International Journal of Research and Review
Vol. 10; Issue: 1; January 2023
Website: www.ijrrjournal.com
Original Research Article *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Patient Characteristics and Prevalence of Single Nucleotide Polymorphism (SNP) V16A MnSOD Gene in Diabetic Retinopathy Patients at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar Bali

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DOI: https://doi.org/10.52403/ijrr.20230170

ABSTRACT

Diabetic Retinopathy (DR) is a microvascular complication of Diabetes Mellitus (DM) caused by hyperglycemia and oxidative stress that is prevalent to the blindness risk. Manganese superoxide dismutase (MnSOD) gene affect the oxidative damage and progression of DR severity. This study aims to determine the association of patient characteristics on the severity and prevalence of Single Nucleotide Polymorphism (SNP) V16A in the MnSOD gene in DR patients at Prof. Dr. I.G.N.G. Ngoerah Denpasar Bali.

Twelve (12) subjects with Non-Proliferative DR and 21 subjects with Proliferative DR were enrolled. Patient characteristics (age, T2DM duration, BMI, blood pressure) were associated to the DR severity using cross-sectional analytic study. While to detect the status of the SNP V16A MnSOD gene using descriptive experimental study. DNA segment amplification was performed using the PCR method, sequencing was performed to detect the base pattern at the location of the SNP.

A domination of the VV genotype in the SNP V16A MnSOD gene was present in all subjects (100%). The majority of subjects were female (51,5%), age range 40-65 years (84,9%), T2DM duration <10 years (60,6%), normal BMI (60,6%). Blood pressure was dominated with non hypertension 60,6%, with a history of oral anti hypertensive. No significant association was found between patient characteristics and the DR severity (p<0,05).

We may conclude that wildtype variant (VV) SNP V16A MnSOD gene was dominated in this finding. There is no significant association between patient characteristics and the severity of DR in Prof. Dr. I.G.N.G. Ngoerah Denpasar Bali.

Keywords: Diabetic Retinopathy, MnSOD gene, SNP V16A

INTRODUCTION

Aging is a physiological process followed by a decrease in the physiological functioning of organs that can lead to a state of illness[(1)]. It is known that Diabetes Mellitus (DM) also accelerates aging which, if not taken seriously, will cause micro and macrovascular complications[(2,3)]. One of the chronic microvascular complications that can occur is Diabetic Retinopathy (DR), which involves damage to the retinal capillaries that are the main cause of blindness worldwide.

The degree of DR development is caused by oxidative stress which is associated with genetic factors and other risk factors

such as age, DM duration, HbA1c, BMI, blood pressure[4-6].

Manganese Superoxide Dismutase (MnSOD) is an enzyme found in mitochondria, acting as an antioxidant that play important role againts oxidative stress. A number of single nucleotide polymorphism (SNP) in MnSOD gene has been described, but only the V16A (rs4880) has been reported a functional significance. Allele V is thought to cause structural changes in the order of MnSOD targets in the mitochondria thereby increasing susceptibility to oxidative stress resulting in the development of DR severity[4,8]. Research on the association of patient characteristics with the severity and prevalence of SNP V16A MnSOD gene in DR patients in the population in Bali is still rare. Based on these, it is necessary to conduct studies that discuss the association of patient characteristics with the severity of DR and to determine the prevalence of SNP V16A MnSOD gene in DR sufferers in Indonesia, especially Bali Province.

LITERATURE REVIEW

The severity of DR is divided into Non-Proliferative Diabetic Retinopathy (NPDR) as an initial phase and Proliferative Diabetic Retinopathy (PDR) as an advanced stages that can develop into Diabetic Macular Edema (DME)[5]. Hyperglycemia is considered as the main cause of retinal microvascular damage due to exposure to reactive oxygen species (ROS) in mitochondrial tissue which will cause damage to the vascular endothelium. Oxidative stress occurs when ROS and antioxidants are out of balance. High ROS will results in cell apoptosis, necrosis, and inflammation of the endothelial blood vessels[4,5,9]. In this sense, the role of antioxidants is to neutralize the increasing number of free radicals and keep cells protected from their toxic effects and contribute to disease prevention[10].

Patient Characteristics On The Severity

The Diabetes Control and Complications Trial concluded that strict control of risk factors can delay the development and slow down the development of DR[11]. DR is the main cause of vision loss in adults with DM aged 20-74 years. Age can be grouped based on classification according to Law Number 13 of 1998 concerning Elderly Welfare, where 65 years old is the last old person[12]. PDR is found in a total of 25% of T2DM patients with a duration of T2DM over 25 years[13]. Research that discusses the patients characteristics with DR severity is important to prognose biomarkers related to aging. This is because biological measures of aging are reflected in functional status, brain health, self-awareness of physical well-being, and organ function[14]. Prevention of a worsening of DR is very important to improve the patient's quality of life.

Single Nucleotide Polymorphism V16A MnSOD Gene In Diabetic Retinopathy Patients

The MnSOD enzyme can act as an antioxidant, which will catalyze the dismutation of superoxide radicals into hydrogen peroxide so that it can protect retinal endothelial cells from oxidative damage. This suggests that the activity of the MnSOD enzyme can be associated with the development of DR[5].

Diabetic Retinopathy has been associated with multiple gene polymorphisms as a risk factor, most studies linking the single nucleotide polymorphism V16A (rs4880) to it. The gene encoding the SNP V16A MnSOD gene is located on the long arm of chromosome 6q25.3 consisting of four introns and five exons, coding for 238 amino acids from 159,669,069 bp to 159,762,281 bp[15,16]. It is characterized by a structural mutation of substituting thiamine (T) for cytosine (C) in exon 2 which translates the amino acid valine (GTT) into alanine (GCT). The substitution results in a β-sheet secondary structure where no hydrogen bonding occurs with Leu-20 (dotted red line) instead of the expected α-helix structure

(Figure 1), which results in lowering the efficiency of enzyme transport into mitochondria, modifying antioxidant defense against ROS. The protein encoded by V will be located on the mitochondrial inner membrane, and this is associated with lower enzyme activity, thereby increasing susceptibility to oxidative stress[5,15,17]. Therefore, the V16A SNP in the MnSOD gene can cause differences in susceptibility among individuals to DR[4].

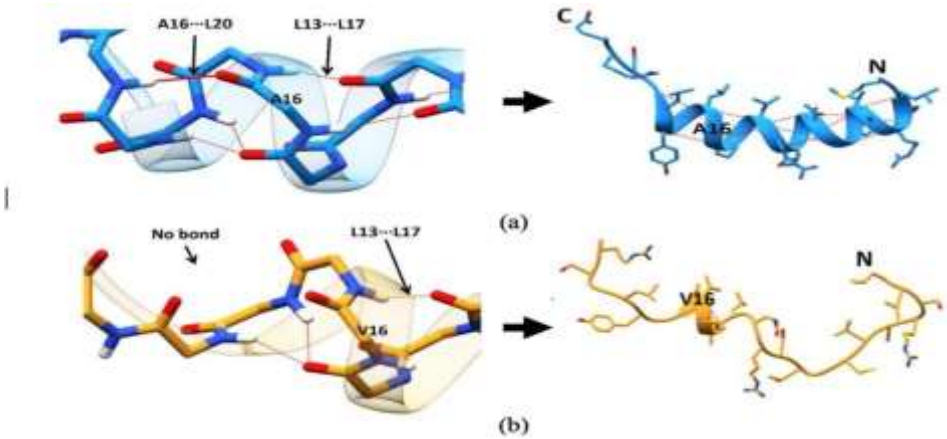

Figure 1 Visual Representation of Essential Hydrogen Bonds in SNP V16A MnSOD Gene[18]

MATERIALS & METHODS

Study Design and Sample Preparation

A cross-sectional analytical study to determine the relationship of patient characteristics to the severity of DR and descriptive experimental study to determine the prevalence of SNP V16A MnSOD gene in DR patients at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar.

The sample size were 33 samples with 12 NPDR and 21 PDR samples. This study usedblood specimens which were stored as biological materials from the Integrated Biomedical Laboratory Unit (LBT) of FK Udayana supplemented by clinical data, originating from T2DM patients who were treated at the Eye Polyclinic at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar, Bali for the period 2020 to 2021.

The data collected includes demographic and clinical parameters. Demographic parameters are age and gender. The clinical parameters recorded were: T2DM duration), body mass index, blood pressure (grouped as normotensive and hypertension if systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg).

The diagnosis of DM has been established in a previous study[19] with criteria for fasting plasma sugar levels >126 mg/dl and 2 hours plasma glucose >200 mg/dl; HbA1c ≥ 6.5%[3]. Diagnosis and grading of DR was performed by Vitreoretinal Specialist Consultant using slit lamp biomicroscopy with a 78 lens. Retinopathy is classified into NPDR and PDR.

Patients with anterior segment abnormalities, increased intraocular pressure, had orbital tumors and degraded DNA samples were excluded. This study was approved by the Udayana University Medical Ethics Committee.

Sample Assessment

The whole blood sample begins with DNA isolation at the LBT Unit of the Faculty of Medicine, Udayana University. The V16A MnSOD SNP gene was evaluated by PCR, using the forward primer: F:5'-CAG CCC AGC CTG CGT AGA CGG- 3' and the reverse primer: R:5'-CTT GGC CAA CGC CTC CTG GTA CTT-3', and the BsaW1 restriction enzyme as described by Jalood et al[20]. Genotyping was performed by three investigators (A.P., D.M., A.E.), regardless of the patients retinopathy status.

STATISTICAL ANALYSIS

The Statistical package for social studies (SPSS version 25.0) was used for data analysis. Descriptive statistics (numbers and percentages for categorical variables) and Fisher-exact tests was used to test the association. The $p \leq 0.05$ was considered statistically significant. Odds ratio (OR) with 95% confidence interval (95% CI) were generated to quantify relationships with each risk factor. Multiple logistic regression was applied to identify the predictor of DR. Prevalence of SNP V16A were identified by using descriptive statistics.

RESULT

The characteristics of the research subjects are listed in Table 1. The classification of obesity by World Health Organization[21], while blood pressure classified based on the 2019 Hypertension Management Consensus[22].

Table 1 Characteristics of the Research Subject

Variable	N (33)
Gender, n(%)	
Man	16 (48.5)
Woman	17 (51.5)
Age, n(%)	
40-65 years	28(84.9)
≥ 65 years	5(15.2)
T2DM duration, n(%)	
≥ 10 years	13 (39.4)
< 10 years	20 (60.6)
Body Mass Index, n(%)	
Normal	20 (60.6)
Overweight	13 (39.4)
Blood Pressure Classification, n(%)	
Hypertension	13 (39.4)
Non Hypertension	20 (60.6)
Severity,n (%)	
NPDR	12 (36.4)
PDR	21(63.6)

Eligible samples were processed by PCR and then electrophoresed. The PCR product (Figures 2) was then sent to PT Genetics Science for sequencing. The Snapgene application is used to read the sequencing results (Figure 3). In the analysis of the SNP V16A MnSOD geneat rs4880, 33 (100%) samples of MnSOD gene found in wildtype (VV).

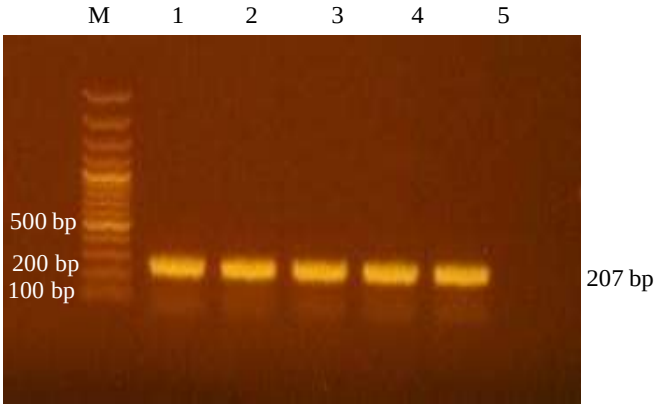

Figure 2 PCR Product of MnSOD Gene On Agarose Gel Electrophoresis 1%, M

Pathway: *marker* 100bp; Lines 1-5: MnSOD gene PCR product with primary *forward* (207 bp)

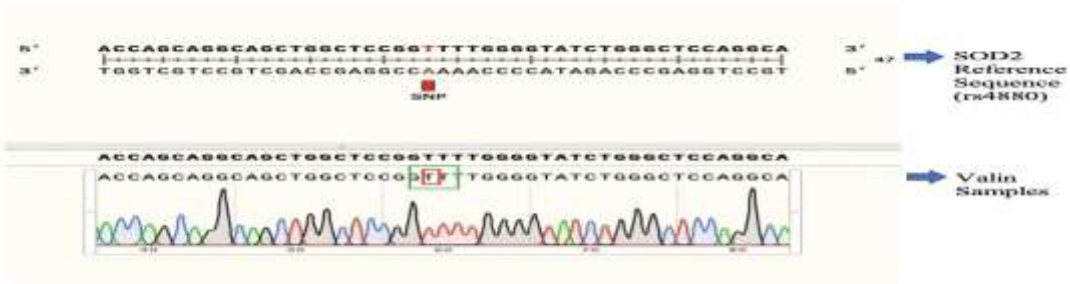

Figure 3 Visualization Of Sequencing Results by Snapgene Application

Association Between the Characteristics Of The Patient Sample And The Incidence Of Diabetic Retinopathy

Furthermore, bivariate analysis was carried

out to determine the association between the patient characteristics (age, BMI, Hypertension and T2DM duration) and the incidence of DR. From the results of the analysis, it was found that there was no significant relationship between patient characteristics polymorphisms and the incidence of retinopathy status. Fisher-exact test confirmed that the difference was no significant (Table 2). Multivariate analysis of logistic regression was performed on 2 variables that qualified (*p*-value 0.25) i.e. T2DM duration and BMI (Table 3).

Table 2 Bivariate Analysis of Relationship of Patient Characteristics with Severity ofDiabetic Retinopathy

Variable	Severity		*p*-value*
	NPDR	PDR	
Age (years)			0.630
40-65	11	17	
≥ 65	1	4	
T2DM Duration (years)			0.067
< 10	10	10	
≥ 10	2	11	
IMT			0.142
Normal	5	15	
Overweight	7	6	
Blood pressure			1.000

* Statistically meaningful if the value of $p<0.05$

Table 3 Multivariate Analysis Using Logistic Regression

Variable	ln	P-value [a]	Exp(B)	CI 95%	
				lower	upper
IMT	1.26	0.143	3.257	0.670	15.837
Duration of T2DM	-1.645	0.072	0.193	0.032	1.161
Nagelkerke R Square				0.248	
Hosmer and Lemeshow Test				*p*-value [b]: 0.140	

[a] Statistically meaningful if the value of $p<0.05$

[b] Statistically meaningful if the value of $p>0.05$

Prevalence of SNP V16A MnSOD Gene in Diabetic Retinopathy Patients at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar, Bali

The entire sample underwent homozygous (wildtype/VV) polymorphism. Bivariate analysis of SNP V16A of the MnSOD gene in patients with T2DM against the severity of DR can not be done because there are cells with a reality frequency value or also known as an Actual Count (F0) of 0 (zero). Table. 4 shows the prevalence of the VV genotype in the overallsample.

Table 4 Prevalence of SNP V16A MnSOD Gene in NPDR-PDR Group

Genotypes	NPDRn (%)	PDRn (%)	Totaln (%)
VV	12 (100)	21(100)	33 (100)
non-VV	0	0	0
Total	12 (100)	21 (100)	33 (100)

DISCUSSION

In the present study, we analized that sex was dominated by women 51.5% (17/33). This is related with the data of the Riskesdas 2018 report, namely the prevalence of DR in T2DM sufferers in Provinsi Bali is dominated by female sufferers (31.1%) higher than men[(23)]. The hormone estrogen is thought to have an effect on the occurrence of DR in T2DM, which is the dominant sex hormone in women. High levels of the hormone estrogen can decrease an leptin which plays a role in suppressing appetite in the hypothalamus, as a result of which food intake is not controlled, so it can cause the accumulation of excess fatty tissue accompanied by high blood sugar levels due to a decrease in sensitivity of peripheral tissues to insulin[(24,25)].

Association of Patient Characteristics to Severity of Diabetic Retinopathy

The age range in this study was 40–72 years old. Age is categorized into 2 groups, namely the age of 40-65 years and more

than equal to 65 years. This grouping is based on ageclassification according to the Law Number 13 of 1998 concerning Elderly Welfare, where theage of 65 years is the final elderly[26]. From the results of the study, 84.9% (28/33) of research subjects aged 40-65 years were obtained. Based on data from the 2018 Riskesdas report, Retinopathy sufferers have the highest distribution in the age range of 45-74 years. In the bivariate analysis, it was stated that there was no significant relationship between age to severity of DR (p = 0.63). This is in line with the study of Balasopoulou et al (2017), which states patients with young onset DM can have a higher degree of DR severity[27].

In this study, the data for BMI obtained the majority on the normal criteria of 60.6% (20/33) in the normal classification. In bivariate analysis, it was stated that there was no significant relationship between BMI and DR severity (p = 0.142). This is in line with meta-analysis research that states the BMI index does not increase the severity of DR (OR=0.99.95% CI 0.97–1.01; p=.25; I 2=79%). In normal BMI levels, PDR can also occur in patients with T2DM, this is because the worsening of DR derajat is more based on glycemic status[28,29].

In this study, the T2DM duration was categorized into groups of less than 10 years andmore than equal to 10 years. This is based on research that states in the first 10 years of Diabetes duration, the prevalence of Retinopathy is low and experiences slow development[30]. From the results of researchers, the majority of them for less than 10 years were 60.6% (20/33). In the bivariate analysis, it was stated that there was no significant relationship between the T2Dm duration and the severity of DR (p = 0.067). These results are in line with the research conducted by Manao et al (2021) which stated that there was no statistically significant relationship between the duration of T2DM and the severity of DR[31]. The delay in diagnosing Diabetes leads to the development of Diabetes complications, one of which is DR. Possible factors contributing against undiagnosed cases of Diabetes is low knowledge and awareness for early medical examination[32].

The majority of blood pressure data in the non-Hypertensive classification is 60.6% (20/33). This relates to the history of taking antihypertensive drugs in some subjects with normal blood pressure classification. In the bivariate analysis, it was stated that there was no significant relationship between blood pressure and the severity of DR (p = 1,000). This is in line with the research conducted by Varghese et al (2016) which stated that blood pressure, especially systolic TD did not show a significant positive correlation with the severity of DR[33].Well-controlled control of glycemic and Hypertension levels has been clinically proven to reduce the risk of macrovascular and microvascular complications in patients with T2DM[34]. Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults, recommends blood pressure goals <130/80 mm Hg in adults with Diabetes for the prevention of further complications[35]. Renin-angiotensin system (RAS) is thought to play a role in the pathological process that results in DR. Angiotensin II induces inflammation, apoptosis, cell growth, migration and differentiation. The polymorphism of angiotensin and angiotensinogen-modifying enzymes has been shown to have an effect on RAS[36].

In the present study study, the strongest determinants for the development of DR were BMI, which patients with the overweight have a 3.3 times higher chance of experiencing DR severity compared to those with the normal (OR:3.257;CI 95%: 0.670 - 15.837; p;0.143; ln:1.26) and T2DM duration which patients with under 10 years have a 0.072 times higher chance of experiencing the severity of DR compared to those more than equal to 10 years (OR:0.072; CI of 95%: 0.032-1.161;

*p:*0.072; ln:-1.645). All these two risk factors have been documented in most of the studies related to DR.

Prevalence of SNP V16A with Severity of Diabetic Retinopathy

The results of the study in Table 4 prevalence of SNP V16A MnSOD gene in Patients DR at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar, Bali as a whole (100%) experienced a wildtype variant (VV) SNP. This is in line with several studies in Indonesia that suggest that the VV genotype is the dominant polymorphism in DR patients, especially in Asia[8,37]. Studies conducted by several researchers, also suggest that VV genotypes were found to be more numerous in patients with DR compared to non-DR[4,5,8,38]. Analysis of subgroups by racerevealed that the SNP V16A of the MnSOD gene influenced the risk of DR in Caucasian and Asian races in the dominant model of the VV genotype. Differences between races may reflect the presence of actual population-specific disease variants, but may also be due to differences in genome structure at the locus between populations[4]. There are also statements from different studies that variants of the VA genotype are more at risk of PDR with OR 8.33 (2.56- 27.13) and are statistically significant[39]. According to the National Center for Biotechnology Information (NCBI) the proportion of V alleles in Asia in particular is the majority at 86.54%. In a meta-analysis study it was suggested that SNP V16A in the MnSOD gene could cause differences between individuals in DR terhadap susceptibility. There has been some controversy over the relationship of SNP V16A to the severity of the DR. In meta-analysis studies, genetic factors including the MnSOD gene with a dominant model (VV) have been known to be related to the severity of DR (OR=0.66; 95% CI=0.48-0.91; p<0.0001). However, these asossiation were found to be weak, inconsistent, and lack of standardization of the DR phenotype in different populations[4].

The limitation of our study is that a the use of secondary data and biologically stored material, limitations on the researcher's data and in number of research samples. In the DR degree variable, the data obtained did not classify the case subjects at each of the NPDR degree stages (very light, light, medium, heavy, very heavy) and the PDR degree stages (mild-medium, high-risk, advanced). Researchers evaluated only one SNP in the MnSOD gene to assess the risk of DR in that gene study and did not consider lifestyle factors from each subject, such as smokin habits, alcohol consumption, and antioxidan intake.

CONCLUSION

We may conclude that there is no relationship between patient characteristics (age, BMI, blood pressure, duration of T2DM) to the severity of DR patients at RSUP Prof. Dr.

I.G.N.G. Ngoerah Denpasar, Bali. Also there was no difference in prevalence in the SNP V16A variant of the MnSOD gene in DR patients at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar, Bali. This is because all study subjects experienced SNP V16A of the wildtype variant MnSOD gene (VV). Understanding the role of the MnSOD gene in the development of DR severity may become an important molecular target for future pharmacological interventions.

Declaration by Authors

Ethical Approval: Approved
Acknowledgement: None
Source of Funding: None
Conflict of Interest: The authors declare no conflict of interest.

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How to cite this article: Andhika Putri Perdana, Desak Made Wihandani, Agus Eka Darwinata et.al. Patient characteristics and prevalence of single nucleotide polymorphism (SNP) V16A MnSOD Gene in diabetic retinopathy patients at RSUP Prof. Dr. I.G.N.G. Ngoerah Denpasar Bali. *International Journal of Research and Review*. 2023; 10(1): 634-642.
DOI: *https://doi.org/10.52403/ijrr.20230170*

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