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Association of *MnSOD-47C/T*GENE Polymorphism (Rs 4880-47C/T) in Sudanese Patient with Diabetic Retinopathy

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Diabetic retinopathy (DR) is the common micro-vascular complication of diabetes mellitus (DM). It is the main cause of blindness among young adults worldwide. Poor glycemic control in addition to longer diabetic duration is the main risk factors for diabetic retinopathy. Many genes have been postulated as candidates for diabetic retinopathy. Little is known about anti-oxidative enzyme gene polymorphism and its association with diabetic retinopathy, mainly for catalase enzyme and manganese superoxide dismutase and glutathione genes. The study aims to assess the role of manganese superoxide dismutase MnSOD *(rs4880)* gene polymorphism in diabetic retinopathy Sudanese patients and its relation with *MnSOD* level. In addition to determine

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the association of Fasting Blood Glucose (FBS), HbA1c and Lipid in the pathogenesis of diabetic retinopathy.

Methodology: The number of subject involved were 130 which, were classified into (n 60) clinically diagnosed as diabetic retinopathy and (n 70) diabetes mellitus without retinopathy as control group, age ranged from (22 – 80) years old, from Makkah Eye Complex. DNA was extracted and PCR product for *MnSOD*, gene segment was digested by **NgoM** enzymes, moreover gene polymorphisms were determined. Serum *MnSOD*, activity and FBS, TG, CHOL and HbA1c level were analyzed using Cobas Int 400 using absorption photometer and immunoassay methods respectively.

Results: The results revealed that retinopathy is more common in female than male by approximately 2 fold =1.9:1. Type II is more common in our population that type 1. The majority of the patients had type II diabetes (128, 98.5%) and only 2(1.5%) patients were type I diabetes mellitus. The activity of *MnSOD*, was significantly higher in DNR when compared with DR (*p*= 0.003). Mean HbA1c and FBG concentration were significantly higher among DR than DNR *p*=0.001 and p=0.001 respectively. In contrast, mean serum CHOL and TG level revealed insignificant differences when compared DR with Diabetic without retinopathy (DNR).The genotyping for *MnSOD-47C/T* showed that the frequency of genotype CC was significantly lower in cases compared with control. Theses Associations for SNPs CCs, MnSOD-47C/T SNP rs4880, decreased risk after correction for multiple testing (OR = 0.088, 95% CI = 0.034-0.224 *p*= 0.001), While the frequency of the CT heterozygote genotype was significantly higher in cases group compare with control, the OR= 3.76(1.41-10.5), *P*=0.006. While frequency of the TT genotype was significantly higher in cases than controls. Theses Associations for MnSOD 47C/T SNP rs4880, increased risk after correction for multiple testing (OR = 5.31, 95% CI = 1.91-14.75, *p*= 0.001). The C allele is observed in 47% of the cases while the T allele – risky allele- observed in 73% of the cases, OR= 0.150(0.079-0.285), *P*=0.001 (Table 6).

Conclusion: The study concludes that there is a significant association between *MnSOD-47C/T (rs 4880)* gene polymorphism and the occurrence of diabetic retinopathy in Sudanese population. There is a significant decrease in *MnSOD* levels and glycemic control in patients with the mutant allele T.

Keywords: Mangenes Superoxide Dismutase MnSOD-47C/T; gene polymorphism (rs4880); diabetic retinopathy; PCR-RFLP.

1. INTRODUCTION

Diabetic retinopathy "is the main of the known" micro and macro-vascular complications of diabetes mellitus. Always affect the age group 20-60 years which, has a burden on the economy and community as it's associated with loss of productivity. Recently, WHO added diabetic retinopathy to the priority list of eye disease as it can be partly prevented and treated. It is a well-known fact that diabetes mellitus is a risk factor for cardiovascular disease [1-3]. Poor glycemic control and longer disease duration are leading cause to the development of vascular complications. Diabetic retinopathy DR occurs both in type 1 and type ɪɪ diabetes and is strictly related to disease duration [4,5]. In Sudan, the prevalence of diabetes mellitus is 3.4% and reaches up to 10% in some communities. The prevalence of diabetic retinopathy was reported to be low as 28.1% in 1989 but this prevalence is increased to 43% in 1995. This increase is attributed to poor glycemic control, long disease duration, old age and hyperlipidemia. It had been reported that Sudanese patients are more prone to develop micro and macro vascular complications. As the main cause for developing vascular complications is hyperglycemia, but this report may indicate the involvement of genetic factors in development of vascular complications as retinopathy [6,7]. One of the chief mechanisms to develop vascular complications is reactive oxygen species (ROS) Production. ROS are generated under physiological condition, it's over productions is involved in different pathological conditions. Including pathogenesis of diabetes vascular complications. Recently, many scientists noticed that some diabetic patients develop macro-vascular complications while others didn't develop any complications in spite of sharing the same level of glycemic control, mode of treatment and matching for age. This finding lead to postulation of genetic

susceptibility to developing diabetes vascular complications [8,9]. ROS is mostly developed in micro vascular cells in retina and the superficial fiber cells, which are highly reactive. the proper regulation of cell functions depends on a certain level of ROS, such as intracellular signal, transcription activation, cell proliferation, inflammation, and apoptosis, but higher amounts of ROS are harmful to macromolecules [10,11]. The main functions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), protection the organisms from oxidative damage [6,8,11]. SOD decomposes superoxide into hydrogen peroxide. CAT catalyzes the decomposition of hydrogen peroxide into water and oxygen, thereby preventing cell damage from high levels of ROS. GPXs are selenoproteins that reduce organic peroxides and hydrogen peroxide through the coupled oxidation of glutathione [10,11].

Genetic variations in the antioxidant genes coding for the SOD, CAT, and GPX enzymes may lead to defect in regulation of their enzymatic activity and alter ROS detoxification. Moreover, genetic variations among enzymes that protect the cell against ROS may increase disease risk [12,13]. According to the high interaction potentiality of ROS with genetic material, polymorphisms in genes coding for antioxidant enzymes may play an important role for inter-individual differences in maintaining the human genome's integrity. Genetic polymorphisms in *SOD*, *CAT*, and *GPX* have been included in proneness to cancer and other diseases [14,16]. These potentially significant genetic variants related to oxidative stress have already been studied extensively, including single nucleotide polymorphisms (SNP) –47C/T in the promoter region of the MnSOD gene (SNP, rs4880), MnSOD–47C/T, polymorphism is one of the most common and important of antioxidant enzymes. Most of these polymorphism result in changes in the levels or the activities of these enzymes, which can lead to reduced protection against oxidative stress. The effect of these variations on the lens has not yet been clarified, so we choose these candidate SNPs to study in our work [14-18].

The aim of the present study was to evaluate the possible association of MnSOD-47C/T gene polymorphisms with diabetic retinopathy, in addition to determine the association of MnSOD-47C/T activity, Fasting blood glucose FBS and HbA1c and Lipid concentration in the pathogenesis of diabetic retinopathy in the Sudanese population.

2. MATERIALS AND METHODS

Methodology: In case-control hospital based study (n 130) subject were enrolled, then classified into (n 60) clinically diagnosed as diabetic retinopathy and (n 70) diabetes mellitus without retinopathy as control group, age ranged from (22 – 80) years old, from Makkah Eye Complex. DNA was extracted and PCR product for MnSOD–47C/T gene segment digested by ***NgoM*** enzyme was used for determination of genotype by (PCR-RFLP).

Serum was obtained to determine the activity of antioxidant enzyme and concentration of FBS, TRG, CHOL, by using absorption spectrophotometer. HBA1c was measured by immunoassay.

SOD genotypes were detected using a multiplex PCR–RFLP method. An C→Tin exon 10 (codon 399) was amplified to form undigested fragments of 107 bp using primers, described in PCR conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 50 s, 63°C for 50 s, 72°C for 50 s, and a final extension step at 72°C for 7 min. The 107 bp PCR products were digested with **NogM** (MBI Fermentas, Burlington, TC) at 37 C for 5 h and analyzed with 2% agarose gels. **NgoM** digestion resulted in one fragment of 107 bp for wild-type (TT); two fragments of 89 and 18 bp for variant homozygous (CC); and three fragments of 107, 89, and 18 bp for heterozygous (TC) (Show Tables 1, 2. 3).

2.1 Ethical Consideration

Ethical consideration Permission of this study was obtained from the local authorities in the area of the study. This study was approved by the College Board Committee. The objectives of the study were explained and written consent was obtained from each participant in this study.

2.2 Statistical Analysis

Statistical data analysis Data were analyzed using SPSS software, version 20.0 for Windows (SPSS). P of <.05 was considered statistically significant. Chi-square ($\chi 2$) test was used to compare the differences between patients and control groups.

Table 1. SNP specification and primer sequences and PCR-RFLP products

Gene	Mutation	Forward	Primer Length	PCR Product	Restriction enzyme	Allele	PCR-RFLP Products
MnSOD	rs4880 47T>C Val16Ala	F5:ACCAGCAG GCAGCTGGCG CCGG:3 R5: GCGTTGATGT GAGGTTCCAG: 3	42bp	107bp	**NgoM**	C/C 107+18 T/C107+8 9+18 T/T 107	89+18bp 89+18+10 7bp 107bp

Table 2. SNPs location and prevalence of risk allele among African population

Gene	Mutation	Prevalence of risk allele	Chromosome
MnSOD	rs4880- 47T>C Val16Ala	14%	6q25.3

Table 3. Manganese superoxide dismutase (Mn-SOD) Primer

Name of Primer	Primer Sequence
(Mn-SOD) (sense)	5": CTGGTCCCATTATC:3",
(Mn-SOD) (antisense)	5: TCGATTCCTACTGTGCAC:3.

3. RESULTS

The baseline clinical and demographic features of the study patients with DR and DNR are shown in (Table 1). In this study, 130 diabetic patients were enrolled, sixty patients with diabetic retinopathy (DR), (46.2%) 60 and on the other hand seventy diabetic without retinopathy (DNR), (53.8)70 as (controls). The overall male to female was 1:1.9 folds. The majority of the patients was 128(98.5%) type II diabetes and only 2(1.5%) patients were type I diabetes mellitus. The eldest patient in this study aged 80 years while the youngest was 22 years. The mean of the cases group versus controls was [59 ± 11.0 vs. 59± 10.5 years; *P*=0.317], (Table 4). The mean duration of diabetes in years was significant among cases than controls [16.5±7.5 vs. 16.5±7.5; *P*=0.005].

The serum of MnSOD activity was significant different from [3.12±0.87 U/mL among controls to 1.53 ± 0.14 U/mL, for case *P*=001] (Table 5).

HbA1c ranged between 6-10 mg/dl and found significantly high among case than control groups [8.20±1.94 vs. 7.2±1.1 mg/dl *P*=0.001].

Fasting blood glucose ranged between 72-282 mg/dl and found significantly high among case than control groups [190.4±45.1 vs. 160.1 ±45.8; mg/dl, *P*=0.001] mean serum cholesterol and triacylglycerol levels were not statistically different between both groups, however cholesterol and triacylglycerol range in this study were 104-246 mg/dl and 25-258 mg/dl respectively (Table 6).

The genotyping for Mn-SOD 47C/T showed that, the frequency of CCalleles was observed in 12(20%) in the cases group compared to 52(74%) in controls, this showed that the genotype CC is a protective allele and OR (95% CI) = 0.088 (0.034-0.224), P=0.001. The frequency of the CT heterozygote genotype was 23(38%) in cases group 10(14%) in control group, the OR= 3.76(1.41-10.05), P=0.005. While frequency of the TT allele which is a risky allele observed in 25(42%) of the cases and 8(12%) of the controls and its risk assessed and confirmed by high OR= 5.31(1.91-14.75), P=0.006. The C allele is observed in 47% of the cases while the T allele – risky allele- observed in 73% of the cases, OR= 0.150(0.079-0.285), P=0.001 (table 7).

4. DISCUSSION

Diabetic retinopathy is the result of metabolic disorder in diabetes and most common cause of blindness in people aged 30-60 years. After 15 years almost all patients with typeI and two thirds of those with type Π diabetes have a risk of retinopathy. In the retina there is increased oxygen uptake and glucose oxidation relative to any other tissue; Consequently this phenomenon

Table 4. Socio-demographic comparison between cases and control

Variables	Patients N=60	Controls N=70	p-value
Patients Gender (%)			
Male	26(43%)	25(36%)	
Female	34(57%)	45(64%)	
Patients Age, years	59 ±11.2	56.0 ±10.5	0.075
Duration of diabetes, years	16.5±7.5	14.7±6.3	0.005
Type of Diabetes (%)			
Type I	2(4)	0(0)	
Type II	58(96)	70(100)	

Significant difference considered as p-value ≤ 0.05

renders retina more vulnerable to oxidative stress, accordingly the present study was carried out to evaluate CAT, GPX and MnSOD gene polymorphisms and level in patients with diabetic retinopathy (DR), and to correlate between gene polymorphism and study variables.

Table 5. Biochemical comparison between cases and controls

Variables	Patients N=60	Controls N=70	p-value
MnSOD U/mL	1.53 ± 0.14	3.12±0.87	0.001

Significant difference considered as p-value ≤ 0.05

The frequency showed that the prevalence of DR in the present study was 60(46.1%), which is similar to previous findings thatI in the India 60(42.7%), and was higher than that in the prospective diabetes studies done in Egypt 28(39.84%), KSA 22(32.84%), UK 25(37%) and Melbourne 26(35.7%). A similarity was observed with Pima Native Americans in Arizona 58(41.8%). The high prevalence of DR in our study and the Arizona study might be attributed to the poor glycemic control that increases the risk for diabetic retinopathy. Moreover, a limited period of poor glycemic control can have a prolonged effect on the incidence of diabetic retinopathy ("metabolic memory") as demonstrated by the Epidemiology of Diabetes Interventions and Complications (EDIC) cohort follow-up. The prevalence of DR in our study was also higher than that in another study conducted in Hong Kong 30(28.4 %) in which, the subjects were recruited from primary health care clinics [18,19,20].

Table 6. Biochemical comparison between cases and controls

Variables	Patients N=60	Controls N=70	p-value
HBA1c, mg%	8.20±1.94	7.2±1.1	0.001
Fasting Blood Glucose, mg/dl	190.4 ±45.1	160.1 ± 45.8	0.001
Cholesterol Level, mg/dl	195.7 ±55.2	184.9 ± 39.1	0.463
Triaylglycerol, mg/dl	156.3 ±88.6	141.3 ± 51.0	0.335

Significant difference considered as p-value ≤ 0.05

The genotyping for MnSOD-47C/T showed that the frequency of genotype CC was significantly lower in cases compared with control, Theses associations for SNPs CCs, MnSOD 47C/T SNP rs4880, decreased risk after correction for

Table 7. Distribution of genotype frequencies of Mn-SOD-47C/T polymorphisms in cases and controls

Genotype Mn-SOD-47 C/T	Patients (%) N=60	Controls (%) N=70	OR (95% CI)	p-value
CC	12(20)	52(74)	0.088(0,034-0.224)	0.001
CT	23(38)	10(14)	3.76(1.41-10.05)	0.005
TT	25(42)	8(12)	5.31(1.91-14.75)	0.006
C allele frequency	0.47	0.114		
T allele frequency	0.73	0.26	0.150 (0.079-0.285)	0.001

Significant difference considered as p-value ≤ 0.05

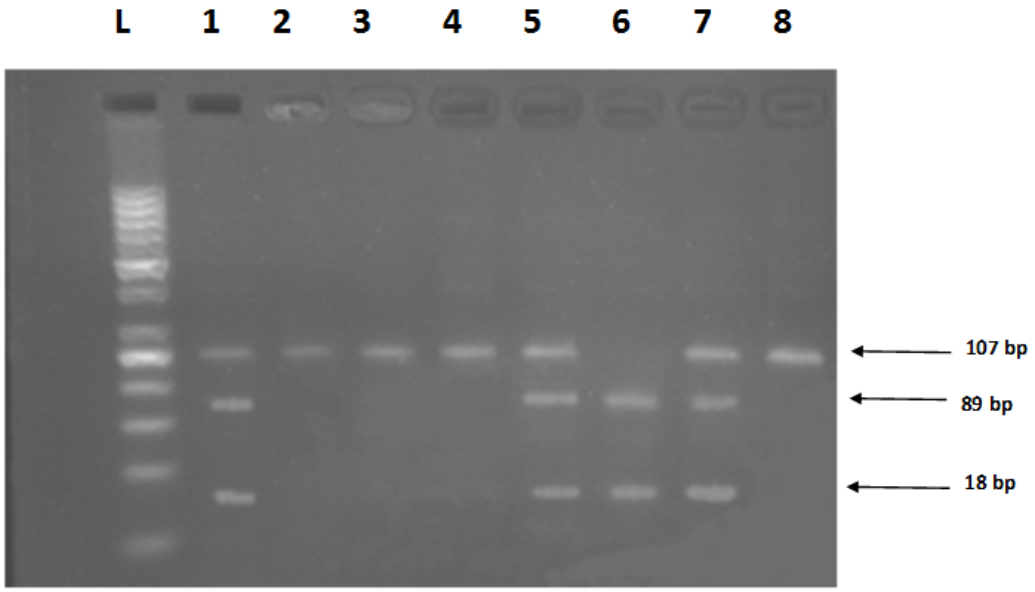

Fig. 1. PCR-RFLP analysis for MnSOD-47/T polymorphism (107 bp)

One fragment of MnSOD 107bp indicates variant homozygous (TT), two fragments of 89 and 18bp for wild homozygous (CC); and three fragments of 107, 89 and 18bp for heterozygous (CT). Column L show the DNA ladder 30 bp; columns 1, 5 and 7 MnSOD heterozygous (CT) genotype; 2-4 and 8 MnSOD variant homozygous (TT); column 6 MnSOD wild homozygous (CC)

multiple testing (OR = 0.088, 95% CI = 0.30 - 0.224, *P*= 0.001), while the frequency of the CT heterozygote genotype was significantly higher in cases group compared with control, the OR= 3.76(1.41-10.50), *P*= 0.006, while frequency of the TT genotype were significantly higher in cases than controls. Theses Associations for SNPs TTs, MnSOD- 47C/T SNP rs4880, increased risk after correction for multiple testing (OR = 5.31, 95% CI = 1.91-14.75, *P*= 0.001). The C allele is observed in 47% of the cases while the T allele – risky allele- observed in 73% of the cases, OR= 0.150(0.079-0.285), *P*= 0.001. Several studies on the *MnSOD* Val/Val genotype showed an increased risk of diabetic retinopathy in type 1 diabetes. However, *MnSOD* Val16Ala polymorphism associated with diabetic retinopathy by Slovenian study. Moreover, other study from Korean reported that V16A polymorphism of the Mn-SOD gene has been associated with diabetic macular edema in type Пdiabetic patients. We hypothesized that genetic variability of Mn-SOD enzymes regulating oxidative stress could be involved in development of microangiopathic complications in people with diabetes. The retina is particularly susceptible to oxidative stress because of its high consumption of oxygen, high proportion of polyunsaturated fatty acids, and expo- sure to visible light, large inter-racial differences in the allele frequency have been reported so far, and only few studies enrolled general population in Caucasians. The frequency of the V allele in general population is around 0.50 in Caucasians. Additionally, limitation study from India showed significant association *of SOD2* + 47C/T gene variants in DR in T2DM. However, one study in Ljubljana from Solovenian, report that The *MnSOD* Val/Val genotype was associated with a higher risk for diabetic retinopathy in patients with type 1 diabetes. Additionally,other study from china was reported that we found that the *SOD1*–47C/T polymorphisms were associated with an increased risk of cataract [18,19,20,21,22].

Serum anti-oxidant activity of MnSOD was significantly lower among DR (*P*=0.001), compared with DNR individuals. This may be attributed to the poor glycemic control of DR patients. Poor glycemic control may be the key factor enhancing AGE formation, which may be associated with lower MnSOD activity in DR [18]. Interestingly, one study in Hungarian patients had reported that an increased frequency of diabetes with catalase deficiency compared with both healthy relatives and the background population [23]. Free radicals' formation in diabetes mellitus and increase over time may play a role in the development of diabetic retinopathy, which is an important complication of the disease [24].

Oxidative stress can influence the expression of multiple genes, including signaling molecules; over expression of these genes may cause mitochondrial dysfunction and peroxidization of the lipid and protein structure, which induce a variety of cellular dysfunctions leading to retinopathy [21]. The levels of oxidized lipids, DNA and proteins are higher in diabetics, suggesting a diminished capacity to reduce toxic reactive oxygen [25]. And the level of glycemic control as measured by HbA1c, fasting blood glucose is a marker for both development and progression of DR. there was significant increased main level of HbA1c, fasting blood glucose concentration in the DR patients when compared with DNR, (P=0.001, P=0.001), contradicted with patient. This was in accordance with findings obtained from another study [18,26,22]. Also of interest, it has been reported that the serum fasting plasma glucose levels correlated well with the progression of DR [18,27]. Finally, poor glycemic control increases the risk for diabetic retinopathy. Moreover, a limited period of poor glycemic control can have a prolonged effect on the incidence of diabetic retinopathy ("metabolic memory") as demonstrated by the Epidemiology of Diabetes Interventions and Complications (EDIC) cohort follow-up [18,21]. The present study showed significant association between hyperlipidemia and DR. But in our study, the lipid profile concentration in the DR patients when compared with DR or DNR was insignificant (P=0.463, P=0.335, and P=0.327). This is similar to a previous study that showed no significant association between hyperlipidemia cholesterol decrease and triglyceride decrease and HDL normal and DR [18,24]. And there was no correlation between diabetic retinopathy and hyperlipidemia. It is strongly recommended that glycemic and lipidemic control be widely promoted and that lipid profile investigations be carried out routinely [18,28,29]. The results should be compared with those of the Wisconsin Epidemiologic Study of diabetic retinopathy which examined the 25-year cumulative progression and regression of diabetic retinopathy (DR) and its relation to various risk factors [18,26,28,29] The Wisconsin study revealed that the higher glycated hemoglobin is associated with increased risk of incidence of PDR.

5. CONCLUSION

The study concludes that, presence of additional minor T alleles results in decreased Catalase activity (MnSOD-47C/T) and hence association with higher level of the glycemic control HbA1c and FBG thus could contributes to pathogenesis of diabetic retinopathy.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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