

## Interleukin-6 gene polymorphism (rs1800796) in patient with diabetic nephropathy among Balinese

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Original Article

### ABSTRACT

#### ARTICLE INFO

##### Keywords:

diabetic nephropathy,  
interleukin-6,  
rs1800796,  
gene polymorphism,  
PCR-RFLP

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DOI: 10.20885/JKKI.Vol14.Iss3.art9

##### History:

Received: July 19, 2023

Accepted: December 8, 2023

Online: December 30, 2023

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**Background:** Diabetic Nephropathy (DN) is the most common complication of diabetes mellitus. Interleukin 6 (IL-6) known as pro-inflammatory cytokine increases extracellular matrix expression and proliferation in DN progression. One single nucleotide polymorphism (SNP) of the Interleukin (IL)-6 gene, rs1800796 play a role in the progression of DN among Asian.

**Objective:** This research seeks to investigate the correlation between IL-6 gene polymorphism (rs1800796) and diabetic nephropathy in individuals diagnosed with type 2 diabetes mellitus (T2DM) among the Balinese population.

**Methods:** This research employed an observational approach through a case-control design involving a total of 60 subjects for each group selected by a simple random sampling method. The occurrence of the rs1800796 polymorphism was identified through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The genotype and allele distribution acquired in this study were assessed using the chi-square ( $\chi^2$ ) test and Hardy-Weinberg equilibrium law.

**Results:** There were no significant differences identified in the occurrence of (DN) based on the genotype or allele distribution of the IL-6 gene rs1800796. Genotype CG-GG had a higher risk of becoming DN with Odd ratio 1.230, but not statistically significant ( $p=0.577$ ). Allele G with OR 1.198 ( $p=0.502$ ) was not significant in causing DN.

**Conclusion:** No significant association was identified between IL-6 polymorphism (rs1800796) and diabetic nephropathy in Balinese individuals with type 2 diabetes mellitus. The lack of association could be due to the presence of other haplotypic connections or might require a larger sample size for conclusive results.

**Latar Belakang:** Nefropati Diabetes (ND) adalah komplikasi umum yang muncul dari diabetes mellitus. Interleukin 6 (IL-6), yang dikenal sebagai sitokin proinflamasi, meningkatkan ekspresi matriks ekstraseluler dan proliferasi dalam perkembangan ND. Perkembangan ND dipengaruhi oleh suatu SNP tertentu, rs1800796, dalam gen IL-6 pada populasi Asia

**Tujuan:** Penelitian ini bertujuan untuk menyelidiki hubungan antara polimorfisme gen IL-6 (rs1800796) dan nefropati diabetes pada individu yang didiagnosis menderita diabetes mellitus tipe 2 di antara populasi Bali.

**Metode:** Penelitian ini menggunakan pendekatan observasional melalui desain kasus-kontrol dengan total 60 subjek untuk setiap kelompok yang dipilih dengan metode pengambilan sampel acak sederhana. Keberadaan polimorfisme rs1800796 diidentifikasi melalui teknik PCR-RFLP. Distribusi genotipe dan alel yang diperoleh dalam penelitian ini dievaluasi menggunakan uji chi-square ( $\chi^2$ ) dan hukum keseimbangan Hardy-Weinberg.

**Hasil:** Tidak ada perbedaan signifikan yang diidentifikasi dalam kejadian ND berdasarkan genotipe atau distribusi alel dari gen IL-6 rs1800796. Genotipe CG-GG memiliki risiko lebih tinggi untuk mengalami ND dengan OR 1.230, tetapi tidak signifikan secara statistik ( $p=0.577$ ). Alel G dengan OR 1.198 ( $p=0.502$ ) tidak signifikan

dalam menyebabkan ND.

**Kesimpulan:** Tidak ditemukan hubungan signifikan antara polimorfisme IL-6 (rs1800796) dan nefropati diabetes pada individu Bali dengan diabetes mellitus tipe 2. Kurangnya asosiasi ini bisa disebabkan oleh adanya koneksi haplotipik lain atau mungkin memerlukan ukuran sampel yang lebih besar untuk hasil yang konklusif.

## INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder group distinguished by increased levels of blood sugar, resulting from abnormalities in both the secretion and effectiveness of insulin. Diabetes mellitus is becoming one of the global health threats because the cases are increasing every year. There were 422 million adults worldwide with diabetes in 2014.<sup>1</sup> Diabetic nephropathy is among the frequently encountered complications associated with diabetes mellitus. About 30-40% of people with DM will develop DN.<sup>2</sup> Recent studies have shown that an inflammatory process involving various cytokines plays a role in disease progression towards DN.<sup>3</sup> Interleukin-6 is a pro-inflammatory cytokine that has a direct impact on the thickening of the glomerular basement membrane, which is a lesion that appears in DN.<sup>2</sup>

One of the mechanisms for regulating inflammatory cytokines in the kidneys of DM patients that is currently being studied is genetic polymorphism. Genetic variations are found in genes that encode inflammatory cytokines, resulting in gene sensitivity to DN by changing its function or expression.<sup>4</sup> There are three polymorphisms in IL-6 that are thought to be associated with DN; SNP rs1800795, rs1800796, and rs1800797.<sup>5</sup> The distribution of gene polymorphisms differs across populations, influenced by factors such as race and ethnicity. In several previous studies, IL-6 gene polymorphisms in SNP rs1800796 influenced the progression of DN in Asian populations.<sup>6,7</sup> The SNP rs1800796 is one of the SNPs located in the IL-6 gene that has the largest proportion in several studies on DN conducted in Asia. The Asian population in these studies is the closest race/ethnicity to the Indonesian population, particularly in Bali.<sup>6,7</sup> Because the difference of races and ethnic groups between Indonesia and other Asian, also the wide variety of ethnic group in Indonesia itself, examining the association between IL-6 gene polymorphism (rs1800796) and diabetic nephropathy among individuals

with T2DM in the Balinese population is deemed essential.

## METHODS

### Study design

This research followed an observational methodology, utilizing a case-control design. This study conducted from January to April 2021 in Biomolecular Laboratory, Faculty of Medicine and Health Science, Warmadewa University.

### Subject and sampling

This case-control study used 60 subjects in each group. The sample used in this study was a Deoxyribo Nucleic Acid (DNA) archive from previous study, which selected using simple random sampling method.<sup>8</sup> Complete data was required for inclusion, while exclusion was based on insufficient DNA quantity for genetic analysis. The case group included type 2 diabetes mellitus patients that has urine albumin-to-creatinine Ratio (uACR)  $\geq 300$  mg/dL and more than 5 years duration of diabetes. The control group consisted of individuals with type 2 diabetes mellitus, characterized by a uACR of  $<300$  mg/dL and a diabetes duration exceeding 5 years.

### Genotyping rs1800796

The SNP rs1800796 of the IL-6 gene was identified through the PCR-RFLP analysis technique. Gene target amplification was conducted using a forward primer 5'-GGAGACGCCTTGAAGTAACTGC-3' and a reverse primer 5'-GGGCTGACTCCATCGCAG-3'. The PCR reaction mixture for each sample contained 12.5  $\mu$ L of master mix Promega®, 1  $\mu$ L of forward primer, 1  $\mu$ L of reverse primer, 2  $\mu$ L of DNA, and 8.5  $\mu$ L of ddH<sub>2</sub>O. The PCR process was conducted using TaKaRa® PCR Thermal Cycler TP650. The DNA amplification commenced with an initial denaturation at 94°C for 5 minutes, followed by cycles of denaturation, annealing, and extension (94°C for 30 seconds, 58°C for 5 seconds, and 72°C for 30 seconds) repeated 37 times. The final extension occurred for 7 minutes at 72°C. Subsequently, post-PCR, each 10  $\mu$ L of PCR product underwent digestion using 1U of BsrBI (Thermo Scientific™) in a 30.5  $\mu$ L reaction volume at 26.3°C for 10 hours. The digestion outcomes were electrophoresed in a 2.0% agarose gel, stained with Invitrogen™ SYBR™ Safe DNA Gel Stain, and

visualized on a UV-transilluminator (Uvidoc HD5, Uvitec Cambridge®).

### Data analysis

The characteristics of the subjects were examined through univariate analysis. The association between the risk of developing diabetic nephropathy and the IL-6 polymorphism (rs1800796) was assessed using Chi Square and odds ratio, accompanied by a 95% confidence interval. The analysis was conducted using SPSS software for Windows 25 with p-value < 0.05 declared significant. Hardy-Weinberg analysis is employed to assess whether the alleles of SNPs within a population adhere to the Hardy-Weinberg equilibrium pattern, or if there are particular factors influencing the distribution of alleles and genotypes.

### Ethical clearance

The ethical clearance in this study provided by the Ethics Committee of The Faculty of Medicine, Udayana University (2490/UN14.2.2.VII.14/LT/2020) for human biological material stored in biomolecular laboratory.

### RESULTS

Table 1 displays the clinical features of diabetic patients. Both groups showed similarity in the distribution of age, history of DM, height, weight, Body Mass Index (BMI), blood glucose, systolic and diastolic blood pressure. However, a notable difference was observed in the prevalence of sex between the two groups ( $p = 0.003$ ). In subjects with diabetic nephropathy there were more males

than females, namely 44 males and 12 females. Whereas in control group the number of male subjects less than females, namely 28 males and 32 females.

The amplified PCR product of IL-6 gene rs1800796, with C alleles as the wild type had a molecular size of 154bp. After digestion by the enzyme, the 154bp yielded two bands at 94bp and 60bp and defined as mutant G alleles (Figure 1). The digestion products reveal prevalence of CC, CG, and GG genotypes in subjects with or without DN (Table 2). Genotypes distribution were in Hardy-Weinberg equilibrium.

Neither genotype distribution nor allele frequencies had significant association with DN shows in table 3. Mutant genotype (GG-CG) frequency with DN was about 52.1%, meanwhile wild type genotype (CC) frequency with DN was about 46.9%. However, no significant association was observed between the genotype and DN from the statistical analysis, with p value 0.577 (OR= 1.230, 95% CI = 0.593-2.550). Based on allele frequency, subject with G alleles that having DN was about 52.9% whereas subject with C allele that having DN is about 48.4%. The statistical analysis shows that allele might be a predictive factor of DN (OR 1.19, 95% CI= 0.707-2.029), but no significant association was found between alleles and the occurrence of DN statistically with  $p = 0.502$ .

### DISCUSSION

Diabetic nephropathy remains the most frequent complication arising from diabetes mellitus. The inflammatory process plays a

Table 1. Patient clinical characteristic

	Type 2 Diabetes Mellitus		p value
	Diabetic Nephropathy	Non-Diabetic Nephropathy	
Gender (M/F)	44/12	28/32	0.003
Age (year)	60.37 ± 10.40	59.77±8.67	0.819
History of DM (year)	10.93 ± 6.02	8.58±5.51	0.505
Height (cm)	164.27 ± 6.87	160.75±8.24	0.332
Weight (kg)	64.88 ± 11.80	63.22±9.91	0.329
BMI	24.02 ± 4.05	24.58±4.29	0.477
Systolic BP	138.01 ± 24.42	128.03±17.51	0.274
Diastolic BP	76.93 ± 10.85	76.42±8.52	0.237
Fasting blood sugar	150.72 ± 47.74	159.88±49.14	0.368

\*p values were calculated by independent t-test for continuous variables and by Pearson's chi square test for categorical variable. DM: Diabetes Mellitus; BMI: Body Mass Index; BP: Blood Pressure.

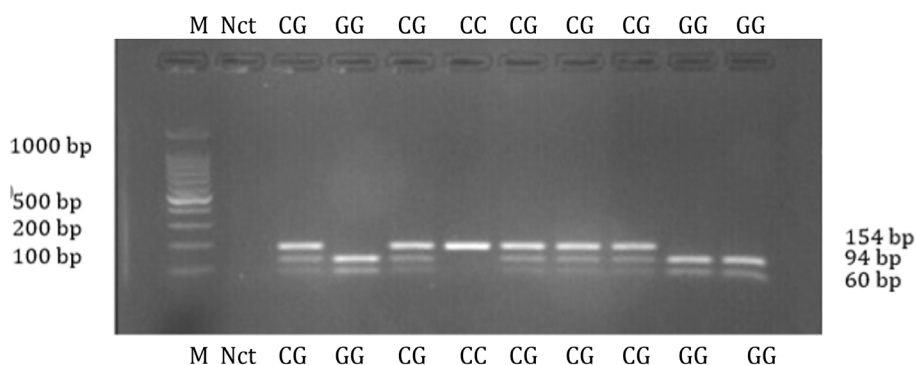


Figure 1. Results of RFLP electrophoresis of the IL-6 gene in patients with diabetic nephropathy using a DNA ladder with 100bp marker in 9 samples. RFLP: Restriction fragment length polymorphism DNA: deoxyribonucleic acid; M: marker; Nct: negative control; bp: base pairs. There is no restriction area in the fifth well. 3<sup>rd</sup>, 9<sup>th</sup> and 10<sup>th</sup> wells show a restriction area below 100bp

Table 2. Genotype distribution and Hardy-Weinberg equilibrium within case and control

Genotype	Type 2 Diabetes Mellitus		p value
	Diabetic Nephropathy	Non-Diabetic Nephropathy	
Wild Type (CC)	23 (38.3%)	26 (43.3%)	0.480
Mutant Heterozygote (CG)	28 (46.7%)	27 (45%)	
Mutant Homozygote (GG)	9 (15%)	7 (11.7%)	
Genotype	Observed	Expected	
CC	49	43	
CG	55	58	
GG	16	19	

Table 3. The association between IL-6 gene rs1800796 with diabetic nephropathy

Genotype	Type 2 Diabetes Mellitus		Odd Ratio	p value
	Diabetic Nephropathy	Non-Diabetic Nephropathy		
CG – GG	37 (52.1%)	34 (47.9%)	1.230	0.577
CC	23 (46.9%)	26 (53.1%)		
Allele			1.198	0.502
G	46 (52.9%)	41 (47.1%)		
C	74 (48.4%)	79 (51.6%)		

crucial role in the progression from DM to DN by increase production of chemokine, inflammatory cell infiltration to the kidney, pro-inflammatory cytokine production, and kidney damage.<sup>3,9</sup> the IL-6 is one of inflammatory cytokine causing glomerular basal membrane thickening which is a characteristic lesion found in DN.<sup>4</sup> High blood sugar level stimulate podocyte to produce IL-6. IL-6 will bind to its receptor, gp130 and IL-6R. This binding results in a autophosphorylation and structural changes from janus kinase (JAK). Thus will activate transcription factor STAT-3 to increase mesangial cell proliferation.<sup>8</sup>

In this study, the case and control sample conditions did not exhibit significant differences

in variables such as age, duration of diabetes, height, weight, systolic blood pressure, diastolic blood pressure, BMI, and fasting blood glucose levels. Although there was an apparent trend showing higher values in age, duration of diabetes, and blood pressure within the case group, these differences did not reach statistical significance. Older individuals, especially those aged 65 and above, are at a higher risk of experiencing DN. The natural aging process of the body results in a gradual decline in kidney function. When diabetes is also present in older individuals, aging kidneys become more vulnerable to the negative effects of prolonged hyperglycemia.<sup>10,11</sup> The duration of diabetes plays a significant role in the incidence

of kidney complications in T2DM. Prolonged exposure to hyperglycemia can result in damage to the small blood vessels in the kidneys, leading to kidney dysfunction. A Danish study conducted over 18 years reported that the overall occurrence of microalbuminuria, observed after 7.3 years, was 12.6% in patients with type 1 diabetes mellitus and 33% in those with type 2 diabetes mellitus.<sup>12</sup> Hypertension can activate the Renin-Angiotensin-Aldosterone System (RAAS), which can result in changes in renal blood flow and sodium retention. This can lead to gradual kidney damage.<sup>13</sup> Hypertension can disrupt endothelial function, the lining of blood vessels that regulates blood flow. Endothelial dysfunction can interfere with the regulation of blood flow and blood pressure in the kidneys, as well as causing inflammation and tissue damage.<sup>14</sup>

The difference finding between previous studies and this study might happen due to number of subjects observed and method dissimilarity. Several factors can impact the study's results, and this includes the intricate process of gene expression that may involve interactions among SNPs within a gene, post-translational modifications, and epigenetic factors. Sample characteristics, such as age, gender, blood pressure, BMI, fasting blood glucose levels, and blood glucose control in the form of HbA1c, can also impact the results.<sup>15-17</sup>

Several factors including the complex process of a gene causing DN might cause difference result in every subject. The IL-6 gene is a gene encoding the IL-6 cytokine. Genes will undergo a series of processes to produce proteins. Once produced, IL-6 will bind to the receptor and undergo a feedback mechanism so that podocytes are able to produce more IL-6. High level of serum IL-6 lead to podocyte hypertrophy and results as injury of the podocyte.<sup>13,14,18</sup> Other cytokines that also contribute to the progression of DN are Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), and IL-1. The TNF- $\alpha$  is co-produced with IL-6 by Monocyte Chemoattractant Protein-1 (MCP-1) activity. A study conducted in Bali shows significant increase of MCP-1 serum among patient with DN.<sup>9</sup> Cytokine TNF- $\alpha$ , IL-1, and IL-6 can cause mesangial cell proliferation, extracellular matrix accumulation, and glomerulosclerosis which are the stages of DN progression. Therefore, it is necessary to examine serum levels of IL-1, TNF- $\alpha$ , and IL-6 in subjects to control the confounding variables, namely IL-1 and

TNF- $\alpha$  which also contribute to the development of DN.<sup>19</sup>

The progression of diabetic nephropathy is also influenced by factors such as gender, blood pressure, and HbA1c concentration.<sup>20</sup> This study identified a significant difference between male and female subjects. The proportion of male subject with DN are higher than the female proportion. Decrease in total testosterone and free testosterone levels in male with type 1 or type 2 DM which can lead to decrease in arterial vasoreactivity and an increase production of pro-inflammatory cytokines. While women have estrogen hormone which can prevent the thickening of the glomerular basement membrane. This causes men are likely experiencing DN when compared to women.<sup>21</sup> In type 2 diabetic patient with hypertension are likely to become DN due to microvascular resistance. Thus will lead increase production of IL-6 via angiotensin II.<sup>22,23</sup> In this study, it was observed that the frequency of diabetic nephropathy did not show a significant difference based on the presence or absence of a history of hypertension.

In Asian populations, three SNPs were found to be associated with the incidence of DN such as rs1800796, rs1800795, and rs1800797. To determine the dominant proportion of IL-6 polymorphisms in Bali, it is necessary to conduct other study on each SNP.<sup>5,24</sup> The difference proportion of gene polymorphisms can be influenced by race and ethnicity. The ethnic composition and racial demographics in Indonesia are distinct from those found in China and Japan. Those might be causing difference proportion of polymorphisms.<sup>25</sup>

A study conducted in Thailand reveals a significant association between an increase in IL-6 serum levels and patients with type 2 diabetes.<sup>26</sup> Increase expression of IL-6 gene also found in DN induced rats.<sup>27</sup> This study found no significant association between IL-6 gene polymorphism rs1800796 and DN in patient with type 2 DM among Balinese. This study is in agreement with other study conducted in Bangladesh that found no significant association between G and C alleles with T2DM progression.<sup>28</sup> A similar study conducted in Boston also failed to find any significant association between IL-6 gene polymorphism rs1800796 in CC, CG, or GG genotype with DN among Caucasian.<sup>29</sup>

A study demonstrates an association between IL-6 rs1800796 polymorphism and DN in Japan

with 454 subjects observed using case-control method. It shows that the GG genotypes are significant in association with DN.<sup>6</sup> A prospective cohort study in China with over 1000 subjects observed shows significant association between GG genotypes and DN progression.<sup>7</sup> Significant finding of G alleles association with DN also showed from a meta-analysis study in Thailand.<sup>5</sup>

One of the strengths of this study lied in its case-control research design. Moreover, this study, carried out in Indonesia, is distinctive as one of the few investigations that involve a case-control study comprising type 2 diabetes mellitus patients, encompassing both those with and without diabetic nephropathy. However, our study came with several limitations. One of them was the sample size being relatively small, even though the results achieved a statistical power of 80% in both codominant and recessive genetic models. Furthermore, our findings may not be applicable to other ethnic populations. Additionally, we did not measure the inflammatory marker, IL-6. Therefore, we were unable to assess how these polymorphisms affect the expression of the IL-6 protein.

## CONCLUSION

Our studies revealed no significant association between IL-6 gene polymorphism rs1800796 and DN in patients with type 2 DM among Balinese. However, further investigations with larger population sizes are necessary to firmly establish the association of IL-6 gene polymorphism with DN and other SNPs analysis to know which SNPs are dominant in Balinese population.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ACKNOWLEDGEMENT

The authors would like to extend their gratitude to the Research Unit of the Faculty of Medicine and Health Sciences, Warmadewa University, for their support in providing the research funding. Additionally, the authors would like to express their appreciation to I Gusti Ngurah Dwija Putra, A.Md.Kes, for his assistance in conducting the PCR-RFLP analysis.

## AUTHOR CONTRIBUTION

Concepts or ideas, design, the definition of

intellectual content by AL; literature search, experimental study, data acquisition and data analysis by GAPRW; statistical analysis by SAA; manuscript preparation by AL; manuscript editing by PANKP; manuscript review by PNC

## LIST OF ABBREVIATIONS

DM: Diabetes Mellitus; DN: Diabetic Nephropathy; IL-6: Interleukin-6; SNP: Single Nucleotide Polymorphism; uACR: urinary albumin-creatinine ratio; JAK: janus kinase; RAAS: Renin-Angiotensin-Aldosterone System; BMI: Body Mass Index; HbA1c: glycated hemoglobin; TNF- $\alpha$ : Tumor Necrosis Factor- $\alpha$ ; MCP-1: Monocyte Chemoattractant Protein-1

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