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Association between A1166C Polymorphism of the Angiotensin II Type-1 Receptor Gene and Type-2 Diabetic Nephropathy in an Indonesian Malay Population

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ABSTRAK

Latar belakang: *nefropati diabetik (ND) adalah penyebab terpenting dialisis di dunia. Penelitian terdahulu menunjukkan keterkaitan antara polimorfisme A1166C gen AT1R dan keadaan hiperfiltrasi glomerulus pada patogenesis ND. Diperlukan penelitian yang bertujuan untuk membuktikan hubungan antara polimorfisme A1166C AT1R dan kerentanan individu yang menderita diabetes melitus tipe 2, khususnya pada populasi Melayu, terhadap ND.* ***Metode:*** *penelitian ini merupakan studi analitik observasional dengan desain kasus-kontrol, melibatkan 120 pasien diabetes melitus tipe 2 (DMT2), 40 pasien untuk tiap kelompok makroalbuminuria, mikroalbuminuria, dan normoalbuminuria sebagai kontrol, dengan metode consecutive sampling. Adanya polimorfisme gen A1166C AT1R diperiksa dengan metode PCR/RFLP.* ***Hasil:*** *alel C mutan ditemukan sebanyak 5% pada kelompok normoalbumnuria, 13.75% pada kelompok mikroalbuminuria, dan 12.5% pada kelompok makroalbuminuria. Genotipe heterozigot AC ditemukan secara signifikan lebih tinggi pada kelompok mikroalbuminuria, dibandingkan dengan kelompok normoalbuminuria. Genotipe AC (OR 3.2 [1.01-10.08], p=0.03) dan alel C (OR 2.8[0.95-8.67], p=0.038) ditemukan lebih tinggi pada kelompok ND, menandakan polimorfisme A1166C gen AT1R sebagai faktor risiko ND pada pasien DMT2 ras melayu.* ***Kesimpulan:*** *terdapat hubungan antara polimorfisme A1166C gen AT1R dengan kejadian ND pada pasien DMT2 di populasi melayu Indonesia. Juga ditemukan bahwa polimorfisme A1166C gen AT1R kemungkinan berperan dalam penurunan fungsi ginjal pada pasien DMT2 sejak tahap awal.*

Kata kunci: *angiotensin II tipe-1 reseptor, polimorfisme, nefropati diabetik.*

ABSTRACT

Background: *diabetic nephropathy (DN) is the leading cause of blood dialysis worldwide and a major etiology of End-Stage Renal Disease cases in Indonesia. Previous studies showed a relevant link between A1166C polymorphism of Angiotensin II Type-1 Receptor (AT1R) gene and glomerular hyper-filtration as a part of pathogenesis of DN. The aim of this study was to elaborate the association between A1166C AT1R polymorphism and susceptibility of individual with type-2 diabetes to DN in Malay Indonesian population.*

Methods: *a case-control study of 120 consecutive patients with type-2 diabetes mellitus (40 patients in each groups for macro-albuminuria, micro-albuminuria, and normo-albuminuria) was conducted for A1166C AT1R gene polymorphism. The A1166C polymorphism of the AT1R gene was determined based on PCR/RFLP.* ***Results:*** *the mutant C allele was found in 5%, 13.75%, and 12.5% in normo-, micro-, and macro-albuminuria patients respectively. The heterozygote AC genotype was found significantly higher in micro-albuminuria, compared to normo-albuminuria group. Heterozygote AC genotype (OR 3.2 [1.01-10.08], p=0.03) and C allele (OR 2.8[0.95-8.67], p=0.038) were significantly higher in DN, indicating A1166C AT1R gene polymorphism as a risk factor for DN in Malay Indonesian population with type-2 diabetes.* ***Conclusion:*** *there was positive association between A1166C AT1R polymorphism and susceptibility of type-2 diabetics to DN in Malay Indonesian Population. It also indicated that the A1166C AT1R polymorphism could play a role in early pathogenesis of DN.*

Keywords: *angiotensin II type-1 receptor, polymorphism, diabetic nephropathy.*

INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD) and blood dialysis worldwide[1]. DN accounts for 22% of ESRD in Indonesia.[2] Playing a significant role for mortality in type-2 diabetes mellitus, DN affects almost 35% of patient with diabetes regardless of their glycemic control.[3] Despite the identification of risk factors, such as duration of diabetes, hypertension, hyperglycemia, chronic inflammation, and genetic components, no clear single etiologic entity of DN has been identified. The understanding of genetic susceptibility in DN, which has been affirmed by previous familial studies, can elucidate the pathogenesis, and lead to better treatment and prevention efforts of DN.[4,5]

The angiotensin II type 1 receptor (AT1R), found mostly in the kidney and vascular smooth muscle,[6] may have a role in the natural history of DN. AT1R is a G-protein coupled receptor,[7] which angiotensin II binds to produce proliferation, vasoconstriction, and cellular effects.[8] Some AT1R gene polymorphism have been identified, and the A1166C polymorphisms is the most studied. The A1166C polymorphism of AT1R gene due to a base replacement of cytosine for adenine at the position 1166 in the 3'untranslated region is related to the posttranslational modification of AT1R mRNA.[9] Previous studies has found that a mutant C allele was associated with an increased filtration fraction response to angiotensin II infusion,[10] thereby indicating the augmented angiotensin II activity in the presence of C allele and thus enabling glomerular hyper-filtration, an early classical finding for micro-albuminuria stage of DN.[11]

Several previous studies have investigated the role of AT1R polymorphism in the involute etiology of DN although they showed conflicting results. Those studies have signified[12] or refuted[13,14] the role of A1166C AT1R gene variant in the natural history of type-2 DN. The inconsistent finding among studies is accredited to the genetic diversity and ethnicity background among various populations. We accordingly opted to explain for the first time the plausible association between the A1166C AT1R polymorphic variants and DN in an Malay Indonesian diabetic population, as there is an absence of the comparable studies in an Indonesian population. The aim of this study was to elaborate the association between A1166C AT1R polymorphism and susceptibility of individual with type-2 diabetes to DN. In an attempt to gather better explanation about the role of the A1166C AT1R polymorphism in natural history of DN, we eleborated DN into its two group stages, which were micro-albuminuria and macro-albuminuria.

METHODS

An observational analytic study with a case-control design was conducted at an outpatient clinic and inpatient wards in Mohammad Hoesin General Hospital Palembang, South Sumatera. All consecutive patients were recruited. Written informed consent were obtained from all patients. The study was approved by the local

Ethics Committee from health research review committee of Mohammad Hoesin General Hospital and Faculty of Medicine Universitas Sriwijaya on January 9th, 2012, with a reference number 004/KEPKRSMHFKUNSRI/2011.

The study population consisted of an Malay Indonesian population with diabetes who had resided in South Sumatera for three generations. The minimum sample needed was 38 patients for each group. The inclusion criteria for case group were subjects with type-2 diabetes aged 40-65 years old, with DN, and normal renal function-defined by estimated Glomerular Filtration Rate (GFR) more than 60 mL/min/1.73m^2. For the control group, the sex-and-age-matched individual with type-2 diabetes with normo-albuminuria (i.e. urinary albumin excretion below 30 µg/mg creatinine) were included. Diagnosis of diabetes was made according to the American Diabetes Association Standard Medical of Care which was adapted to Indonesian National Consensus on Type-2 Diabetes Mellitus.[15,16] The diagnosis of nephropathy was based on at least two consecutive occasions of micro-albuminuria (urinary albumin excretion 30-300 µg/mg creatinine) or macro-albuminuria (urinary albumin excretion above 300 µg/mg creatinine) as a minimum criterion in non-oliguric patients with diabetic retinopathy without any other possible cause.[16] In order to exclude the cause of proteinuria other than diabetes, those with evidence of previous renal disease that could be the secondary causes of albuminuria such as glomerulonephritis, nephro-ureterolithiasis, chronic infections, malignancy, and obstructive uropathy were not eligible to participate in the study.

Genetic Analysis

2 mL of peripheral blood, were drawn by venipuncture in the upper limbs and placed into tubes containing 5% EDTA. Genomic DNA was then isolated from leukocytes and DNA was extracted by standard techniques.[17] A1166C polymorphism of the AT1R gene were determined by polymerase chain reaction (PCR or restriction fragment length polymorphism (RFLP) using modified method of previous studies.[18]

For the detection of A1166C polymorphism in 3' untranslated region, the primer used were 5'GCA CCA TGT TTT GAG GTT-3' for sense primer and 5'CGA CTA CTG CTT AGC ATA-3' for antisense primer. The PCR product was 527 base pairs (bp) fragment. After digesting with DdeI RFLP enzyme for 3 hours, the PCR products was detected on 2% agarose gel electrophoresis stained with ethidium bromide (Biorad, Hercules, California USA). Wild type allele, allele A, generated one fragment corresponding to size 527 bp. Two fragments of 417 bp and 110 bp were observed as mutated allele C. The heterozygote genotype would generate three fragments of 417, 110, and 527 bp.

Statistical Analysis

Values were presented as mean and standard deviations. The fisher exact test or chi square test was used for analysis of the categorical variables, and ANOVA was used for continuous variables with normal distribution. The association between A1166C AT1R gene polymorphism and DN susceptibility was assessed by calculating the odds ratios. P-values of less than 0.05 were considered to be significant. Statistical analysis was performed using SPSS 16.0 for Windows.

RESULTS

We generated the genotype of total 120 patients with type-2 diabetes mellitus, there was 40 patients for each groups for macro-albuminuria, micro-albuminuria, and normo-albuminuria. Demographic, clinical, and laboratorial characteristics of all patients are shown in **Table 1**. There were no significant difference among groups' demographic and clinical characteristics. The patients with DN, both micro and macro-albuminuria, had significantly lower hemoglobin level. They also tend to have significantly higher level of serum creatinine.

The allele frequency and the genotype distribution of A1166C polymorphism in the groups of patient according to the clinical profile (macro-albuminuria, micro-albuminuria, and normo-albuminuria) are shown in **Table 2**. The heterozygote genotype was found to be significantly higher in patients with micro-albuminuria compared to normo-albuminuria. There was no CC genotype found. Based on

their clinical diagnosis as shown in **Table 3**, it was found that AC genotype (OR 3.2 [1.01-10.08], p=0.03) and C allele (OR 2.8[0.95-8.67], p=0.038) were significantly higher in DN.

DISCUSSION

According to our knowledge, this study was the first to investigate AT1R polymorphisms in the Indonesian with type-2 diabetes, specifically in the Malay ethnic group. The corroboration for the role of this gene in the pathophysiology of DN comes from studies in diabetics who had mutated C allele of A1166C AT1R that asserted the infusion of angiotensin II which leads to increased filtration fraction,[10,19] an early pathogenesis hallmark of micro-albuminuria stage of DN.

AT1R is a G-coupled protein that is found mostly on kidney and systemic vasculature.[6] It mediates the effects of angiotensin II on vasoconstriction and cell transport of molecules. In the glomerulus of the kidney, the expression of AT1R was localized mostly in the podocytes.[20] An in vivo study on nephropathy-induced female Brown Norway mice observed an overexpression of AT1R correlated to proteinuric state.[21] The study also highlighted the inhibition of AT1R which improved the proteinuria mainly caused by the reduction of functional protein on slit diaphragm. Glomerular slit diaphragm is a membrane-like structure of glomerular basal membrane that functions as a barrier which averts

Table 1. Characteristics of the patients

Variables	With DN		Without DN	p
	Micro-albuminuria	Macro-albuminuria	Normo-albuminuria	
Sex, n (%)				
- Male	12 (30)	15 (37.5)	15 (37.5)	0.721
- Female	28 (70)	25 (62.5)	25 (62.5)	
Diabetes duration (years), mean (SD)	5.98 (3.20)	7.98 (3.81)	6.57 (4.09)	0.052
Age (years), mean (SD)	52.60 (9.19)	54.80 (5.81)	54.03 (4.89)	0.352
Body Mass Index (kg/m^2), mean (SD)	21.28 (1.43)	21.53 (2.33)	21.86 (1.65)	0.372
SBP (mmHg), mean (SD)	135.75 (11.07)	137.50 (15.48)	126.27 (24.01)	0.012*
DBP (mmHg), mean (SD)	83.25 (7.64)	85.75 (9.0)	81.75	0.92
Hemoglobin (g/dl), mean (SD)	12.35 (0.84)	12.54 (0.97)	12.95 (1.24)	0.032*
HbA1C (%), mean (SD)	8.91 (2.11)	8.76 (2.34)	7.86 (1.94)	0.062
Blood Urea (mg/dl), mean (SD)	31.58 (11.34)	33.20 (12.42)	28.75 (8.09)	0.182
Creatinine (mg/dl), mean (SD)	0.73 (0.19)	0.92 (0.18)	0.64 (0.11)	0.002*

Note: SBP, systolic blood pressure; DBP, diastolic blood pressure; 1fisher exact test; 2one way ANOVA; *p<0.05

Table 2. Allele frequency and genotype distribution of A1166C polymorphisms of AT1R by clinical profile

	Normo-albuminuria n=40 (N)	Micro-albuminuria n=40 (Mi)	Macro-albuminuria n=40 (Ma)	p	OR (95% CI)
Genotype Distribution					
- AA	36 (90)	29 (72.5)	30 (75)	0.04*	Mi to N = 3.41 (1.03-11.86)
- AC	4 (10)	11 (27.5)	10 (25)	0.07	Ma to N = 3.01 (0.85-10.54)
- CC	0 (0)	0 (0)	0 (0)	0.5	Mi to Ma = 1.13 (0.41-3.08)
Allelic Frequency					
- A	76 (95)	69 (86.25)	70 (87.5)	0.05	Mi to N = 3.02 (0.92-9.95)
- C	4 (5)	11 (13.75)	10 (12.5)	0.08	Ma to N = 2.7 (0.81-9.04)
				0.5	Mi to Ma = 0.89 (0.35-2.24)

Note: chi square test, *p=<0.05; Mi: Micro-albuminuria; Ma: Macro-albuminuria; N: Normo-albuminuria. 1Odds Ratio for heterozygote AC genotype/ mutant C allele between selected groups.

Table 3. Allele frequency and genotype distribution of A1166C polymorphisms of AT1R by clinical diagnosis

	DN, n=80	Without DN, n=40	p	OR (95% CI)
Genotype Distribution				
- AA	59 (73.8)	36 (90)	0.03	3.2 (1.01-10.08)
- AC	21 (26.2)	4 (10)		
- CC	0 (0)	0 (0)		
Allelic Frequency				
- A	139 (86.9)	76 (95)	0.038	2.8 (0.95-8.67)
- C	21 (13.1)	4 (5)		

Note: chi square test, *p=<0.05; 1Odds Ratio for heterozygote AC genotype/ mutant C allele.

plasma proteins leakage into urine.[20] In patients with DN, the slit diaphragm damage is a focus of novel targeted therapy.[22]

Previous studies found that A1166C allele frequencies varied widely, depending on ethnic heterogeneity. Moradi et al[13] reported the prevalence of the C allele in Iranian (15.3%). Our study did not find any mutant homozygote CC genotype. Doria et al[18] which conducted a research in USA and Fradin et al[12] which was conducted in Caucasian French found 9% and 3% CC genotype respectively. Our study found that the mutated C allele frequency was 13.1% in patients with type-2 DN, which was similar to the studies in Iranian type-2 DN patients (13.2%)[13] and lower than that observed in French Caucasian (23.9%).[12]

Our study showed that significant difference in genotype distribution was only found between micro-albuminuria and normo-albuminuria, and there was no significant difference in macro-albuminuria. Altogether, these findings are interpreted to support the hypothesis that the A1166C polymorphisms might play a role in the early pathogenesis of DN.[10,11] There was also no significant difference in allele frequency among three groups. Similar findings were identified in study by Chang et al[14] which found no significant difference in allele frequency among normo-, micro-, and macro-albuminuria Taiwan Chinese type-2 DN patients.

A different findings were identified, when comparing heterozygote AC genotype and C allele distribution in DN to non-DN group. Heterozygote AC genotype and C allele was 3.2 times and 2.8 times in DN. These findings support the hypothesis that A1166C polymorphism was a risk factor for the susceptibility of type-2 diabetics to DN. A similar result was found in French Caucasian.[12] Intriguingly, different results were found in two other studies in Asian[13,14] which identified null association between A1166C polymorphism and type-2 DN.

There is a chance that the negative findings in mutant homozygote CC genotype is the caused of a selective loss in this study. Further studies in larger sample size is needed. In spite of this limitation, our study was the first published attempt to elucidate the association between AT1R polymorphism and DN in Indonesian population which provides definitive support for the role of AT1R polymorphism in type-2 DN.

CONCLUSION

In conclusion, A1166C AT1R gene polymorphism is a risk factor for DN among Indonesian Malay type-2 diabetics. Our study also indicated that A1166C AT1R polymorphism could play a role in early pathogenesis of DN.

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