more insulin. However, when β cell is no longer adequate to produce insulin as compensation for increased insulin resistance, blood glucose levels will increase and lead to chronic hyperglycemia. Hyperglycemia increases extracellular matrix (ECM) expression, leading to renal function deterioration (Decroli, 2019; Galicia-Garcia *et al.*, 2020).

ACR and eGFR are clinical assessments of renal function in T2DM. The ACR is a parameter for diagnosing DN. Meanwhile, the eGFR monitors renal function relating to end-stage renal diseases

complication (ESRD). This investigation revealed that the mean urine ACR value in the diabetic nephropathy group was statistically higher than the control group (Table 2). We used urine ACR levels ≥ 30 mg/gr Cr as the parameter for diabetic nephropathy diagnosis. Urinary ACR value can identify albuminuria by calculating the ratio of urine albumin to urine creatinine. The urine ACR in this study was calculated using random spot urine sampling. It is because a previous study reported that this sampling was more representative and practical in assessing albuminuria associated with complications of diabetes mellitus than a 24-hour urine sample (Elfiani *et al.*, 2020). Albuminuria in patients with diabetes mellitus provides an early indication of kidney damage (Seidu, Barrat and Khunti, 2020). Increased urinary albumin excretion in diabetic nephropathy occurs due to intraglomerular hyperfiltration or hypertension (Umanath and Lewis, 2018). Albuminuria in DN patients is also associated with a decrease in podocyte density, causing the loss of barrier to preventing urinary protein loss (Podgórski *et al.*, 2019).

Moreover, the mean value of the Estimated Glomerular Filtration Rate (eGFR) in the DN group tended to be statistically lower than the control group (Table 2). eGFR is calculated from serum creatinine concentration as a marker of endogenous glomerular filtration (Seidu, Barrat and Khunti, 2020). The hyperglycemia will induce media and intima layer thickening in afferent arterioles. Then, it causes decreased elasticity of blood vessels and control of glomerular pressure. In addition, hyperglycemia causes afferent arteriolar dysfunction. Next, it leads to increased glomerular pressure, hyperfiltration, and reduced serum creatinine levels. High glomerular pressure will result in proteinuria. In addition, it can decrease the glomerular filtration rate due to mesangial denaturation or glomerular necrosis. Finally, it leads to end-stage kidney damage (Ohishi, 2018; Yamazaki, Hitomi and Nishiyama, 2018).

The Correlation between Angiotensinogen rs699 and the Incidence of Diabetic Nephropathy

This research reported that the population's CC genotype was wild-type, and the T allele was minor. In addition, our studies' proportion of genotype frequency deviated from the Hardy Weinberg equation, which may be due to the small number of samples (Table 3). Furthermore, there was no correlation between Angiotensinogen rs699 and the incidence of diabetic nephropathy in this paper (Table 4). Studies in Slovenian, Caucasian, Mexican Americans and subgroups of Asian populations also revealed similar results (Rahimi, 2016; Makuc *et al.*, 2017; Tziastoudi, Stefanidis and Zintzaras, 2020). However, Several studies in Indian, Turkish and Tunisian populations showed an association between angiotensinogen rs699 and the risk of diabetic nephropathy in individuals with type 2 diabetes mellitus (Ahluwalia *et al.*, 2009; Rahimi, 2016). The different results in various populations may be due to ethnic heterogeneity and sampling method (Makuc *et al.*, 2017).

In addition, the CT genotype acted as a protective factor against the incidence of diabetic nephropathy but was not statistically significant (Table 4). However, a study in India showed that CT genotype was a risk

factor for diabetic nephropathy and CC genotype was a protective factor. In addition, this study found that the T allele acted as a protective factor against diabetic nephropathy and the C allele acted as a risk factor among type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels in the Jambi Malay ethnicity. Still, it was not statistically significant (Table 4). Research in the Slovenian population also reported that T and C alleles of angiotensinogen rs699 did not associate with the risk of diabetic nephropathy (Makuc *et al.*, 2017). However, a study in India indicated that the T allele acted as a risk factor for diabetic nephropathy, and the C allele is a protective factor (Ahluwalia *et al.*, 2009).

Some genotype and phenotype studies reported the association between Angiotensinogen rs699 and ESRD. A prior study in Egyptian reported that the T allele of rs699 was associated with an increased risk of ESRD (El-garawani *et al.*, 2021). The review article on the role of the RAAS gene in DN reported that the TT genotype was associated with susceptibility and faster progression to ESRD in T2DM patients (Rahimi, 2016).

The authors used ARMS-PCR specific for angiotensinogen rs699 in this investigation. The ARMS-PCR method is faster, more reliable, and affordable than other methods. The method is appropriately performed with limited laboratory resources (Puspasari *et al.*, 2021). In addition, primer angiotensinogen rs699 was adapted from El-Garawani *et al.* (El-garawani *et al.*, 2021). We performed In Silico analysis to measure the primer sequence and PCR product size. Furthermore, PCR conditions optimization was adapted to our laboratory resources.

The limitations of this study were the relatively small number of samples and no conformational sequencing. However, the sample number in this study was proper with the minimum number of samples size. In addition, we did an in-silico analysis to measure the primer sequence and PCR product specific for angiotensinogen rs699.

CONCLUSION

Angiotensinogen rs699 is not a risk factor for diabetic nephropathy among type-2 diabetes mellitus patients with uncontrolled postprandial glucose levels in the Jambi Malay ethnicity. Further study should use a larger sample and analyses other genetic variations.

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