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Angiotensin-converting Enzyme Genetic Polymorphism rs4343 as Risk of Diabetic Nephropathy in Jambi-Malay Population

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Background: Diabetic nephropathy (DN) is one of the frequent complications of type II diabetes mellitus (T2DM) in Jambi province. Controlling blood glucose and blood pressure does not guarantee DN prevention, since genetic factors may also contribute to this disease. Multi-ethnic studies showed that one of the strongest genetic factors associated with DN was single nucleotide polymorphism rs4343 of angiotensin-converting enzyme (ACE) gene. Study regarding phenotype-genotype association of *ACE* rs4343 and DN has not yet been performed in Jambi Province, which is dominated by Malay ethnicity. This study was conducted to reveal the association between *ACE* rs4343 and the risk of DN in the Jambi-Malay population.
Materials and methods: This was a cross-sectional study involving 75 subjects (44 with DN and 31 without DN) who suffered from T2DM and hypertension. DN was defined as albumin to creatinine ratio (ACR) ≥30 mg/g. Genotyping was performed with one-step tetra amplification refractory mutation system-polymerase chain reaction (PCR) using specific primer for *ACE* rs4343. Bivariate and multivariate analyses were performed to analyze the genetic risk for DN.
Results: The bivariate analysis showed the proportion of DN subjects was higher than non-DN within the AG genotype (11:1) than within the AA (33:30) genotype. This difference was statistically significant (p=0.012; OR (95% CI): 10.00 (1.22-82.15)). Multivariate analysis showed that AG genotype (p=0.047; OR (95% CI): 10.04 (1.03-97.31)) and uncontrolled blood pressure (p=0.001; OR (95% CI): 6.72 (2.08-21.71)) were the risk factors of DN in the Jambi-Malay population.
Conclusion: Polymorphism of *ACE* rs4343 is a risk factor of DN in the Jambi-Malay Population.

Keywords*: rs4343, angiotensin-converting enzyme gene, diabetic nephropathy, Malay, Jambi*

Introduction

Based on Indonesia National Health Survey prevalence of type II diabetes mellitus (T2DM) has increased in Indonesia, including in Jambi Province.[1,2] An epidemiology study held in Jambi reported the diabetic nephropathy (DN) was more frequent than peripheral microvascular complications and macrovascular complications in T2DM.[3] DN can lead to

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end-stage renal diseases, which affect patient's quality of life and burden of health costs.[4] The environmental factors in interaction with genetic predisposition play a role in hemodynamic and metabolic changes in T2DM, which may lead to the phenotype of DN.[5,6] Although blood glucose and blood pressure are clinically controlled to prevent DN, the importance of genetic predisposition screening has also been a concern recently. This screening provides a better understanding of the pathophysiology, promises better management and predicts the prognosis of DN progression especially with patients who have risk genotype.[7]

Genome-wide study and meta-analysis report that the genetic predisposition which increases the risk of DN is single nucleotide polymorphism (SNP) located in the gene associated with lipid metabolism, glucose metabolism, angiogenesis, inflammation and oxidative stress, renal structure and function, and the renin-angiotensin-aldosterone system (RAAS).[8-11] One of them are genetic variants located in angiotensin-converting enzyme (ACE) gene.[8-12] The rs4343 is a genetic variant of *ACE* which has the strongest association with ACE activity.[11] Previous studies reported that patients who had AG and GG of this synonymous SNP had higher ACE levels and activity than the ones who had A allele.[13,14] ACE is one of the components of RAAS, the most well-known studied axis which has a main role in DN initiation and progression. ACE contributes to DN pathophysiology through its activity to activate angiotensin II. This protein induces increased vascular pressure which enhances perturbation in anatomical structure, such as thickening of glomerular basement membrane, extracellular matrix expansion, mesangial cell and podocytes abnormality, all of which lead to DN phenotype.[15,16]

The effect of SNP in diseases is mainly influenced by ethnicity.[17] Previous studies reported a controversial result of *ACE* rs4343 as a risk of DN. South Indian and Chinese patients with T2DM who had allele or genotype polymorphism for this gene have been reported to have an increased risk of DN and early renal insufficiency.[18-20] Meanwhile, a study in North India reported a lack of association between this SNP and the risk of DN.[21]

To the best of our knowledge, study regarding phenotype-genotype association of *ACE* rs4343 and DN in T2DM has not yet been performed in Jambi Province, which is dominated by Malay ethnicity. This study was conducted to reveal the association between *ACE* rs4343 and the risk of DN in the Jambi-Malay population. In conjunction with the main aim, an analysis of uncontrolled blood pressure and blood glucose as risk factors for DN was also performed in this study.

Materials and methods

Study Design and Subjects Recruitment

This study was a cross-sectional study included 75 subjects who suffered from T2DM and hypertension. The inclusion criteria were age 30-70 years old, suffered from T2DM for at least 5 years based on medical records, being Jambi-Malay ethnic, and residing in Jambi Province. The exclusion criteria were subjects who suffered urinary tract infection based on clinical examination and routine urine analysis, had a history of other renal diseases, and were immunocompromised, as well as pregnant and breast-feeding women. Subjects were selected from 3 general hospitals namely Raden Mattaher General Hospital, Baiturrahim General Hospital and Mayang Medical General Hospital located in Jambi Province. The study was conducted during January 2020 to December 2021.

The diagnosis of T2DM was based on criteria from the Association of Indonesian Endocrinologists 2021.[22] Subjects with fasting serum glucose ≥126 mg/dL or 2-hour postprandial serum glucose ≥200 mg/dL were defined as T2DM and hypertension was defined if blood pressure ≥140/90 mmHg, which was taken at least twice measurement. All data was listed in medical records. Kidney function parameters, namely albumin to creatinine ratio (ACR) and glomerular filtration rate (GFR), were measured in all subjects.

The protocol of this study has been validated and approved by the Ethic Research Committee from The Faculty of Medicine and Health Sciences, Universitas Jambi (No. 3075/UN21.8/PT.01.04/2022). All subjects signed informed consent after receiving detailed information regarding the aims and procedures of the study, and the rights as participants of the study.

Blood Pressure and Laboratory Examination

The blood pressure was the mean of blood pressure taken twice with the same well-trained medical staff using a calibrated sphygmomanometer. Before measurement, subjects had to rest for 5-10 minutes and the measurement was taken in a sitting position. Subjects who had mean of systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg were categorized as uncontrolled blood pressure.[22] For laboratory examination, all subjects

had to fast for 8-10 hours. Five mL of blood sample was then taken from the peripheral vein by well-trained medical staff for measurement of serum creatinine and fasting serum glucose levels. After 2 hours of meals, 2 mL blood sample was also taken to measure the 2-hour postprandial serum glucose. The glucose oxidase peroxide amino-antypirin (GOD-PAP) method was used for the measurement of serum glucose with Glucose GOD FS 10 kit (ProLiNE, Cikarang, Indonesia) and TRX7010 clinical chemistry analyzer (Tokyo Boeki, Tokyo, Japan). Uncontrolled fasting serum glucose was defined as fasting serum glucose >130 mg/dL and uncontrolled 2-hour postprandial serum glucose was defined as 2-hour postprandial serum glucose ≥180 mg/dL.[22] Serum creatinine level was measured for calculation of the estimated GFR based on the chronic kidney diseases epidemiology collaboration (CKD-EPI) equation 2021 without race[23], enclosed gender coefficient and age. The colorimetric enzymatic hydrolase assay using Creatinine PAP FSR kit (ProLiNE) and TRX7010 clinical chemistry analyzer was performed to measure serum creatinine level. Urine samples were collected for urine albumin and creatinine measurement. Urine albumin was measured based on immunoturbidimetric method using Albumin in Urine/CSF FS kit (ProLiNE) and urine creatinine was measured based on enzymatic colorimetric method using creatinine FS (ProLiNE) kit. Both urine albumin and creatinine levels were used for ACR calculation. DN was defined as ACR ≥30 mg/g.

Genotyping

The deoxyribonucleic acid (DNA) was extracted from the buffy coat using a DNA extraction kit for human blood (Purelink Genomic DNA Mini Kit, Invitrogen, Waltham, MA, USA). Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure the quality and quantity of the extracted DNA. Then, genotyping was performed with one-step tetra amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) using specific primers for *ACE* rs4343.[24] The primer sequences consisted of one pair of outer primers (FO [5′ CATTTCTGAAATTCTCTGAGCTCCCCT 3′] and RO [5′ TTTAGGAAAATGAAGGGACCCAAGTGC 3′]) and one pair of inner primers (Fi [5′ CATATTTGACGAATGTGATGGCCCCA3′] and Ri [5′CC AATAACAGGTCTTCATATTTCCGGTAC 3′]). The PCR cycling conditions were initial denaturation of 94°C for 1 minute, followed by 35 cycles of denaturation (94°C for 35 seconds), annealing (61°C for 45 seconds), and extension (72°C for 35 seconds), and final extension of 72°C for 4 minutes. Negative control of each PCR reaction and twice repetition for at least 20% random sample was performed for quality control of one-step tetra ARMS-PCR. Then, electrophoresis using 2.5% agarose gel with 100 mV for 45 minutes was performed to visualize the PCR product. The size of G and A alleles were 134 bp and 202 bp, respectively.

Data Analysis

Bivariate analysis was performed to analyzed baseline subject characteristic data. The association between *ACE* rs4343 and DN was analyzed bivariate and multivariate. Multivariate analysis was performed enclosing the blood pressure and blood glucose as covariate variables with binary logistic regression backward methods. SPSS 25 (IBM, Armonk, NY, USA) was used for data analyses.

Results

Characteristics of Subjects

Seventy-five T2DM subjects participated in this study consisting of 44 subjects who suffered from DN and 31 subjects who did not suffer from DN. The DN group was slightly older than the non-DN group. The difference in frequency of males and females in both groups was not statistically significant. Renal function test showed the DN group had a significantly higher ACR and a significantly lower GFR than the non-DN group. The frequency of subjects who had uncontrolled blood pressure and serum glucose was higher in the DN group than in the non-DN group. However, only the blood pressure was significantly different between the DN and non-DN groups (Table 1).

The Genotyping and Genotype Distribution

One-step tetra ARMS-PCR results were visualized using agarose gel electrophoresis. The different base pairs are signed by different alleles. The G and A alleles were visualized as 134 bp and 202 bp PCR products, respectively. The general product size was 280 bp (Figure 1). The genotype distribution of *ACE* rs4343 in this study was shown in Table 2. In addition, the Hardy-Weinberg equilibrium (HWE) calculation was also performed. The most frequent genotype in this study was AA. GG genotype was not observed in this study. The minor allele was G with a frequency of 16 %. *ACE* rs4343 in this population did not deviate from HWE.

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Table 1. Baseline subjects' characteristics.

Characteristic	DN (n=44)	Non-DN (n=31)	*p*-value
Age (years), median (min-max)	52 (29-67)	50 (22-60)	0.470[c]
ACR (mg/g), median (min-max)	252.96 (30.68-14,403.00)	12.44 (0.00-28.44)	<0.001[c]
GFR (mL/min/1.73 m^2), median (min-max)	63.55 (13.80-124.00)	95.10 (35.60-134.80)	0.002[c]
Gender, n			
Male	19	25	0.503[a]
Female	11	20	
Blood pressure, n			
Uncontrolled (systolic ≥140 and/or diastolic ≥90 mmHg)	32	12	<0.001[a]
Controlled (systolic <140 and diastolic <90 mmHg)	7	24	
Fasting serum glucose, n			
Uncontrolled (>130 mg/dL)	28	16	0.112[a]
Controlled (≤130 mg/dL)	14	17	
2-hour postprandial serum glucose, n			
Uncontrolled (≥180 mg/dL)	40	4	0.183[b]
Controlled (<180 mg/dL)	24	7	

[a]Chi-square; [b]Fisher exact test; [c]non-parametric test with Mann Whitney test.

ACE rs4343 Genotype as Risk of DN

The bivariate analysis of *ACE* rs4343 and DN in this study showed that subjects with AG genotype had a significantly higher risk of suffering from DN than the ones with AA genotype. Allele analysis showed that subjects with G allele had a significantly higher risk of suffering from DN than the ones with A allele (Table 3).

The binary regression logistic backwards was performed as a multivariate analysis and the last model was followed by genotype, uncontrolled blood pressure and GFR. This analysis reported that genotype polymorphism of ACE rs4343 and uncontrolled blood pressure were statistically significant risks for DN in this study. Subjects with AG genotype and uncontrolled blood pressure had a higher risk of suffering from DN (Table 4).

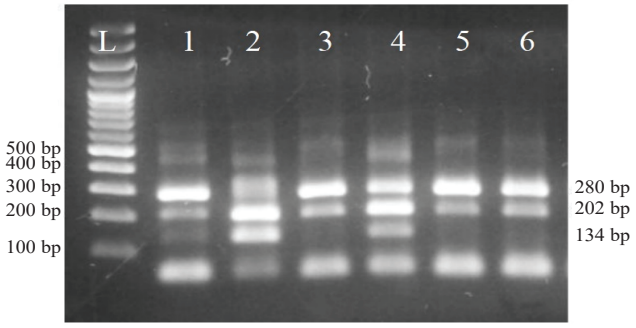

Figure 1. Visualization of tetra ARMS-PCR products for *ACE* rs4343 A/G after electrophoresis. L lane: 100 bp ladder; Lane 1, 2, and 4: Heterozygote AG; Lane 3, 5, 6: Homozygote AA.

Discussion

In the present study, age and gender were not statistically different between DN and non-DN subjects due to subject matching. Although the proportion of uncontrolled blood sugar was higher in the DN group than the non-DN group, this difference was not statistically significant. This study reported that the proportion of uncontrolled blood pressure was significantly higher in the DN group than the non-DN group. The previous meta-analysis study reported that both uncontrolled blood pressure and blood glucose were strongly associated with the progression of DN. This discrepancy may relate to different sample criteria and the use of HbA1C as a marker of glycemic control. HbA1C reflects blood glucose level over the past 2-3 months and slightly interferes with recent dietary intake. This is in contrast to fasting or 2-hours postprandial blood glucose, which are much more interfered with by recent dietary intake.[25,26] Higher blood pressure, especially in T2DM, enhances RAAS activation, which increases glomerular capillary pressure. This hemodynamic change activates profibrotic and proinflammatory cytokines, which hasten the structural changes and decline of kidney function, which leading to DN.[6,15,16]

Table 2. Genotype distribution of *ACE* rs4343.

Genotype	Observed Value	Expected Value	χ^2 (DF)	*p*-value	MAF
AA	63	63			
AG	12	11	0.567	0.451	0.16
GG	0	1			

χ^2 value with degree of freedom (DF)=1; MAF: Minor allele frequency.

The present study revealed that SNP rs4343 (AG genotype or G allele) of *ACE* was a risk factor for DN in the Jambi-Malay population and this risk was higher in multivariate analysis with uncontrolled blood pressure as covariable. This finding was similar to a phenotype and genotype study in Northern Han of China which reported that the AA genotype was a protection factor for suffering nephropathy in T2DM patients.[18,19] A Study in South India reported that *ACE* rs4343 was the risk factor for T2DM and DN, and the risk for DN was higher for subjects younger than 46 years old. The AA genotype was protective and the GG genotype was a risk factor for DN.[20] Contrary to those studies, a study in North India demonstrated that SNP rs4343 of *ACE* alone was not a statistically significant risk of DN but the haplotype of this SNP with other SNP (rs4311 and rs13447447) of *ACE* was a risk factor for DN with A allele as protection factor.[21]

The pathophysiology of DN and hypertension and the cross-talk between them have a strong association with ACE levels and activity in the circulation and the kidney. The genetic variance in ACE level also contributes to this cross-talk. ACE is an enzyme involved in RAAS. ACE is produced by epithelial and endothelial cells in the kidney, lungs and blood vessels. ACE converts Angiotensin I to Angiotensin II, which controls hemodynamic balance. Higher levels and activity of ACE are observed in hypertension and hyperglycemic conditions. Overactivity of ACE causes an increase in angiotensin II, leading to an increase in glomeruli capillary pressure, stimulation of mesangial proliferation and rearrangement of podocytes. All of this causes functional and structural changes in the kidney and leads to DN.[15,16] The ACE level and activity are mainly controlled by genetic factors, the SNP rs4343 was the genetic factor which has the strongest association with it.[13] This SNP is a synonymous mutation, in which A is substituted to G in the intron gene (Thr776Thr). Functional studies reported that synonymous mutation may alter the mRNA folding that decreases protein stability and transcription level, thus playing a role in ACE level and activity. The AG and GG genotypes tend to have increased ACE levels and activity.[13,14]

The minor allele in the present study was the G allele, which is similar to that reported in other populations, although homozygote GG was not observed. This may be due to the limited subject number, but this recent study meets a minimal sample size. The HWE was used as quality control of genotyping SNP in Genome Wide Association Study (GWAS), the SNP which did not departure from HWE was included in GWAS analysis. The Hardy-Weinberg calculation in the present did not depart from HWE equation. It may reflect the small bias for genotyping method, the small bias for the false-positive rate to identify the association of genotype-phenotype or may meet HWE assumption for the genetic population (random mating, the absence of natural selection, no gene flow and autosomal locus).[27,28] The frequency of minor alleles in the present study was 16%. Based on NCBI database, this frequency is lower than the ones in other populations.[29]

Table 3. Association between *ACE* rs4343 genotype and DN.

Genotype	DN (n)	non-DN (n)	*p*-value	OR (95% CI)
Additive model				
AG	11	1	0.012	10.00 (1.22-82.15)
AA	33	30	ref	
Allele				
G	11	1	0.049	7.50 (0.92-61.19)
A	44	31	ref	

Fisher exact test was performed in the bivariate analysis due to an expected value below 5 in more than 20% of the cells.

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Table 4. Multivariate analysis; predict diabetic nephropathy based on genotype and another factor.

Variable	B	SE	Adjusted *p*-value	Adjusted OR	95% CI
Additive genotype					
AG	2.31	1.16	0.047	10.04	1.03-97.31
AA	ref				
Uncontrolled blood pressure	1.90	0.60	0.001	6.72	2.08-21.71
GFR	-0.020	0.011	0.059	0.98	0.96-1.00

Good fitness was analyzed with Hosmer and Lemeshow, the model above fulfilled the Hosmer Lemeshow For Good Fitness with p-value>0.05. B: model coefficient; SE: standard error; OR: odds ratio; CI: confidence interval; ref: reference genotype.

The genotyping method used in the present study was one-step tetra-ARMS PCR. This technique was reliable, cost-effective, simple and faster than polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) or high-resolution melting (HRM)-PCR, especially with limited resources.[30,31] Four specific primers were used, consisting of outer forward and reverse forward primers to gain general product and inner primers which are allele-specific for the SNP.[24] The National Center for Biotechnology Information (NCBI) database was used to measure the location of primer sequences and estimated PCR product by performing *in silico* analysis (Supplementary 1). Then, the thermocycling condition and PCR mixture were adjusted to yield the best electrophoresis visualization.

The present study was a preliminary, single-center study which involved a limited number of subjects. To strengthen evidence of this genetic variant as screening for DN risk factors, further studies involving a larger number of subjects from multi-ethnic populations with other variables including other genetic variants and other modifiable risk factors are needed. Although genetic variant is an unmodifiable factor for disease susceptibility, knowing it promises a better understanding of the disease's pathophysiology and promotes better treatment for patients. Knowing the risk is the first step, and performing early, more aggressive treatment may prevent the disease or its complications.

Conclusion

Polymorphism of *ACE* rs4343 is a risk of DN in Jambi-Malay Population. T2DM subjects with AG genotype or G allele have a higher risk of suffering from DN than the ones with AA genotype or A allele.

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Authors Contribution

EE and AP were involved in concepting and planning the research. EE, CWA and CM performed the data acquisition/collection. AP, NNAS and CWA calculated the experimental data and performed the analysis. AP, ZA, NS, IE, S, E, CWA, CM drafted the manuscript and designed the figures. E, AP, CWA, NNAS aided in interpreting the results. All authors took part in giving critical revision of the manuscript.

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