Nigeria Journal of Biotechnology and Life Sciences Vol 1 Issue 2 *May 2024*
ISSN: 2014-0281

Identification of Single Nucleotide Polymorphisms in Extra Cellular Superoxide Dismutase 3 Gene in Type 2 *Diabetes mellitus*

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ABSTRACT

Diabetes is a group of metabolic disorders characterized by hyperglycemia in which glucose is underutilized due to defects in insulin secretion, insulin action, and or both. The prevalence, morbidity and mortality due to diabetes mellitus have been on increase worldwide. New methods and technologies for the diagnosis and management of the disease are therefore needed to halt and reverse the trend. The study was aimed to identify a single nucleotide polymorphism (SNP) in the extra-cellular superoxide dismutase 3 (EC-SOD3) genes in type 2 diabetes mellitus patients in MMSH, Kano. A total of 40 Type 2 Diabetes Mellitus (T2DM) patients and 10 control subjects were enrolled in the Diabetic clinic of Murtala Muhammad Specialist Hospital Kano. Questionnaires were given to the study subjects and blood sample collection and processing were done using standard procedures. Anthropometry, Body Mass Index (BMI), serum fasting glucose and lipid profiles were determined. Amplification of the EC-SOD3 gene was done using PCR and genotyping was done using PCR-based direct DNA sequencing. From the result of the PCR base direct DNA sequencing, new variants of EC-SOD 3 gene were identified. The new variants were CTT and TCT, which were identified in the untranslated region 3! Just 81 base pair after the stop codon. Result of biochemical parameters in patients with the new variants shows no significant difference in lipid profiles and slight increase with mean serum glucose to that of test Diabetes participant. The identified variants of EC-SOD3 gene in this study were checked in ensemble SNP data base for identification but no record was found which indicated they were not previously identified. The present study suggests that EC-SOD polymorphism can be associated with T2DM.

Keywords: *superoxide dismutase, SNP, type 2 diabetes.*

INTRODUCTION

All forms of diabetes have very serious effects on human health. In addition to abnormal metabolism of glucose (e.g., hyperlipidemia, glycosylation of proteins, etc.), there are a number of long-term complications associated with the disease. These include cardiovascular, peripheral vascular, ocular, neurologic and renal abnormalities, which are responsible for morbidity, disability and premature death in young adults (American diabetes Association, 2018)

Type 2 diabetes is due primarily to lifestyle factors and genetics. A number of lifestyle factors are known to be important to the development of type 2 diabetes, including obesity, lack of physical activity, poor diet, stress, and urbanization (Wikipedia, 2020) The world is experiencing a definite surge in the prevalence of diabetes mellitus from 366 million people in 2011, which is expected to increase to 522 million by 2030 (Gokulnath et al, 2013). Use recent information…. We have data report of IDF 2020

In Africa, the estimated prevalence of diabetes is 1% in rural areas, up to 7% in urban Sub-Sahara Africa, and between 8 – 13% in more developed areas such as South Africa and in population of Indian Origin. The prevalence in Nigeria varies from 0.65 in rural Mangu (North) to 11% in urban Lagos (South) (Sonny, et al., 2011). Optimal blood pressure for prevention of cardiovascular (CV) events in patients with Type 2 diabetes mellitus (T2DM) remains uncertain and there is concern for increased risk with low diastolic blood pressure (DBP). This study analysed the association between blood pressure and CV outcomes in high-risk patients with T2DM (Brain et al., 2018).

There is a clear need to understand the genetic basis for the regulation of food intake, energy expenditure, and variations in energy balance in various individuals. In the long run, it may be more beneficial to develop treatments based on these genetic mechanisms than to rely on the use of will power to modify lifestyle. We know from the natural history of diabetes complications that when patients are first diagnosed, they may already be marked progression of microvascular and macrovascular complications (Sophia et al., 2017).

Oxidative stress plays a pivotal role in cellular injury from hyperglycemia. High glucose level can stimulate free radical production. Weak defence system of the body becomes unable to counteract the enhanced ROS generation and as a result condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress. Superoxide dismutase (SOD) is the antioxidant enzyme that catalyses the dismutation of superoxide anion (O-2) into hydrogen peroxide and molecular oxygen (Patrik et al., 2017).

Superoxide reacts rapidly with nitric oxide (NO), reducing NO bioactivity and producing the oxidative peroxynitrite radical. SOD, a major defender against superoxide, in the kidneys during the development of murine diabetic nephropathy and down regulation of renal SOD (SOD 1 and SOD 3) may play a key role in the pathogenesis of diabetic nephropathy. The elevated level of SOD is shown to reduce oxidative stress; decrease mitochondrial release of cytochrome C and apoptosis in neurons; and, in mice, prevent diabetes-induced glomerular injury, thus suggesting a major role of SOD in the regulation of apoptosis (Elia et al, 2018).

Increased oxidative stress as well as reduction in antioxidant capacity could be related to the complications in patients with diabetes such as oxidative DNA damage and insulin resistance. Due to decrease in antioxidant potential of plasma, complications of diabetes increase which include cardiovascular disease, nerve damage, blindness, and nephropathy. Thus, the increasing incidence of diabetes is a significant health concern beyond the disease itself (Styskal. et al, 2012).

METHODOLOGY

This is a cross sectional descriptive study using convenient sampling technique. A total of fourty (40) Type 2 Diabetic mellitus attending diabetic clinic of Murtala Muhammad Specialist Hospital and Tenth (10) as apparently healthy individuals as control were randomly recruited for the study.

Ethical approval was sought from the Ethical Committees of Kano stste ministry of health. Informed consent was also sought from all subjects in accordance with the ethical guidelines of the Ethics Committees of the said state ministry of health, kano. The nature of the study was fully explained to the subjects in an appropriate language before inclusion in the study and collection of blood sample. A questionnaire was developed for the study and was administered to each subject including medical history, social habits, health status and familial history of diabetes.

Anthropometric measurement- Height (m) was measured using a standard hospital scale with the subject barefooted. Body weight (kg) was taken with the subject in light underwear using standard hospital scale. Waist circumference (cm) was measured at the level of the naval with the subject standing and breathing normally. Body mass index (BMI) was calculated as weight (kg)/ height (m2) and these parameters were recorded according to Nutall (2015).

Study protocol: Subjects that volunteered to take part in the study were instructed to be on their normal diet and observe an overnight fasting before sample collection for fasting glucose.

Sample Collection, Processing and Preservation: Five milliliters (5mLs) of blood sample were collected from each subject through the ante-cubital vein after an overnight fast lasting between 10 – 12 hours. The sites of collection was aseptically cleaned with 70% alcohol swab and allowed to dry before blood was collected. Three (3) milliliters of the blood was transferred into fluoride oxalate bottles for immediate fasting glucose analysis and the remaining harvested plasma was stored at - 200C for future analysis. The remaining 2mLs of whole blood was transferred into an ethylene diamine tetra-acetic acid (EDTA) bottle for DNA Extraction and Polymerase chain reaction (PCR) for each subjects.

Analytical Methods

Serum glucose was determined using glucose oxidase method by Trinder (1969), serum lipid profiles for total cholesterol, triglyceride, HDL-C were determined using the method by wybenga et al., (1970), while LDL-C was determine using friedewald equation.

DNA Extraction: DNA was isolated from peripheral blood samples, using QIAamp DNA Blood Mini Kit (QIAGEN, USA) following the manufacturer instruction.

SOD 3 gene Amplification (DNA Amplification)

The amplification was done using Promega PCR Master Mix (Promega M7502). PCR was carried out in a 25 µl reaction mixture, including 2.0 µL of DNA, 0.51µl each of forward primer CGGGCGACTTCGGCAACTT and reverse primer: GAGAGGGCTGCGGGGAGAC that were purchased. The primers were adopted from similar studies not synthesize, PCR program were performed with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The amplicon products were determined using a 1.5% agarose gel electrophoresis.

Agarose Gel Electrophoresis procedure

The gel was prepared using 1.5g of agarose powder dissolved in 100mills of tris acetate buffer and boiled in micro oven. It was then cooled to 60 0C and small amount of ethidium bromide as intercalating agent (to allowed visualization using UV light) was added, the mixture were mixed. The mixture was poured on caterage for solidification and comb was put on the caterage containing the mixture for the formation of holes. The solidified gel was put inside electrophoretic tank containing the buffer inside. The ladder that will calibrate the different size of the gene was put at the first hole, each amplicon (3µl) were mixed with loading dye (Bromophenol or 5X DNA loading) and dispensed inside the hole after which the electrophoretic tank was closed and run using 110V for 35minutes. The gel was taken and visualized using gel documentation system.

After running the electrophoresis and in visualized using UV light, out of 50 samples processed for PCR, only 37 seen to be amplified.

SEQUENCING OF PCR PRODUCT

The analysis of the EC-SOD genotypes was performed by polymerase chain reaction (PCR) followed by DNA sequencing. The following specific primers that been purchased were used: Forward primer: 5'- Primers CGGGCGACTTCGGCAACTT Reverse: GAGAGGGCTGCGGGGAGAC, DNA samples were amplified by PCR.

The amplified samples (37) were sent for Sequencing, only 27 were able to be sequenced out of which 24 were diabetic subjects and 3 control subjects. The sequenced products were compared with the sequence extracted from National Centre of Biotechnology Information (NCBI) (from internet) for SOD 3 gene. The sequence indicate the start codon (ATG) and stop codon (TGA) of SOD 3 gene. The positions of the two site mutations detected of the sequenced products compared with that of internet were CCT at (251 amino acid) and TTT at (260 amino acid) which led to mutations (new variants) of CCT to CTT (251 amino acid) and TTT to TCT(260 amino acid) respectively at the 3'UTR region, as seen in figure 3.1. And only 4 samples were found to have mutations out of the 27 samples been sequenced, which are within diabetics subject not control.

Statistical analysis

The data obtained were presented in the form of tables and figures. The results were analyzed using Microsoft excel broad sheet and statistical software for social sciences (SPSS) version 20.

The results of anthropometry and biochemical parameters obtained from diabetic patients were compared with those of controls using student t-test. Oneway anova statistical method posthoc test (HSD) Turkey was used to determine the differences in mutant diabetics, non-mutant diabetics and non-diabetics (control). All statistical differences were analysed at $p < 0.05$.

Results

The results of biochemical parameters for T2DM and controls were shown in Table 1. There were significant differences in serum glucose, total cholesterol, LDL-Chol and triglyceride between T2DM and controls group. Mean serum HDL-C (1.31±0.39 mmol/L) was significantly lower in patient than in controls (2.17±0.33 mmol/L) (p=0.010).

Table 1: Serum Lipid profiles and blood glucose levels (Mean ± SD) in Diabetic Patients and Non-Diabetics

Subject	N	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	GLUC (mmol/L)
Non-Diabetics	10	4.94±0.43	1.01±0.34	2.17±0.33	2.27±0.48	^{a}4.36±0.66
Diabetics	40	5.36±0.58	1.71±0.80	1.31±0.39	3.22±0.66	^{b}9.62±1.87

TC=Total Cholesterol, TG=Triglyceride, HDL-C=High density lipoprotein, GLUC=Glucose, LDL-C=High density lipoprotein (P<0.05)

The results of Serum Lipid profiles and blood glucose levels for diabetic patients in mutant and wild type (diabetic and non- diabetic) were shown in Table 2. The mean serum glucose in mutant diabetics (9.78±2.24) has no significant difference when compared with wild type (non- mutant diabetics) (9.12±1.70), but they all have a great significance compared with non-diabetics (Control). There were no significant differences in BMI, serum total cholesterol, LDL-Chol and triglyceride between mutant and wild type group (of non-mutant diabetics and control). A significance difference were observed in serum Glucose and Age, were by mutants have higher values compare to non-mutant diabetics and highest significant than non-diabetics (control).

Table 2 Serum Lipid profiles, AGE, BMI and blood glucose levels (Mean ± SD) in Mutant and Wild type.

Genotype	AGE (Yrs)	BMI (kg/m²)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	GLUC (mmol/L)
Mutant CTT	^{a}64.25 ±11.79	23.09±4.23	4.95±0.35	1.75±0.42	1.82±0.36	3.57±0.66	^{a}9.78±2.24
Wild type: Diabetes n=20	^{a}57.75 ±9.90	25.59±6.24	5.39±0.67	1.87±1.03	1.34±0.40	3.20±0.73	^{a}9.12±1.70
Non-diabetic	^{b}22.33 ±3.21	25.89±6.83	5.27±0.32	1.59±0.41	1.47±0.59	3.08±0.67	^{b}4.37±0.35

TC=Total Cholesterol, TG=Triglyceride, HDL-C=High density lipoprotein, GLUC=Glucose, LDL-C=Low density (P<0.05)

The results for the analysis of the variant of EC-SOD gene in T2DM and control is presented in Figure 1 and 2, respectively. Samples were analysed for the presence of EC-SOD3 gene which is to have a molecular weight of 430bp.

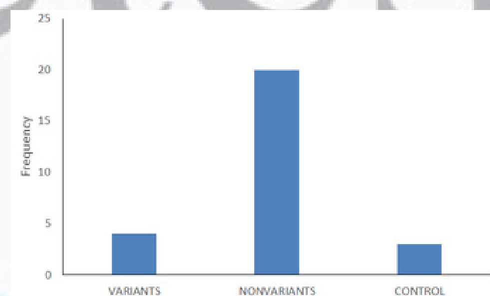

Figure 1 : Prevalence of the variant of EC-SOD gene in T2DM and control

The expected size of the PCR product is 430 bp and was analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide. DNA molecular size 100markers in base pairs is shown on the left. The an amplified samples may be either as a result of destruction of nucleic acid by the DNase in the course of sample handling / preparation, or may be the omission of the primer specific to the target gene during master mix preparation. This may applied to all the remaining Figure with gene not been amplified.

After running the electrophoresis and in visualized using UV light, out of 50 samples processed for PCR, only 37 seen to be amplified.

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Genotype	AGE (Yrs)	BMI (kg/m^2)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	GLUC (mmol/L)
Mutant **CTT**	^{a}64.25 ±11.79	23.09±4.23	4.95±0.35	1.75±0.42	1.82±0.36	3.57±0.66	^{a}9.78±2.24
Wild type: Diabetes n=20	^{a}57.75 ±9.90	25.59±6.24	5.39±0.67	1.87±1.03	1.34±0.40	3.20±0.73	^{a}9.12±1.70
Non-diabetic	^{b}22.33 ±3.21	25.89±6.83	5.27±0.32	1.59±0.41	1.47±0.59	3.08±0.67	^{b}4.37±0.35

TC=Total Cholesterol, TG=Triglyceride, HDL-C=High density lipoprotein, GLUC=Glucose, LDL-C=Low density (P<0.05)

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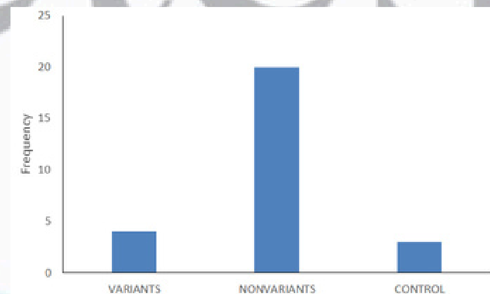

Figure 1 : Prevalence of the variant of EC-SOD gene in T2DM and control.

The presence of the corresponding EC-SOD3 gene (430p) was observed on the amplified samples(Figure 2).

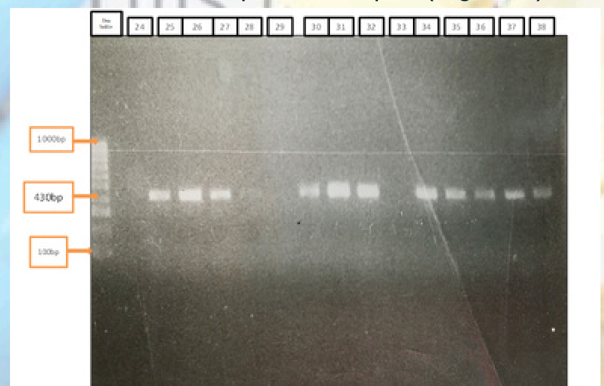

Figure 2.0 Gel electrograph showing the presence of EC-SOD3 gene.

DISCUSSION

The mean age of the T2DM patients and controls were 58.17 ± 10.63
years and 22.00 ± 1..69 years, respectively and this shows that the mean age of diabetic is greater than that of the control and this is similar to what other research found out (Sudesna et al, 2017). This indicate that the development of type2 diabetes is associated with age showing that type 2 diabetes is commoner among older people than the young ones and this also support the assumption that oxidative stress is associated with development of type2 diabetes as the older individual are exposed to more oxidative stress than the control (Ray et al, 2018). From table 4.1, there is significant difference between age in type 2 diabetes in comparison with non-diabetes, as older people are more front to diabetes which hold true looking at similar research conducted in Iraq (Faiza et al, 2017) shows the mean age of the T2DM patients and controls were 41.32 ± 11.39 years and 40.13 ± 10.28 years, respectively. The age of onset in T2DM patients was 44.05 ± 11.20 years. Likewise from the same table, the mean BMI of Diabetic is 24.35±5.27kg/m2 and that of the control is 22.80±4.61kg/M2. This shows that there is no significant difference between the mean BMI of diabetic and that of non-diabetics which is contrarily differed from what is obtained in other similar study like that obtain by a study in Iraq (Zinah A.U and Mahmood S.A., 2011) where they obtained a value higher among type2 diabetic compare to the non-diabetics.

With the sequenced result in table 4.3 above, we identified that individuals carrying CTT and TCT have an increased risk of type 2 diabetes mellitus in comparison to the non-diabetics, this can be seen from our present studies in which there is no significant difference in serum fasting glucose level of the in identified mutants (9.78±2.24) and that of non-mutant diabetics (9.12±1.70), but great significance was observed in comparison with wild type non-diabetics (4.37±0.37). The two mutants detected that of CTT and TCT in each sample were indicated in the sequence of the SOD3 gene at 3! Utr region which is unusual when compare to the other different types of SNP findings which are mostly found in the codon region (Yangi, 2016).

Most striking findings of this investigation is the emergence of direct correlation between the mean serum glucose and severity of the genotype of mutant i.e. serum glucose was found to be increased with SNP of this mutant CTT and TCT (9.78±2.24 mmol/L), whereas mean serum glucose (4.37±0.37 mmol/L) has been found to be depressed in the wild type of non-diabetics. Also from the table 4.3 there were no significant differences in serum total cholesterol, triglyceride, HDL-cholesterol, LDL-Cholesterol and BMI between mutants and wild type groups. Mean serum HDL-C (1.18±0.36 mmol/L) was significantly lower in mutant than in wild type of non-diabetics (1.47±0.59 mmol/L) (p=0.016), this may be due to the fact that, most of the patients are on follow up and medication.

Present studies still shows that sample of the diabetics for genotype of mutant have high mean value of AGE (68.00±7.25yrs) when compare with wild type (54.80±1.54yrs), this is in line with pathophysiology of human, as the age is increasing, there is tendency for body to be associated with so many risk factor among which is diabetics (Fatima et al., 2017).

Also in all the reported findings of EC-SOD polymorphism in association with T2DM were founds in codon region unlike our study which is in utr region 3!. This study was subject to a limitation. The power of this study could not enable us to establish a protective role or other wise of the two (2) mutation detected in four (4) samples. To the best of our knowledge these mutation detected in the utr region of SOD gene has not been reported. Further studies using larger sample size is recommended to evaluate the association between EC-SOD polymorphisms and the development of T2DM.

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