



Genetic variant of TGF- β associated with decreased renal function in type II diabetes mellitus patient: single center pilot study in Indonesia

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ABSTRACT

Submitted: 2020-06-04
Accepted : 2020-12-28

The interaction between genetic factors, blood glucose and hypertension plays a role in the onset of diabetic kidney disease (DKD) in type II diabetes mellitus (T2DM). Genetic variation of TGF- β 1 is associated with renal complication in T2DM with varying results between ethnicities. The Jambi Malay ethnic, which is the majority ethnic in Jambi Province, is an area that reports an increased prevalence of T2DM with DKD as the most frequent microvascular complications. In addition, previous study reported controlling blood glucose not associated with DKD indicating genetic may have play a role in DKD in this population. Studies related to genetic variation and decreased kidney function in T2DM patients has never been performed in this ethnic group. This study aimed to investigate the role of TGF- β genetic variation as risk factor for decreased renal function in T2DM patients from Jambi Malay ethnicity. We conducted a cross sectional study involving 70 patients with T2DM. The inclusion criteria for renal complication based on a decrease in the glomerular filtration rate (GFR) of less than 60 mL/min/1.73. The genotyping method used was amplification refractory mutation system polymerase chain reaction (ARMS-PCR) for TGF- β 1 rs1800470 T/C. Bivariate and multivariate analysis was performed to analyze phenotype and genotype association. The result of bivariate analysis showed T2DM patients with genotype CT (p:0.006; OR:0.125; 95% CI:0.027-0.575) and CC (p:0.007; OR:0.104; 95% CI:0.020-0.546) or C allele carrier (p:0.003; OR:0.117; 95% CI:0.027-0.500) had lower risk for decreased renal function than TT genotype. Multivariate analysis that included blood pressure and age variables showed the same finding for CT (p:0.007; OR:0.086; 95% CI:0.014-0.508) and CC genotype (p:0.022; OR:0.115; 95% CI:0.018-0.731). It is concluded from this study that T2DM patients with genotype CT, CC and carrier allele C have a lower risk for suffering kidney complications than genotype TT.

ABSTRAK

Interaksi antara faktor genetik, kadar gula darah dan hipertensi berperan dalam timbulnya komplikasi penyakit ginjal diabetes (PGD) pada pasien diabetes melitus tipe II (DMT2). Variasi genetik TGF- β 1 berhubungan dengan komplikasi ginjal pada pasien DMT2 dengan hasil yang bervariasi antar etnik. Etnik Melayu Jambi yang merupakan etnik mayoritas di Provinsi Jambi, merupakan daerah yang melaporkan peningkatan prevalensi DMT2 dan penyakit ginjal diabetes sebagai komplikasi mikrovaskular terbanyak. Penelitian sebelumnya juga melaporkan kontrol gula darah tidak berkaitan dengan kejadian PGD. Hal ini mengindikasikan kemungkinan peran variasi genetik pada kejadian PGD dipopulasi ini. Penelitian terkait variasi genetik dan penurunan fungsi ginjal pada pasien DMT2 belum pernah dilakukan pada etnis ini. Tujuan penelitian ini adalah mengkaji hubungan antara variasi genetik TGF- β 1 dengan kejadian PGD pada etnis Melayu Jambi. Penelitian potong lintang ini melibatkan 70 pasien DMT2. Kriteria inklusi penurunan fungsi ginjal berdasarkan penurunan laju filtrasi glomerulus (GFR) kurang dari 60 mL/min/1.73. Metode genotyping yang digunakan adalah *Amplification Refractory Mutation System Polymerase Chain Reaction* (ARMS-PCR) untuk TGF- β 1 rs1800470 T/C. Analisis bivariat dan multivariat dilakukan untuk menganalisis hubungan fenotipe dan genotipe. Hasil analisis bivariat menunjukkan subjek DMT2 dengan genotipe CT (nilai p:0,006; OR:0,125; 95% CI:0,027-0,575) dan CC (nilai p:0,007; OR:0,104; 95% CI:0,020-0,546) memiliki risiko yang lebih rendah dalam penurunan fungsi ginjal dibandingkan genotipe TT. Analisis multivariat model regresi logistik yang memasukkan variabel tekanan darah dan usia juga menunjukkan hasil yang sama untuk CT (nilai p:0,007; OR:0,086; 95% CI:0,014-0,508) and CC (nilai p:0,022; OR:0,115; 95% CI:0,018-0,731). Dapat disimpulkan bahwa subjek DMT2 dengan genotipe CT, CC dan karier alel C dari TGF- β 1 rs1800470 memiliki risiko lebih rendah menderita komplikasi ginjal dibandingkan genotipe TT.

Keywords:
decreased renal function;
diabetic kidney diseases;
genetic variant;
TGF- β 1;
type 2 diabetes mellitus;

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INTRODUCTION

The increasing prevalence of type II diabetes mellitus (T2DM) worldwide including in Indonesia leads to increasing diabetic kidney diseases (DKD). Diabetic kidney diseases caused decline renal function, which burden patient quality of life and health cost.^{1,2} Based on Indonesian National Health Surveillance, Jambi Province is one of regions in Indonesia that reported the phenomenon. The prevalence of T2DM had increased from 1.1 % in 2013 to 1.4 % in 2018.³ Diabetic kidney diseases prevalence is higher than other microvascular complication in this region.⁴

In many studies across the world population, controlling blood glucose level and blood pressure as modifiable risk of DKD does not always prevent the onset of decreased renal function in T2DM patients.⁵ The study in Malay Jambi ethnic reported similar result.⁶ The new biomarker and genetic variant may play role in this event. Genetic factors are risk factors that cannot be modified, but early screening of these factors can provide information about the pathophysiology of DKD and promises hope for better treatment and prognosis.^{5,7}

Hyperglycemia in DM induced oxidative stress and chronic inflammation which induced transforming growth factor beta (TGF- β) expression. The TGF- β 1 cause deposition of extra-cellular matrix leads to renal fibrosis and decreased renal function. Previous study reported that TGF- β 1 level is the marker for degree of progressive decreased in renal function in T2DM.⁸⁻¹⁰

Genetic variation of TGF- β 1 rs1800470 T/C is missense mutation. The mutation cause changes of amino acid, in which Leucine substitute to Proline in codon 10 of TGF- β 1 gene. The substitution associated with alteration of TGF- β 1 level or function.^{11,12} In line to the functional study, this genetic variant associated with risk of chronic kidney diseases (CKD) and end stage renal disease (ESRD)

for all causes include microvascular complication of T2DM. This is unique because the study reported difference in allele risk for CKD and ESRD, which may be influenced by ethnic factors.¹³⁻¹⁹

Previous study reported higher prevalence of DKD than other microvascular complication in T2DM, blood glucose level as modifiable risk factor cannot always be used as clinical marker to prevent the progressive decreased renal function in DKD among Jambi Malay population. It may indicate that the genetic variants have play role as risk for that event in this population. Furthermore, to the best of our knowledge association of TGF- β genetic variation rs1800470 T/C with decreased renal function in T2DM patient has never been conducted in Jambi Malay population. This study aimed to investigate the role of genetic variation in the incidents of decreased renal function in T2DM patients in Jambi Malay population.

MATERIALS AND METHODS

Study design

This study was approved by the Ethic Research Committee, the Faculty of Medicine and Health Sciences, Universitas Jambi with number 2163/UN21.8/PG/2020. This research was a cross sectional study involving 70 patients with T2DM. The inclusion criteria were patients who had suffered from T2DM for at least 5 years, age 35-67 years old and Jambi Malay ethnic. All subjects signed an informed consent. The exclusion criteria were patient who had suffered from urinary tract infection based on clinical examination and routine urine analysis, had a history of other renal diseases, pregnant women and immunocompromised patients.

Patients with T2DM were diagnosed based on plasma fasting blood glucose >126 mg/dL and or 2 h post prandial plasma glucose > 200 mg/dL as listed in medical records. We also measured serum creatinine and blood pressure in

all subjects. All subjects signed informed consent forms after receiving detailed explanation about the study objectives and design. The research protocol was arranged based on the declaration of Helsinki.

Blood pressure and laboratory 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 min. Subjects with a 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 10 h of fasting. This blood sample was used to measure serum creatinine levels and fasting blood glucose plasma levels. The 2 h post prandial plasma glucose was measured 2 h after glucose loading (75 g glucose loading). The plasma glucose level 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 2 h post prandial plasma fasting glucose ≥ 180 mg/dL were classified as uncontrolled plasma 2 h post prandial plasma fasting glucose.²⁰

The decreased of renal function was determined based on GFR less than 60

mL/min/1.73. The GFR was calculated based on chronic kidney disease epidemiology collaboration (CKD-EPI) equation, based on estimated creatinine serum, gender equation coefficient and age. Serum creatinine levels were also measured with enzymatic colorimetric Jaffe methods performed by PRODIA laboratories.

Genotyping and data analysis

Deoxyribonucleic acid (DNA) was extracted from peripheral vein buffy coat using commercial DNA blood extraction kit from MacroGen®. Quality and quantity of DNA were measured using nanodrop with nanodrop index at least ~ 1.8 . Genotyping was performed by two step tetra Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) which showed general product and allele specific product. Primer design was adapted from Perrey *et al.*²¹ Primer sequences and its product are showed in TABLE 1.

The PCR mixture were 5 μ M generic primer; 5 μ M C allele or T allele specific primer; 5 μ M for each internal control primer P53F (Genetica Sciences®); 12,5 μ L PCR master mix (Go taq green, Promega®); nuclease free water 8, μ L; DNA template 1 μ L. The thermocycler (Biorad®) condition was 95 °C for 7 min as initial denaturation and 1 min as denaturation; 60 °C for 1 min as annealing; 72 °C as extension for 1 min and 7 min as final extension. The PCR product then visualized with 2.5% agarose gel for 45 min with 100 mV.

TABLE 1. Primer and the product size

Primer		Fragment
Generic primer (sense/reverse primer)	5´-TCCGTGGGATACTGAGACAC-3´	
C allele specific primer (antisense/forward primer)	5´-GCAGCGGTAGCAGCAGCG-3´	241 bp
T allele specific primer (antisense)	5´-AGCAGCGGTAGCAGCAGCA-3´	241 bp
Internal control primer 1 (P53F)	5´-TGCCCTGTGCAGCTGTGGGTTGATT-3´	
Internal control primer 2 (P53R)	5´-GCCCCAGCTGCTCACCATCGCTATC-3´	

In silico analysis was performed to measured primer sequences and the size of its 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 internal primer band appearance, nihil band appearance in control negative (no DNA template was added in control negative PCR mixture) and certain fragment size of PCR product for allele. As much 10% of sample was performed twice for genotyping to ensure the result and all the second genotyping process showed consistency with the first one.

Bivariate analysis test was performed for baseline subject characteristic 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

Baseline subject characteristic

A total of 70 patients who met research criteria were involved in this study. Grouping of subjects based on GFR (cut off point for GFR) was less than 60 mL/min/1.73. Subjects characteristic are showed in TABLE 2. Patients T2DM with decreased renal function were older and had lower GFR than patients T2DM without decreased renal function.

TABLE 2. Baseline subject characteristic

Characteristic	T2DM with decreased renal function (n=22)	T2DM without decreased renal function (n=48)	p
Age (years)	54.22±5.55	49.39±7.91	0.012*
Glomerular filtration rate (mL/min)	36.33±14.56	94.67±19.58	<0.001*
Gender			
• Male	10	18	0.528
• Female	12	30	
Blood pressure (mmHg)			
• Uncontrolled blood pressure	10	6	0.002*
• Controlled blood pressure	12	42	
Fasting plasma glucose (mg/dL)			
• Uncontrolled FPG	13	26	0.700
• Controlled FPG	9	22	
2 h PP plasma glucose (mg/dL)			
• Uncontrolled 2 hours PP plasma glucose	19	41	0.916
• Controlled 2 PP plasma glucose	3	7	

Numeric scale data were analyzed using t-test; All the numeric data were normally distributed; Chi-square test was performed for categoric scale; *Statistically significant, $p < 0.05$; DM refers to diabetes mellitus; FPG refers to fasting plasma glucose; PP refers to post prandial.

We found a higher frequency of controlled blood pressure was in T2DM without a statistically significant decrease in renal function. The frequency of uncontrolled fasting and blood glucose 2 h post prandial was higher in DM without decrease in renal function, but the difference was not statistically significant (TABLE 2).

Genotype distribution

The PCR product visualization is showed in FIGURE 1. The T and C size of allele fragment is 241 bp, the difference based on specific primer for each allele. Internal control for PCR reaction is the appearance of p53 fragment product (180 bp).

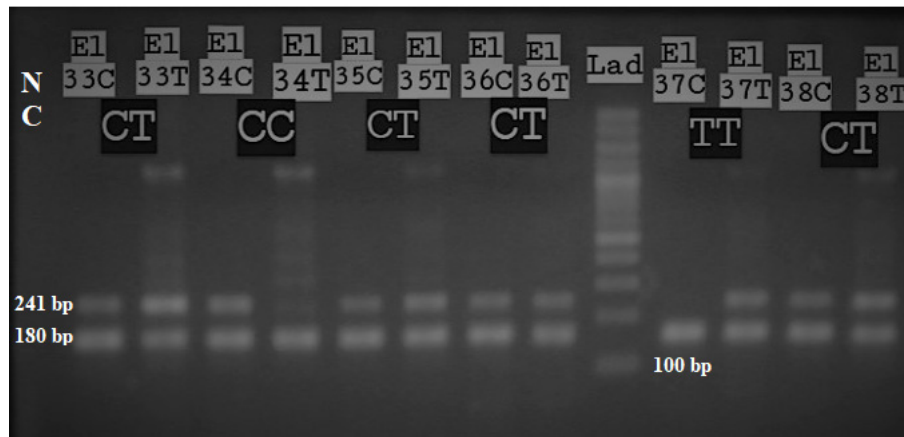


FIGURE 1. Two step electrophoresis of ARMS-PCR TGF- β rs1800470. The size of allele C and T fragment were 241 bp, the lowest fragment was internal control p53 (180 bp). Sample number 33, 35, 36, 38 had heterozygote CT. Sample number 34 had homozygote CC and sample number 37 had homozygote TT. Negative control (NC) before sample 33 showed no band appearance.

TABLE 3. Genotype distribution

Genotype	Observed value	Expected value	X ² (DF)	p	MAF
CC	23	24			
CT	36	34	0.25	0.62	0.41
TT	11	12			

X²value with degree of freedom (DF)=1; MAF=minor allele frequency

Genotype distributions in our population are showed in TABLE 3. Genotype of TT was reported as polymorphic genotype in the population. Percentage of minor allele in our

population was 41%, T allele was the minor allele. The proportion of genotype frequency in our studies were not deviated from Hardy Weinberg equation, $p > 0.05$ (TABLE 3).

Association of genotype with decreased renal function

TABLE 4 shows the result of bivariate analysis. The frequency of subjects who had CC and C allele were lower in T2DM with decreased renal function group. The frequency of subject who had CT,

CCCT genotype were higher in T2DM without decreased renal function group. This difference is statistically significant.

Odds ratio value showed CT, CC, CCCT genotype and C allele had lower susceptibility for suffering decreased renal function. This association was statistically significant (TABLE 4).

TABLE 4. Bivariate analysis of association between genotype and decreased renal function

Genotype	T2DM with decreased renal function (n=22)	T2DM without decreased renal function (n=48)	p	OR (95% CI)
Additive model				
TT	8 (36.4)	3 (6.3)	ref	
CT	9 (40.9)	27 (56.3)	0.006 ^{b*}	0.125 (0.027-0.575)
CC	5 (22.7)	18 (37.5)	0.007 ^{b*}	0.104 (0.020-0.546)
Recessive/dominant model				
CTTT	17 (77.3)	30 (62.5)	ref	
CC	5 (22.7)	18 (37.5)	0.172 ^a	0.490 (0.154-1.557)
TT	8 (36.4)	3 (6.3)	ref	
CCCT	14 (63.6)	45 (93.8)	0.003 ^{b*}	0.117 (0.027-0.500)
Allele				
T	25 (56.8)	33 (34.4)	ref	
C	19 (43.2)	63 (65.6)	0.012 ^{a*}	0.398 (0.192-0.826)

^aChi square; ^bFisher exact test was performed; *Statistically significant, p < 0.05; OR refers to odds ratio; CI refers to confident interval; Ref refers to reference genotype.

TABLE 5. Multivariate analysis of association between genotype and decreased renal function

Variable	B	SE	Adjusted p	Adjusted OR	95% CI
Additive model					
TT		ref			
CT	-2.458	0.908	0.007	0.086	0.014-0.508
CC	-2.163	0.944	0.022	0.115	0.018-0.731
Uncontrolled blood pressure	1.557	0.691	0.024	4.744	1.223-18.396
Age	0.122	0.057	0.034	1.129	1.009-1.264
Recessive model					
TT		ref			
CCCT	-2.335	0.848	0.006	0.097	0.018-0.510
Uncontrolled blood pressure	1.518	0.681	0.026	4.565	1.203-17.327
Age	0.118	0.056	0.036	1.126	1.008-1.258

Hosmer and Lemeshow test was performed to analyzed the good fitness in both model; p for all model 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.

Multivariate analysis with logistic regression model showed consistent trends with bivariate analysis. The subject with CT, CC, and CCCT had lower susceptibility for suffering decreased renal function than TT genotype. The genetic OR as a protective factor in multivariate analysis was lower than OR in bivariate analysis when adjusted for uncontrolled blood pressure and increasing age. The association was statistically significant. In addition, uncontrolled blood pressure and increasing age also increase The susceptibility to decreased renal function in T2DM (TABLE 5).

DISCUSSION

The age, duration of suffering T2DM, blood pressure, and blood glucose level are classic risk factors that play a role in decreased renal function in T2DM patients. Controlled blood pressure and controlled blood glucose level do not always prevent this complication Genetic factors thought to be involved.^{2,5,22} Our

research reported the older age, blood pressure, and TGF- β genetic variant were associated with decreased renal function in T2DM.

Uncontrolled blood pressure in T2DM patients increases glomeruli capillary pressure, mechanical stress of mesangial cell, podocytes, and RAAS activation. It induces a concerted release of proinflammatory and profibrotic cytokine including TGF- β , which accelerates the progression of decreased renal function.⁵ Higher frequency of uncontrolled blood glucose level in T2DM patients without decreased renal function may indicate that there are other factors that played role in T2DM with decreased renal function. One of those factors is the involvement of genetic factors, which was also found in this study. The TGF- β genetic variant rs1800479 is one of the risk factors for decreased renal function in T2DM.

In our population, the polymorphic genotype showed opposite than the association of phenotype-genotype studies.¹³⁻¹⁹ We reported TT genotype

and T allele as polymorphic form in this study. This difference may be influenced by ethnicity and the difference of subject criteria for recruitment. Although our research sample was small in number, the observed value of our genotype did not deviate from Hardy-Weinberg equation. We believe that our genotyping method has a very small bias in addition to the Hardy-Weinberg law assumptions for population genetic.

Genotype and phenotype study in our population reported the statistical significance of CT, CC genotype, and C allele carrier of TGF- β rs1800470 as protective risk for decreased renal function both in bivariate and multivariate analysis. Previous study reported this genetic variant associated with CKD and ESRD for all caused include T2DM renal complication. The difference allele risk was found between those studies. Studies in Spanish and German population report that the TT genotype was more susceptible to ESRD than CC and TC genotype for all caused than another genotype. Same linkage trends was also found in ESRD caused by microvascular complication of T2DM.^{13,14} Our study also reported similar genotype risk.

In contrast to the result of several studies in in Egypt, India, and Mexican which reported that C allele and CC genotype increased risk of suffering diabetic nephropathy with or without ESRD in T2DM patient.¹⁵⁻¹⁷ One meta-analysis of 9 case-control studies reported the CC genotype of this genetic variant associated with higher risk of CKD in Asian.¹⁸ Another recent meta-analysis study in Chinese population reported CC genotype increased risk of suffering T2DM renal complication by 1.14-1.67 than other genotype.¹⁹

The TGF- β 1 rs1800470 T/C genetic variant is an exon variant that plays a role in protein coding. This missense mutation causes the amino acid to code Leucin to proline, different amino acid changing polarity of the protein and

influence the trafficking or exporting the intracellular protein.²³ Previous study also reported the difference genotype associated with different TGF- β 1 level.^{11,12} TGF- β 1 level associated with T2DM renal complication through its role as inducer of excess extracellular matrix cause glomerulosclerosis and lead to decreased renal function.⁸⁻¹⁰ This may explain contribution of this genetic variant as risk for decreased renal function in T2DM.

Genotyping methods used for TGF- β 1 rs1800470 T/C genetic variant was two steps ARMS PCR. This technique allows fast, reliable and low cost for SNP genotyping than PCR-RFLP or HRM-PCR.^{24,25} this is the kind of a proper genotyping technique that can be used in limited laboratory resources. This method uses generic primer and specific allele primer. The quality control of PCR reaction is based on visualization of PCR product of p53 gene (one of house-keeping gene) and negative control for contamination exclusion, then 10% of sample genotyping twice. The primer was adapted from Perrey *et al.*²¹ then *in silico* analysis was performed to measure the primer sequences and PCR product size. PCR condition optimization was based on our laboratory resources.

Limitation of this study was single center pilot study and single genetic variant measurement which influenced decreased renal function of T2DM patient. Further research with larger sample, multicenter, more comprehensive genetic and nongenetic analysis are needed to determine this genetic variant as genetic screening for decreased renal function in T2DM patient. Genetic screening serves as a promising step that can promote the better treatment and prognosis.

CONCLUSION

We report that T2DM-affected subjects who carry the TC, CC, or C allele of TGF- β 1 rs1800470 have a lower

susceptibility to suffering decreased renal function. Further research with larger samples and comprehensive analysis of genetic and non-genetic factor are needed to strengthen the role of this genetic variant as risk for decreased renal function in T2DM.

ACKNOWLEDGEMENTS

We extend our thanks to the Faculty of Medicine and Health Sciences, Universitas Jambi for funding support of this research through “Penelitian Dana PNPB *number: 197/UN21.18/PG/SPK/2020 Skema Dosen Pemula*”. We also thank all the laboratory technicians of Faculty of Medicine and Health Sciences, Universitas Jambi and Prodia for helping in metabolite and genotyping measurement.

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