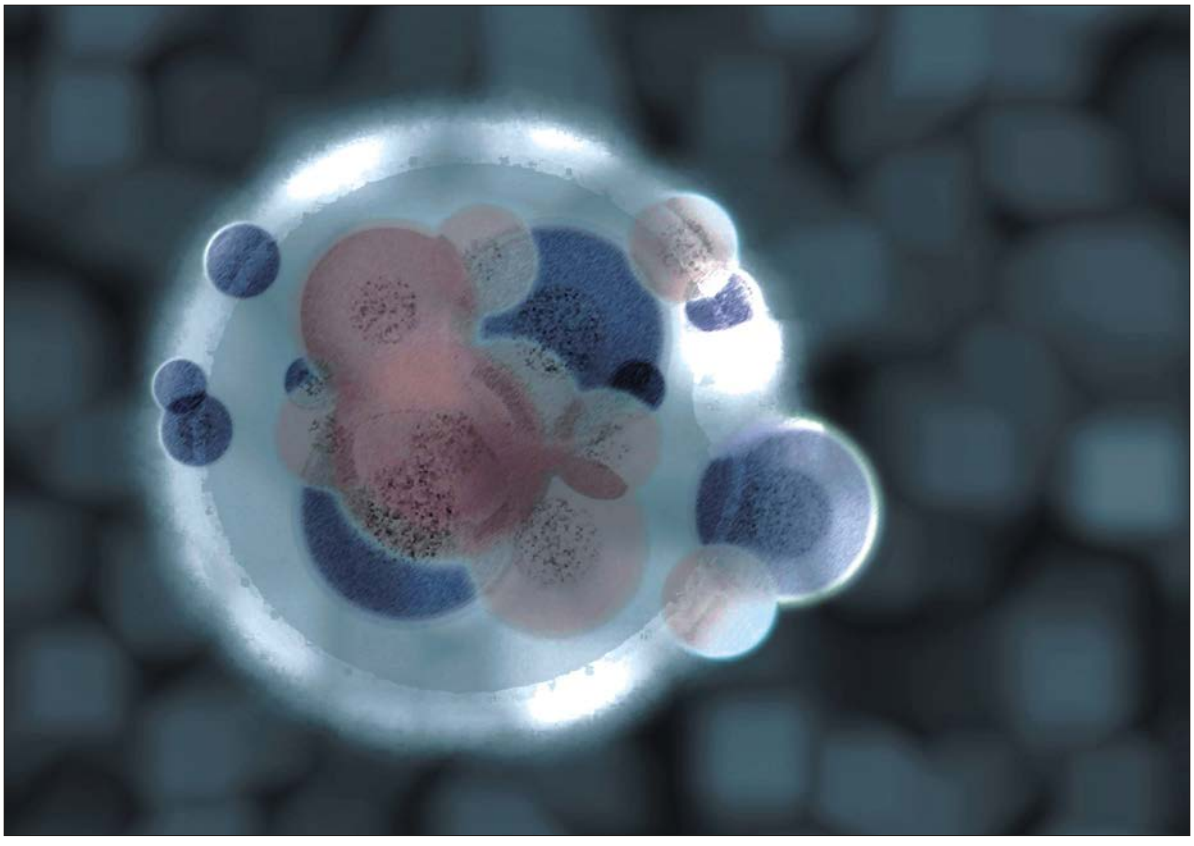

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# A Study on Catalase Activity and its Genetic Polymorphism in Diabetes Mellitus Patients

K. Dhanapal, N. Selvan and V. Dhananjeyan
Centre for Biotechnology, Muthayammal College of Arts and Science,
Rasipuram-637408, Tamilnadu, India

**Abstract:** This study aimed to investigate on catalase activity and genetic polymorphism in diabetes patients, the blood sample were collected from 20 type II diabetic patients attending the outpatient department and admitted to the RMMCH. The age of patients ranged from 40-65 years of both sexes. Reactive oxygen species generated by hyperglycemia modify structure and function of lipids, proteins and other molecules. This study was undertaken to investigate the association between gene polymorphisms of selected antioxidant enzymes. The 20 type 2 diabetes mellitus patients were analyzed for SNP in catalase gene. Restricted fragment length polymorphism studies showed 80% of the patients were TT genotype and 20% were of heterozygous genotype. No AA genotype was observed in our group of study. Observed that patients with T allele had significantly lower fasting plasma glucose, RBC catalase activity and HbA1c where as heterozygous had higher FPG, RBC catalase activity high and HbA1c. It is suggestive of possible association of heterozygocity with poor glycemic control and high catalase activity. It has been observed that the level of enzyme was found to be significantly high in heterozygous group. High (concentration) activity may be secondary to high oxidative stress as a result of poor glycemic control. In heterozygous patients the association of high catalase activity with this SNP should be carefully considered because early study has reported that there is no statistically significant difficult association of CAT alleles and this activity.

**Key words:** Blood samples, estimation of hemoglobulin, RFLP analysis, PCR amplification, estimation of catalase, estimation of blood glucose

## INTRODUCTION

The incidence of diabetes mellitus increases from 2% in the fourth decade to 10-15% in the sixth decade. It is estimated that over 16 million Americans are already caught with diabetes and 5.4 million diabetics are not aware of the existing disease. Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels (Gavin *et al*., 1999). The incidence of diabetes mellitus increases from 2% in the fourth decade to 10-15% in the sixth decade. It is estimated that over 16 million Americans are already caught with diabetes and 5.4 million diabetics are not aware of the existing disease. A general observation says that about 90-95% of people suffering from diabetes are type 2; about 80% are overweight. It is more common among people who are older; obese; have a family history of diabetes (Panzram, 1988). The complications are directly associated with hyperglycemia, regardless of the different pathogenesis of type 1 or 2 diabetes. The complications can be divided into 2 types (DCCT Research Group, 1993) (1) acute complications and (2) chronic complications. Hydroperoxidases (catalases and peroxidases) protects the body against harmful peroxides. Catalase is a hemoprotein containing four heme groups and is found in blood, bone marrow, mucous membrane, kidney and liver. It uses hydrogen peroxide as electron acceptor and electron donor. Its function is assumed to be the destruction of hydrogen peroxide:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Catalase is a common enzyme found in nearly all organisms which are exposed to oxygen where it functions to catalase the decomposition of hydrogen peroxide to water and oxygen (Goth, 1991).

Sources of catalase, normal aerobic cells contain catalase activity although, a few do not, such as the

**Corresponding Author:** K. Dhanapal, Centre for Biotechnology, Muthayammal College of Arts and Science, Rasipuram-637408, Tamilnadu, India

bacterium bacillus popilliae, mcoplasma pneumonia, the green alga euglena, several parasitic helminthes (e.g., the liver fluke) and the blue-green alaga gloeocapsa. A few anaerobic bacteria, such as propionibacterium shennani, contain catalase. In animals catalase is present in all major body organs, being especially concentrated in liver. Catalse in erythrocytes may help protect them against $H_2O_2$ generated by dismutation of O generated by haemoglobin autoxidation. Since, $H_2O$ diffused readily, erythrocytes can also protect other tissues against oxidative damage b absorbing $H_2O_2$. The brain, heart and skeletal muscle contain lower level of catalase than liver (Fita *et al*., 1986).

Gene polymorphism, we still have to elucidate the fact that some patients with DM develope vascular complications but this cannot be seen in the others with the same level of disease control (Fumeron *et al*., 2006) We have focused on the genes encoding superoxide dismutases and catalase. Specifically, we have focused on SNPs for their likely functional role: SOD1 + 35A/C (ref-SNP ID: rs2234694) which is located adjacent to the splice site (exon3/intron3 boundary), SOD2 Ala16Val (refSNP ID: rs4880) which has been suggested to alter protein structure (Kinnula *et al*., 2004) and function (C/T substitution in exon 2, codon position 2, amino acid position 16) and catalase-21A/T which is located inside the promotor region just proximal to the start site.

## MATERIALS AND METHODS

**Sample collection:** Blood samples were collected from 20 type II diabetic patients attending the outpatient department and admitted to the RMMCH. The age of the patients ranged from 40-65 years of both sexes. Blood was collected in EDTA tube for estimation of glycolated haemoglobin and in fluoride oxalate tube for estimation of blood glucose. All estimations were carried out within 4-6 h of collecting the sample.

**Hemoglobin A1C test:** Fifty microliter of blood was added to 200 µL reagent 1. Mixed well and left for 15 min at room temperature. The hemolysate was used for further assay. The column was allowed to drain completely to waste. Fifty microliter of hemolysate was pipetted on the upper filter. Two hundred microliter of reagent 2 was pipette and allowed to the column to drain to waste. Again 2 mL of reagent 2 was pipette and the column was allowed to drain the waste. This was placed over a collecting tube and 4.0 mL of reagent 3 was pippeted and, the elute was collected. The elute, was shaken well and the absorbance of HbA1c was measured against distilled water at 415 nm. For Hb total, 50 µL of hemolysate was added to 12 mL of reagent 3 and shaken well. The absorbance was read against distilled water at 415 nm.

**Isolation of genomic DNA from whole blood:** Three hundred microliter of whole blood was taken in 1.5 mL glass tube to that 900 µL of cell lysis buffer was added. The samples were kept in ice for 10 min and centrifuged for 10 min at 8000 rpm at 4°C the pellets were resuspended in 900 µL of cell lysis buffer and keep in ice for 5 min. Samples were centrifuged for 10 min at 8000 rpm at 4°C and the supernatant was collected and incubated at 65°C for 30 min. The discarded samples were centrifuged for 10 min at 8000 rpm at 4°C and the supernatant was collected and added thrice volume of ethanol to supernatant and keep in ice for 5 min. The tube was again centrifuged for 10 min at 8000 rpm at 4°C the supernatant was discarded. Dried pellet and dissolved in 20 µL of TE Buffer extracted DNA was quantified using UV spectrophotometer against standard at 260 nm. The DNA was stored at -20°C until the amplification (Table 1).

**Catalase gene PCR amplication:** PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although, some techniques allow for amplification of fragments up to 40 kb in size. Master mix containing Taq polymerase, dNTP, MgCl2, forward prime, reverse primer, template DNA and distilled water were pipette into a 200 µL PCR tube according to the table given in Table 2. The target DNAs were amplified by 30 cycles according to the Table 3.

Table 1: Isolation of genomic DNA from whole blood cell components and storage conditions

| Components | Quantity (mL) | Storage |
|---|---|---|
| Cell lysis buffer | 125 | RT |
| DNA Extraction buffer | 15 | -20°C |
| Ethanol | 30 | RT |
| TE Buffer | 1 | RT |

Table 2: Catalase gene PCR amplification components

| Components | Volume | Volume | Final concentration |
|---|---|---|---|
| 5X Master Mix | 10 µL | 5.0 µL | 1X |
| Forward Primer | 3.0 µL | 1.5 µL | 0.4 µm |
| Reverse Primer | 3.0 µL | 1.5 µL | 0.4 µm |
| Template DNA | 1.0 µL | 1.0 µL | 5-100 ng |
| Distilled water | Up to 50 µL | Up to 25 µL | |

Table 3: PCR cyclic condition

| Operation | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 3-5 min | 1 |
| Denaturation | 95 | 30 sec | 30 |
| Annealing | 55 | 30 sec | 30 |
| Elongation | 72 | 30 sec | 30 |
| Final elongation | 72 | 5-10 min | 1 |

Held at 4°C

**RFLP analysis:** The single nucleotide polymorphism catalase 45 to detect the RFLP process. The HinfI restriction enzyme used. The RFLP product conditions to give 37°C at 16 h, this result was given by Table 4.

**Analysis of PCR products by submarine gel electrophoresis:** DNA is a negatively charged molecule due to its phosphate groups, when subject to electric field it moves towards anode through a matrix of agarose. The nucleic acid fragments separated can be visualized directly by Ethidium Bromide (EB) staining. EB is a intercalating fluorescent dye which binds with DNA. This EB-DNA complex fluoresces when exposed to ultraviolet light. 1% of agarose gel was prepared in 1×TBE buffer. It was bored in a water bath until the gel became translucent. It was removed from the water bath when the temperature was about 60°C added Ethidium bromide at the final concentration of 0.5 μg $mL^{-1}$. The comb was removed carefully and samples, controls and ladder were loaded into the wells (Fig. 1).

**Estimation of catalase:** The activity of catalase in the human blood cells was determined by the method of Sinha (1972). The 0.9 mL of phosphate buffer, o.1 mL of hemolysate and 0.4 mL of hydrogen peroxide was added. The reaction was arrested after 15, 30, 45 and 60 sec by adding 2.0 mL of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was read at 620 nm.

Table 4: Detection details of SNP catalase 45 T>G

| Restriction enzymes | HinfI |
|---|---|
| RFLP conditions | 37°C/16 h |
| RFLP product length | |
| In presence a allele | 203 bp a 47 bp |
| In presence of T allele | 250 bp |

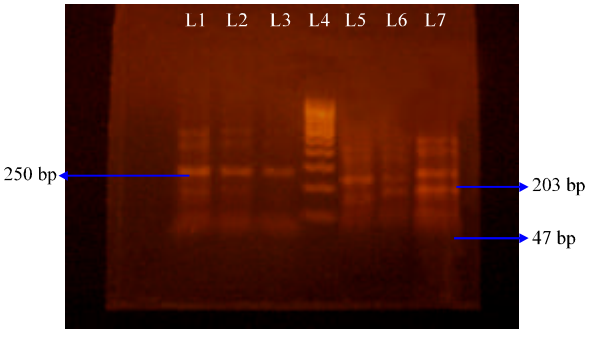


Fig. 1: Agarose gel showing the amplification 250 bp sized product. L1-L3: T allele (contain DNA fragment of length 250 bp this represent TT genotype); L4: 100 bp DNA ladder (marker); L5-L7: Hetrozygous (contain 3 DNA fragments of length 250, 203 and 47 bp which denotes heterozygous allele)

**Estimation of blood glucose:** Blood glucose was estimated b the method of o-toluidine using 0.1 mL of freshly drawn blood was immediately mixed with 1.9 mL of 10% TCA to precipitate the proteins and then centrifuged. One milliliter of the supernatant was mixed with 4 mL of 0-toludine reagent and was kept in boiling water bath for 15 min. The green color developed was read colorimetrically at 620 nm. A set of standard glucose (20-100 μg) were treated simultaneously along with reagent blank. Glucose concentration is expressed as mg $dL^{-1}$ blood.

**Estimation of hemoglobin:** Twenty microliter of hemolysate was added to 5 mL of drabkins reagent, mixed well, kept for 10 min and read at 540 nm along with cyanomethemoglobin standard solution and reagent blank. The hemoglobin content of hemolysate was measured. The hemoglobin content was expressed as g $dL^{-1}$ of hemolysate.

## RESULTS

Restricted fragment length polymorphism analysis represented at Table 4. In this studies showed 80% of patient in the study group had TT (homozygous) genotypes and 20% had TA (heterozygous) genotypes. No AA genotypes were present in our group of study (Table 5). Then the comparison parameters like age, BMI, duration and blood pressure, no significant difference was observed between homozygous and heterozygous group. When compared to homozygous genotype heterozygous group had a significantly high level of fasting plasma glucose level (Table 6). The glycated haemoglobin (HbA1c) a measured of glycemic control was found to be significantly elevated in heterozygous group, when

Table 5: Restricted fragment length polymorphism studies

| Restriction endonuclease enzyme | No. of patients | Amplicon length (bp) | Genotype |
|---|---|---|---|
| HinfI | 24 | 250 bp | T allele 80% |
| | NIL | 203 bp 47 bp | A allele NIL |
| | 6 | both | Heterozygous 20% |

Table 6: Baseline characteristics of type 2 diabetes patients

| Parameter | T allele | Heterozygous | p-value |
|---|---|---|---|
| Age | 36.0±7.4 | 38.0±8.5 | NS |
| BMI weight in kg/hight in $m^2$ | 26.1±1.4 | 25.4±1.8 | NS |
| Duration of diabetes (in years) | 3.4±2.20 | 3.7±2.80 | NS |
| Systolic BP | 126.0±14.2 | 131.0±15.8 | NS |
| Diastolic BP | 86.0±4.80 | 84.0±5.9 | NS |
| FPG | 111.0±57.2 | 220.0±7.64 | <0.05 |

Table 7: HbA1c catalase enzymatic activity

| HbA1c | Homozygous | Heterozygous | p-value |
|---|---|---|---|
| HbA1c | 8.800±1.4 | 9.400±1.3 | <0.05 |
| Catalase | 5.608±0.398 | 5.973±0.219 | <0.05 |

compared to TT genotype. Catalase enzyme activity was measured by Sinha (1972). It has been observed that the level of enzyme was found to be significantly high in heterozygous group (Table 7).

## DISCUSSION

India has highest prevalence of diabetes in world. The Incidence of diabetes mellitus is increasing, especially in developing countries like India. A cross-sectional clinic based study on 2312 children and adolescents (aged b18 years; 45% males) from 96 pediatric diabetes centers in Australia, China, Hong Kong, Indonesia, Japan, Malaysia, Philippines, Singapore, South Korea, Taiwan and Thailand was conducted. Clinical and management details were recorded and finger-pricked blood samples were obtained for central glycated hemoglobin (HbA1c), Craig *et al.* (2006). We are following the same method to perform in glycemic method.

The increasing incidence is due to environmental in influence and genetic susceptibility of individuals. Genetic susceptibility of analysis by doing single nucleotide polymorphism study can give an idea about the susceptibility of an individual to develop diabetes mellitus.

In the present study, 30 type 2 diabetes mellitus patients were analysed for SNP in catalase gene. It has been observed that 80% of the patients were TT genotype and 20% were of heterozygous genotype. No AA genotype was observed in our group of study.

Nineteen normotensive subjects with type 2 DM, 37 hypertensive (diastolic blood pressure 90 mm Hg or more) subjects with type 2 DM and 25 normotensive control subjects with normal glucose tolerance were selected for this study. Superoxide dismutase (SOD), catalase and basal-stimulated PON activities were measured by the methods of different researchers, other lipid parameters were determined using an autoanalyzer described by Sozmen *et al.* (1999).

In present study, it has been observed that patients with T allele had significantly lower fasting plasma glucose, RBC catalase activity and HbA1c where as heterozygous had higher FPG, RBC catalase activity high and HbA1c.It is suggestive of possible association of heterozygocity with poor glycemic control and high catalase activity.

Reactive oxygen species generated by hyperglycaemia modify structure and function of lipids, proteins and other molecules taking part in chronic vascular changes in Diabetes Mellitus (DM). Low activity of scavenger enzymes has been observed in patients with DM. Protective role of scavenger enzymes may be deteriorated by oxidative stress. This study was undertaken to investigate the association between gene polymorphisms of selected antioxidant enzymes and vascular complications of DM described by Flekac *et al.* (2008). In this present investigation we are used in same method.

High (concentration) activity may be Secondary to high oxidative stress as a result of poor glycemic control. In heterozygous patients the association of high catalase activity with this SNP should be carefully considered because earlier study has reported that there is no statistically significant difficult association of CAT alleles and this activity.

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