Possible association of CAT gene-21A/T on diabetic retinopathy in Sudan

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Abstract

Background: Catalase (CAT) is one of the active anti-oxidative enzymes that its deficiency has been related to diabetic retinopathy. The aim of the current study was to determine whether *CAT*-21 A/T polymorphism is associated with the development of diabetic retinopathy or not among Sudanese patients with type 2 diabetes mellitus.

Methods:

A case-control study was conducted at Makkah Eye Complex, Khartoum, Sudan. Fifty patients with diabetic retinopathy (cases) and 50 diabetic control were included in this study. The single nucleotide polymorphism (SNP) in the promoter region of *CAT* gene -21A/T was genotyped using polymerase chain reaction and restriction fragment length polymorphism endonuclease *Hinf*1 enzyme (PCR-RFLP) in cases and control.

Results:

The TT genotype frequency of the *CAT*-21A/T was significantly higher in cases compared with control (*P*=0.043), and showed that a risk effect in diabetic retinopathy patients (OR=2.56, 95% CI=1.01-6.45). While the AA genotype showed a protective consequence (*P*= 0.011, OR=0.33, 95% CI= 0.13-0.79) in control. The allele frequency of the variant T allele was significantly different in the cases group (*P*=0.003, OR=2.35, 95% CI=1.33– 4.16).

Conclusion

The TT genotype and T allele frequency of *CAT*-21A/T polymorphism may be associated with an increased risk of diabetic retinopathy in this setting.

Keywords: Catalase, *CAT – 21A/T*, Polymorphism, Diabetes, Diabetic Retinopathy, Sudan

Introduction

Diabetes mellitus is a chronic devastating disease, with an escalating prevalence annually, in 2013 it affects 382 million people and by 2035 epidemiologist expect 592 million people will suffer from diabetes globally [1]. Diabetes has several systemic complications involving diabetic retinopathy, which is the most common diabetic complication that may lead to blindness [2]. In Sudan, diabetes mellitus is prevalent in 2.6% of the population; 67% of these diabetic patients had a long-term diabetes complication, diabetic retinopathy was reported among 43% of them [3, 4]. The long duration of diabetes, elder age and poor glycemic control are risk factors for diabetic retinopathy [5]. In addition to these known risk factors for diabetic retinopathy, there is a growing body of evidence indicating that, oxidative stress may associate with diabetic retinopathy [6,7]. It is well-known that, oxidative stress, arise from the imbalance between excessive generation of free radicals _like hydrogen peroxide (H_2O_2) _ and their eliminations by anti-oxidant mechanisms [8]. Recently, genetic variability of the anti-oxidative enzyme has been reported as a mechanism for the development of oxidative stress [9].

Catalase (CAT, EC 1.11.1.6) enzyme is a cytosolic antioxidant enzyme that converts hydrogen peroxide (H_2O_2) to water (H_2O) and a molecular oxygen (O_2) [10]. CAT is a homo-tetramer peptide encoded by *CAT* gene which composed of 13 exons and located on chromosome 11p13 [11]. It is expressed almost in all body cells [12]. It can dispose of more than 80% of the cellular H_2O_2 [10]. The *CAT* gene polymorphism -21A/T (rs7943316) is one of the most common single nucleotide polymorphisms (SNP) in the 5′-untranslated region of the *CAT* gene, located just proximal to the starting codon [13]. This *CAT* gene polymorphism may modify the enzyme expressional profile and activity, hence; increase the oxidative stress inside the cell.

Recently, scientist and clinician pay attention to the oxidative stress and their role in the pathogenicity of complex disease like diabetes mellitus and its complications. To the best of our knowledge, no published paper examines the association between the *CAT*-21A/T gene polymorphism with diabetic retinopathy. The objective

of this study was to examine possible association of the *CAT*-21A/T gene polymorphism with the risk of development of diabetic retinopathy in type 2 diabetic Sudanese patients.

Materials and Methods

A case-control study was conducted at Makkah Eye Complex in Khartoum Central Sudan during June to December 2013. Makkah eye complex is a charity tertiary hospital offer specialized ophthalmological services for the Khartoum state population. We approached all type 2 diabetic patient who attend the diabetic retina clinic for the first time or for a follow-up to participate in the study. Signed informed consent was obtained from all participants, and then each participant was asked about the purpose of the visit, his/her age, diabetes duration, history of hypertension, drug history and social history. Weight and height were then measured and body mass index (BMI) was calculated. All the patients were examined by expert senior Ophthalmologist who has experience in retina exceeding 20 years. Retina examination is done by slit-lamp with assisted lenses, after mydriasis by using Cyclogyl (Alcon) eye drop. Abnormalities and clinical findings were reported in the patients' sheet by the same ophthalmologist. Diabetic retinopathy ranked according to the American Academy of Ophthalmology (AAO) scale [14]. All patients and their diabetic control were Sudanese nationalities. Patients with a known renal disease, pregnant women and hypertensive, were excluded from the study.

Blood sampling

Fasting venous blood (5 ml) were drawn from the ante-cubital vein from each participant. Blood collected in EDTA containing vacutainer and fluoride vacutainer. Measurement of blood glucose done immediately according to standard procedures. Other laboratory measurements including, haemoglobin, blood urea, serum creatinine and cholesterol levels were measured using standard methodology.

DNA extraction and PCR analysis

Genomic DNA was extracted from 200 µl of whole venous blood using Vivantis Kits according to the manufacturer's instructions. Extracted DNA was quantified by using a NanoDrop ND-2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Genotyping of the polymorphic site was performed by polymerase chain reaction (PCR) amplification and restriction endonuclease digestion of the PCR products (PCR/restriction fragment-length polymorphism, PCR–RFLP). The polymorphic region of -21A>T, rs7943316 was amplified by PCR using primer pair (forward 5/-AATCAGAAGGCAGTCCTCCC-3/, reverse 5/-TCGGGGAGCACAGAGTGTAC-3/) to amplify a 250-bp fragment. The ready-mix PCR tube (Maxime PCR PreMix) (*i*-Taq) purchased from *i*NTRON Biotechnology and used in PCR analysis. Briefly, the reaction mixtures (25 µL) contained a 5 µl of 50 ng of genomic DNA, 10 µmol/L of each primer, 2.5 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, reaction buffer (10x), gel loading dye and 2.5 U *i*-Tag™ DNA polymerase (5U/µl) (*i*NTRON Biotechnology). The amplification protocol consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s; a final extension was conducted at 72 °C for 10 min [13].

Restriction enzyme analysis

The resulting PCR product was digested with the restriction endonuclease *Hinf* I (Promega, USA) at 37 °C for 4 hours according to the manufacturer's instructions and digestion products were visualized by UV camera and analyzed after performing electrophoresis in 3% agarose gel stained with ethidium bromide (0.5 Ag/ml). Tris–Borate–EDTA (TBE) buffer (0.5) was used as running buffer. Restriction enzyme digestion resulted in two fragments length were 177 and 73 bp which indicate wild type AA, three fragments of 250, 177 and 73 bp indicate heterozygous AT and one fragment of 250 bp indicate the variant homozygous TT, figure 1. All Molecular biology analysis were performed in Al-Neelain University Medical Research Center.

Statistical analysis

Data were entered into the computer using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0 and double-checked before analyses. To compare mean values of continuous variables, the Student's *t*-test were used. Chi-square (X^2) or Fischer's exact test (two-sided) was used to compare categorical variables and to test the association between the genotypes and alleles in relation to the cases and control and test for deviation of the genotype distribution from Hardy – Weinberg equilibrium (HWE). The odds ratio (OR) and 95% confidence intervals (CI) were obtained to measure the strength of the association between genotype and alleles in cases and control. A *P*-value of < 0.05 was considered significant in all tests.

Ethics

This study was approved by Al-Neelain Research Ethics Review Board, Sudan.

Result

Cases and control were well-matched in their socio-demographic characteristics, table 1. The mean (SD) for duration of diabetes [16.3(9.1) vs. 10.6(0.9) years; *P*=0.201] and serum creatinine [0.93 (0.28) vs. 0.72 (0.25) mg/dl; *P*=0.002] were significantly higher in cases compare with control, respectively.

Among the 50 cases, 23 (46%) were diagnosed as severe non-proliferative diabetic retinopathy with mild macular edema, 10(20%) had mild non-proliferative diabetic retinopathy, 6(12%) had non-proliferative diabetic retinopathy with clinical significance macular edema and 11(22%) had proliferative diabetic retinopathy with macular edema.

The distributions of the *CAT-21A/T* genotypes were in accordance with the Hardy-Weinberg Equilibrium among the cases (p=0.31) and the control (p=0.12). The frequencies of the genotypes and alleles of *CAT*–21A/T

polymorphism in the diabetic retinopathy and control groups are shown in Table 2. Cases group showed that 11(22%) were wild-type for the AA genotype, 21(42%) were heterozygous for the AT genotype and 18(36%) were variant homozygous for the TT genotype. There were significant differences between the cases and control groups in the *CAT* A/A and T/T genotype [*P*=0.011; and *P*=0.043], respectively. The risk estimation analysis revealed a possible risk effect with the variant genotype TT [OR=2.56, 95% CI=1.01-6.45], while the AA genotype showed a possible protective effect [OR=0.33, 95% CI= 0.13-0.79] in control. When compared to diabetic control, the allele frequency of the variant T allele of *CAT*–21A/T was significantly different in the diabetic retinopathy group (*P*=0.003, OR=2.35, 95% CI=1.33– 4.16).

Table 3 shows the distribution of genotype frequencies of *CAT*-21A/T polymorphism in diabetic control and patients with diabetic retinopathy after stratification to the diabetic retinopathy subtypes. When compared to diabetic control, the genotype frequency of the AA of *CAT*-21A/T was significantly different in all diabetic retinopathy subtypes. The AT frequency was significantly different in all subtypes of diabetic retinopathy except severe non-proliferative diabetic retinopathy with mild macular edema type. While the genotype frequency of the TT of *CAT*-21A/T was significantly different in mild non-proliferative diabetic retinopathy group (*P*=0.007; OR=0.148, 95% CI: 0.031–0.706), table 3.

Discussion

In this study, association was found between *CAT*-21A/T gene polymorphism and the development of diabetic retinopathy among type 2 diabetes. For the best of our knowledge, this is the first study examined the association between the *CAT*-21A/T and diabetic retinopathy. However, three reported studies were examined the association between the *CAT* gene polymorphisms and diabetic retinopathy. Two of these studies examined the *CAT*-262C/T, one study in Russia examined the *CAT*-1167C/T polymorphism, and in the other study no associations were found [15-17]. Although, this *CAT*-21A/T polymorphism has been studied in other diseases

and found to be associated with cerebro-vascualr-accident (CVA) and asthma, no study up-to-date examined the association of *CAT-21A/T* polymorphism with development of diabetic retinopathy [18, 19].

The *CAT-21A/T* polymorphism is located in promoter region just proximal to the starting codon [13]. This polymorphism in such sensitive position supposed to affect the affinity of the transcription factors to the promoter region. Sequentially, decrease the transcriptional and expressional profile for the *CAT* gene, which leads to decrease catalase enzyme levels. Given the well-studied *CAT*-262C/T gene polymorphism located in the promoter region, patients carrying the variant TT genotype expressed low catalase enzyme activity [9]. That may lead to the development of oxidative stress, which is involved in pathogenicity of many diseases including diabetic retinopathy [20]. Interestingly, low catalase enzyme activity has been described before in-patient with diabetic retinopathy [21].

Catalase enzyme is a cytosolic enzyme that one molecule of it can decompose one million of cellular hydrogen peroxide in one second [7]. Therefore, it is essential for neutralizing the cellular reactive species, especially in cells with high oxygen consumption like retina. Doubtless, the retina is vulnerable to oxidative stress, as it has high polyunsaturated fatty acids content and exposed to environmental visible and non-visible lights [22, 23]. Oxidative stress induces retinal-pericyte cell apoptosis, which in-turn result in retinal vascular disease, a hallmark of diabetic retinopathy [24].

Our study suggested that *CAT*-21A/T gene polymorphism were associated with the development of diabetic retinopathy. As this was a preliminary study with a small sample size of patients and control, further study with larger sample size is needed to endorse this association and confirmed further by quantifying the catalase enzyme activity in relation to genotyping.

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Competing interests

The authors declare that they have no conflict of interests.

Authors' contribution

HZH and SEA carried out the study, performed the statistical analysis and drafting the manuscript. MS did the retina examinations, diagnosis of the patients and participated in the design of the study. MIE design the study, participate in the drafting of the manuscript and co-ordinate the all study. All the authors read and approved the final version.

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Table 1. Socio-demographic, clinical and biochemical characteristic of patients with diabetic retinopathy and control

Variables	Diabetic retinopathy (*n*)=50	No Diabetic retinopathy (*n*)=50	*P*-value
Age, years	58.7(11.1)	55.9(10.4)	0.200
Male gender	18(36)	13(26)	0.280
Duration of diabetes, years	16.3(9.1)	10.6(0.9)	0.201
Body mass index, kg/m^2	25.8(5.4)	27.9(6.9)	0.106
Fasting blood glucose, mg/dl	170.9(44.3)	158.9(41.2)	0.215
Serum cholesterol, mg/dl	196.6(54.3)	184.0(39.2)	0.214
Serum creatinine, mg/dl	0.93(0.28)	0.72(0.25)	0.002
Urea, mg/dl	32.6(16.4)	31.2(15.3)	0.675

Values are mean (SD) or number (%) as applicable.

Table 2. Distribution of genotype frequencies of ***CAT-21A/T*** polymorphism in patients with diabetic retinopathy and control

Genotype ***CAT*-21A/T**	**Diabetic retinopathy** ***n*=50**	**No Diabetic retinopathy** ***n*=50**	**OR (95% CI)**	***P*-value**
AA	11(22)	23(46)	0.331(0.13-0.79)	0.011
AT	21(42)	18(32)	1.287(0.57-2.88)	0.539
TT	18(36)	9(18)	2.562(1.01-6.45)	0.043
A allele frequency	0.43	0.64		
T allele frequency	0.57	0.36	2.35 (1.33-4.16)	0.003

Table 3. Distribution of genotype frequencies of ***CAT-21A/T*** polymorphism in control and patients with different diabetic retinopathy subtypes

Genotype	Control N=50(%)	NPDR+ CM N=23(%)	PDR+ CSM N=11(%)	Mild NPDR N=10(%)	NPDR+CSM N=6(%)
CAT-21 A/T					
AA	23(46)	1(15)[a]	3(11.1)[b]	6(41.6)[c]	2(22.2)[d]
AT	18(32)	11(30)	3(33.3)[e]	2(41.6)[f]	4(77.7)[g]
TT	9(18)	11(55)	5(55.5)	2(16.6)[h]	0(0)

[a]OR: 0.024 (0.003-0.187) p=<0.001; [b]OR: 0.075 (0.021-0.273) p=<0.001; [c]OR: 0.160 (0.058-0.443) p=<0.001; [d]OR: 0.049 (0.011-0.224) p= <0.001; [e]OR: 0.113 (0.031-0.417) p=<0.001; [f]OR: 0.074 (0.016-0.341) p=<0.001; [g]OR: 0.155 (0.048-0.500) p=0.001; [h]OR: 0.148(0.031-0.706) p=0.007.

NPDR+CM, severe non-proliferative diabetic retinopathy with mild macular edema; PDR+ CSM, proliferative diabetic retinopathy with macular edema; Mild NPDR, mild non proliferative diabetic retinopathy; NPDR+CSM, non-proliferative diabetic retinopathy with clinical significance macular edema. Data were expressed as number (%).

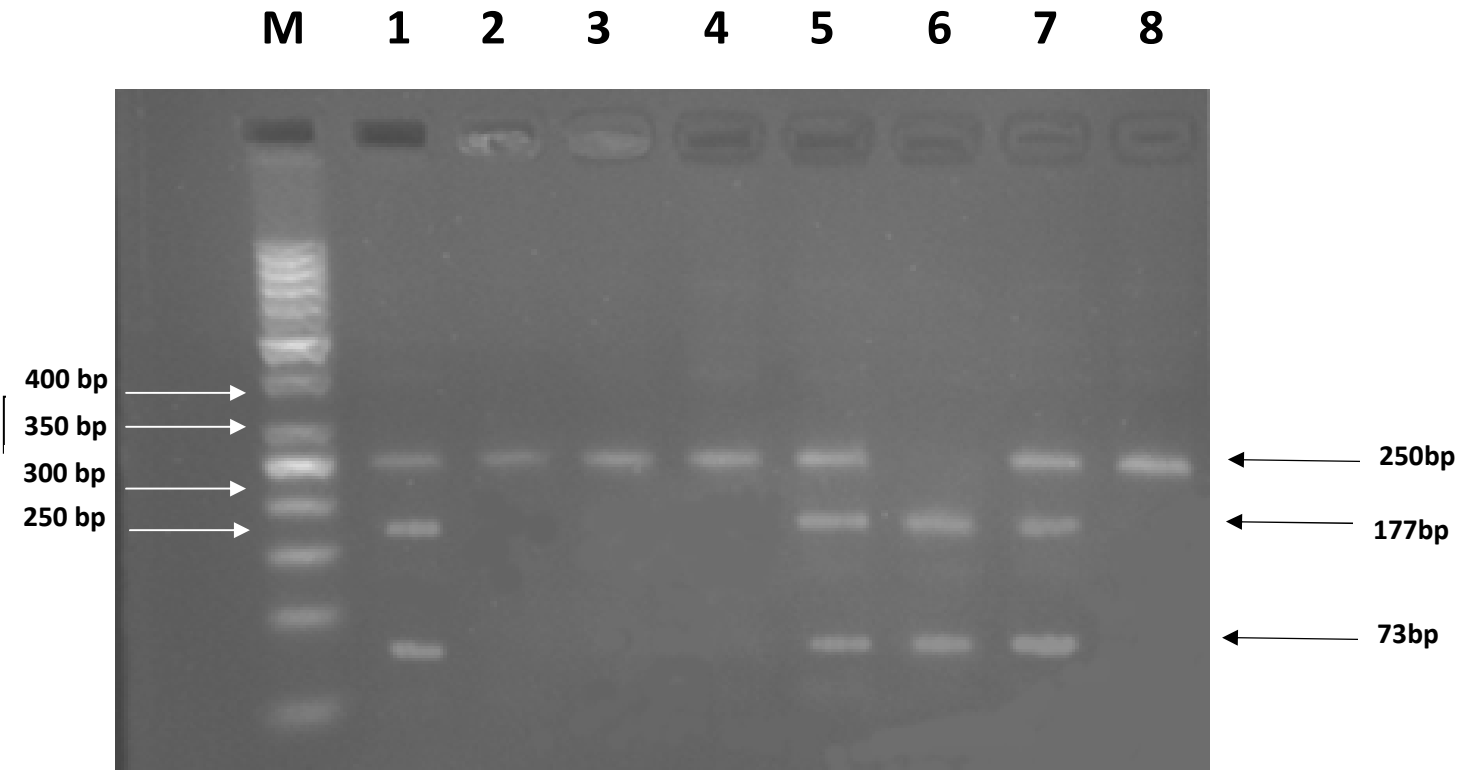

Figure 1. PCR-RFLP analysis for CAT-21 A/T polymorphism (digested with *Hinf*1). One fragment of *CAT* 250bp indicate variant homozygous (TT), two fragments of 177 and 73bp for wild homozygous (AA); and three fragments of 250, 177, and 73bp for heterozygous (AT). lane M show the DNA ladder 50 bp; lanes1, 5 and 7 CAT heterozygous (AT) genotype; 2-4 and 8 CAT variant homozygous (TT); lane6 CAT wild homozygous (AA).