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Full Length Research Paper

Association of Genetic Polymorphism of Superoxide Dismutase and Catalase and It's their Enzyme Activity with Type 2 Diabetes in Vindhya Region Population

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ABSTRACT

Type 2 diabetes mellitus is a metabolic disorder distinguished by enlarged production of free radicals and oxidative stress. Oxidative stress can be described as a disparity between the removal of free radical generation and modify activity levels of antioxidant enzymes. Antioxidant enzymes mess about essentials characters in cellular defense and may be apply as significant biomarkers for Type 2 diabetes. The aim of the present study was undertaken to evaluate three genetic polymorphisms viz. SOD1 35A/C, SOD2 4 7C/T, and Catalase 262 C/T and it's their enzyme activity in 450 Type 2 diabetes cases and 470 healthy controls from Vindhya region,The antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT) in Type 2 diabetes patients and healthy subjects in a Vindhya region population. DNA was isolated from blood samples and genotyping was done by PCR-RFLP. Data were analyzed by using Prism software (v 5.01) and expressed as Mean ± SE. All other biochemical parameters showed highly significant association in Type 2 diabetes cases (P = 0.001). In Vindhya region population, SOD1 3 5A/C variant was monomorphic. Genotype/allele frequencies of SOD2 47C/T polymorphism and carriage rate of 'C' allele showed significant association (p <0.05, p< 0 .001; OR 2.434). Genotype/allele frequencies of Catalase 262 C/T and carriage rate showed no association although the odds ratio of Catalase 262 C/T 'C' allele indicated a 1.362 times higher risk of Type 2 diabetes. SOD2 'CT' and Catalase 262 C/T'CC' genotypes showed maximum association with biochemical parameters. Haplotypes/gene-gene interaction analysis in controls and cases showed that SOD2 47C/T and Catalase 262 C/T were in linkage disequilibrium (D: 0.168; r 2: 0.10) and individuals with this combination had a 1.273 times higher risk [OR; CI (95%)] of developing Type 2 diabetes. Blood lysates were processed and levels of antioxidant enzymes viz. SOD and CAT were measured by spectrophotometric method. The activity levels of SOD and CAT were significantly lower in Type 2 diabetes patients than in healthy subjects (P<0.05). Thus, we conclude that it is essential to assess the combinatorial association of gene variants with Type 2 diabetes in order to identify risk haplotypes in a population. Analysis of data also showed that enzyme activity levels decreased with increasing age both in normal and Type 2 diabetes conditions. Both SOD and CAT level showed significant decrease in diabetic females compared than in diabetic males. Assay of enzyme activity levels in erythrocytes could be used as markers to distinguish individuals predisposed to Type 2 diabetes

Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder derived from oxidative stress (OS) due to disablement in antioxidant enzymes (AEs) and their scavenging activities against reactive metabolites (RMs). Oxidative stress is a circumstance of exuberance production and inefficient removal of RMs by endogenous AEs which include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione S-transferase (GST). RMs reacts with lipids, proteins, and other molecules of human body resulting in different damaging pathways. Functional polymorphisms in AEs cause diminish or no activity of antioxidant

enzymes leading to insulin resistance, β -cell dysfunction, impaired glucose tolerance, Type 2 diabetes, and related complications [1]. The endogenous control of cellular RM levels is made by SOD enzymes. SODs have the capacity to dismutate the superoxide to hydrogen peroxide (H 2 O 2) which is further catalyzed to form H 2 O and O 2 by enzymes such as catalase (CAT). The Super Oxide Dismutase enzyme family contains CuZn-SOD (SOD1; EC 1.15.1.1) located in cytosolic, nucleus, and lysosomes, Mn-SOD (SOD2; EC 1.15.1.1) located in mitochondrial matrix, and EC-SOD (SOD3; EC 1.15.1.1), bound tomatrix and extracellular space proteoglycans. These genes are located on different chromosomes, SOD1 on 21q22.11, SOD2 on 6q25.3, and SOD3 on 4p15.3-p15.1 [2, 3]. CAT, EC 1.11.1.6 is present in the peroxisomes and exists as a dumbbell-shaped tetramer of four identical subunits. Several SNPs in the CAT gene have been reported, most of these are associated with acatalasemia [2]. In the present study, three single nucleotide polymorphisms (SNPs) were selected SOD1 35A/C (rs2234694), SOD2 47C/T (rs4880/rs1799725), and their association with Type 2 diabetes was evaluated in a vindhyan region population [2, 3]. According to Diabetes Atlas, 2011, 366 million people are affected with diabetes worldwide, and the number is likely to reach 552 million by the year 2030 with the largest increase in regions dominated by developing economies. The current reports revealed that India has 61.3 million diabetics which are expected to rise to 101.2 million by the end of 2030 [4, 5].Free radicals and reactive oxygen species (ROS) have been incriminate in a wide diversity of degenerative diseases including cancer, cardiovascular diseases, diabetes and its complications [6]. Growing evidence indicated that oxidative stress increases during diabetes due to overproduction of ROS and decreased efficiency of antioxidant defences which starts very early and worsens over the course of disease [7]. During the development of diabetes, oxidation of lipids, proteins and DNA increases with time [8]. Implication of oxidative stress in the pathogenesis of diabetes is suggested due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation and decreased ascorbic acid levels. The antioxidant enzymes viz. superoxide dismutase (SOD), glutathione peroxidase (GPxs) and catalase (CAT) contribute to eliminate superoxides, hydrogen peroxide and hydroxyl radicals [9-14]. Considering the fact that oxidative stress contributes towards T2DM and its complications, the present study was undertaken to assess the levels of antioxidant enzymes in T2DM patients in vindhyan region population.

Materials and methods

Study Design

The study population consisted of 470 controls with no history of cardiovascular disease, diabetes, hypertension, cancer, or any infectious diseases and 450 patients with type 2 diabetes mellitus, belonging to vindhyan region population. The patients included were characterized based on the alteration in blood sugar level (>126mg/dL). The samples from the subjects were collected into EDTA coated tubes and the informed consent was obtained. Clinical data including information on duration of diabetes, presence of any complication, history of other disorders, age, gender, lipid profile, blood sugar level, and systolic and diastolic blood pressure were collected using a questionnaire. Ethical clearance was obtained for this study.500 of peripheral blood collected was equally distributed in 0.5 M EDTA (pH- 8.0) and plain vials for enzyme assays and biochemical estimations respectively. Blood in plain vials were kept on ice for 30-60 min for separation of serum. Thereafter, serum was collected in fresh vials after centrifugation for 10 min at 3000 rpm and 4ºC.

DNA Extraction

Genomic DNA was extracted from the frozen blood by phenol-chloroform method [15, 16]. For DNA extraction, 500μL of the blood was used and the isolated DNA dissolved in TE was stored at −20∘C.Thequalityofthe DNA was checked in 1% percent agarose (Hi-Media, Mumbai) gel electrophoresis and quantified using UV spectrophotometry (Hitachi,Japan).

Anthropometric Analysis

Body mass index (BMI) and waist hip ratio (WHR) were calculated by measuring height, weight, and waist circumference. Standing body height (to the nearest 0.5 cm) was measured with a commercial stadiometer. A digital scale, with an accuracy of 100 g, was used to measure body weight (BW) in Kg. BMI (Kg) was calculated by dividing weight (in kilograms) by the square of height (in meters), as a measure of total adiposity. The waist circumference (WC) was measured in a horizontal plane midway between the inferior margin of the ribs and the superior border of the iliac crest on subject at the end of exhalation. Hip circumference (HC) was measured at the fullest point around the buttocks with a metallic tape. The measurements were taken thrice and the mean was taken in all cases. WC (cm) was divided by HC (cm) to calculate WHR. Systolic and diastolic blood pressures were also measured using standard technique [17].

Biochemical estimations

Estimations of plasma glucose (mg/dl) and lipid profile, viz., total cholesterol (TC), triglycerides (TGL), high density lipoproteins (HDL), and serum creatinine (SCRT) were done using commercially available Ecoline kits (Merck) by double beam spectrophotometer (Shimadzu, Japan) at 500, 560, and 510 nm, respectively. Low density lipoproteins (LDL) and very low density lipoproteins (VLDL) were calculated by standard formulae [18]. LDL and VLDL were calculated using following formula [18]:

LDL ——— Total Cholesterol – (Triglyceride/5 HDL)

VLDL ——— Total Cholesterol – (LDL HDL)

Preparation of erythrocyte lysates

The EDTA blood was centrifuged at 2789 rpm (1000 xg) for 10 minutes at 4°C. The upper yellow plasma was taken out without disturbing the white buffy layer of leukocytes. Plasma was stored at –80°C for biochemical tests while the white buffy layer was discarded. The remaining lower layer contained erythrocytes (red blood cells) which were lysed with four times its volume of ice-cold HPLC-grade water and centrifuged at 10,000 rpm (12857 xg) for 15 minutes at 4°C. The supernatant was used for different antioxidant enzyme assays. Protein content was estimated in blood lysate by Biuret method [19].

Antioxidant enzyme assays

Superoxide dismutase assay: SOD activity was measured by spectrophotometric method with minor modifications [20, 21]. Two reaction setups viz. experimental and reference were run in parallel. The experimental tubes contained 0.2 ml Nitro blue tetrazolium (NBT), 0.2 ml Phenozinemethosulphate (PMS), 1.1 ml sodium pyrophosphate buffer and 20 µl enzyme sources while reference tubes had all reagents except the enzyme source. Both the reactions started simultaneously after addition of 0.2 ml nicotine adenine dinucleotide, reduced (NADH). After 90 seconds, 1.0 ml glacial acetic acid was added to each tube to stop the reaction and 20 µl enzyme sources was added to reference tubes. The absorbance was read at 560 nm. The difference in A560 between reference and an experimental reaction was the inhibition of NBT reduction by enzyme source. The SOD enzyme activity was defined as the amount of enzyme causing half the maximum inhibition of NBT reduction and was expressed as units/mg protein.

Catalase assay: Catalase activity was measured by spectrophotometric method as described by Aebi et al [22] with minor modifications [19], the decomposition of H2O2 was determined at 240 nm. According to this method, the reaction mixture containing 0.01M phosphate buffer (pH-7.0), 0.02M H2O2 and 20 µl blood lysate were mixed thoroughly and absorbance was taken at 240 nm after every 30 secs for 3 min. One catalase unit was defined as the amount of enzyme that decomposed 1 µmol of H2O2 per min at 37ºC. The results were expressed as units/mg protein.

PCR Analysis of SOD1 (+35A/C) Gene [17]

PCR analysis was carried out using a thermal cycler (EppendorfMastercycler, Germany). Approximately 120 ng of genomic DNA was incubated in a total reaction mixture of 20μL containing both the forward primer 5 CTATCCAGAAAACACGGTGGGCC3 andthereverseprimer5 TCTATATTCAATCAAATGCTACAAAACC 3 (~10picomoles) (GenScript Corp., USA),200 μM deoxynucleotide triphosphate,10x PCR buffer pH-8.3 containing MgCl2 15mM, and 5 units of Taq DNA polymerase (New England Biolabs, Beverly). DNA was initially denatured at 95◦C for 4min prior to amplification. The PCR amplification conditions were as follows: 35 cycles consisting of 1min denaturation at 94◦C, 50sec annealing at 64◦C, and 1min extension at 72◦C. The final extension included 7 mins at 72◦C.The PCR product (278bp) was confirmed by 1.5 % agarose (Hi-Media, Mumbai) gel electrophoresis. The amplified product was used for further restriction fragment analysis.

PCR Analysis of SOD2 (+47C/T) Gene [17]

PCR analysis was carried out using a thermal cycler (EppendorfMastercycler, Germany). Approximately 120 ng of genomic DNA was incubated in a total reaction mixture of 20μL containing both the forward primer (5'-GCTGTGCTTTCTCGTCTTCAG -3') and the Reverse primer (5'-TGGTACTTCTCCTCGGTGACG-3') (~10picomoles) (GenScript Corp., USA),200 μM deoxynucleotide triphosphate,10x PCR buffer pH-8.3 containing MgCl2 15mM, and 5 units of Taq DNA polymerase (New England Biolabs, Beverly). DNA was initially denatured at 95◦C for 4 min prior to amplification. The PCR amplification conditions were as follows: 35 cycles consisting of 1min denaturation at 94◦C, 50sec annealing at 64◦C, and 1min extension at 72◦C. The final extension included 7 mins at 72◦C.The PCR product (278bp) was confirmed by 1.5 % agarose (Hi-Media, Mumbai) gel electrophoresis. The amplified product was used for further restriction fragment analysis.

PCR Analysis of CAT (+262C/T) Gene [17]

PCR analysis was carried out using a thermal cycler (EppendorfMastercycler, Germany). Approximately 120 ng of genomic DNA was incubated in a total reaction mixture of 20μL containing both the forward primer **Forward primer**- (5`-CTGATAACCGGGAGCCCCGCCCTGGGTTCGGATAT-3`)

Reverse primer- (5`-CTAGGCAGGCCAAGATTGGAAGCCCAATGG-3') (~10picomoles) (GenScript Corp., USA),200 μM deoxynucleotide triphosphate,10x PCR buffer pH-8.3 containing MgCl2 15mM, and 5 units of Taq DNA polymerase (New England Biolabs, Beverly). DNA was initially denatured at 95◦C for 4 min prior to amplification. The PCR amplification conditions were as follows: 35 cycles consisting of 1min denaturation at 94◦C, 50sec annealing at 64◦C, and 1min extension at 72◦C. The final extension included 7 mins at 72◦C.The PCR product (278bp) was confirmed by 1.5 % agarose (Hi-Media, Mumbai) gel electrophoresis. The amplified product was used for further restriction fragment analysis.

Restriction Enzyme Analysis [17] of SOD 1(+35A/C) Gene

The 278-bp product was digested with HhaI enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was undigested and generates 278 bpproducts. The mutant homozygous genotype (TT) was digested and generates the 207bp and 71bp products. However, heterozygous genotype (CT) was partially digested and generates 278bp, 207bp and 71bp products. Samples were analyzed by electrophoresis using 2 % agarose gels to analyze the genotype pattern of the gene.

Restriction Enzyme Analysis [17] of SOD 2 (+47C/T) Gene

The 207-bp product was digested with BsawI enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was undigested and generates 207 bpproducts. The mutant homozygous genotype (TT) was digested and generates the 167bp and 40bp products. However, heterozygous genotype (CT) was partially digested and generates 207bp, 167bp and 40bp products. Samples were analyzed by electrophoresis using 2 % agarose gels to analyze the genotype pattern of the gene.

Restriction Enzyme Analysis [17] of CAT(+262 C/T) Gene

The 190-bp product was digested with MspI enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was digested and generates 157 bp and 33bp products. The mutant homozygous genotype (TT) was undigested and showed the 190bp product. However, heterozygous genotype (CT) was partially digested and generates 190bp, 157bp and 33bp products. Samples were analyzed by electrophoresis using 2 % agarose gels to analyze the genotype pattern of the gene.

Statistical analysis:

Statistical analysis was performed by Prism software (v.5.01). Comparison of various groups was performed using the Students't-test. All data were expressed as Mean ± SD. P <0.05 was considered statistically significant.

Table 1.Sequences of used primers and used restrictases.

SNP	sequence of used primers	restriction endonuclease	restriction fragments
SOD1 35 A/C	5'CTATCCAGAAAACACGGTGGGCC 3'	HhaI	C allele 71 bp and 207 bp A allele 278 bp
	5'TCTATATTCAATCAAATGCTACAAAAC3'		
SOD2 47 (C/T)	5'GCTGTGCTTTCTCGTCTTCAG 3'	BsaWI	C allele 267 bp T allele 183 bp and 84 bp
	5'TGGTACTTCTCCTCGGTGACG3'		
CAT -262 C/T	5'-AATCAGAAGGCAGTCCTCCC-3'	HinfI	A allele 203 bp and 47 bp T allele 250 bp
	5'-TCGGGGAGCACAGAGTGTAC-3'		

Result

Selection of samples

In our sample population (n=920), 470 normal healthy controls (51%) and 450 Type 2 Diabetes cases (49%) were selected for the study. Out of 470 normal healthy controls, 51% were males (n=239) and 49% were females (n=231). Out of 450 Type 2 Diabetes cases, 62% were males (n=279) and 38% females (n=171).

DNA quality assessment by agarose gel electrophoresis and PCR reactions

Qualitative analysis of isolated DNAs were routinely carried out by visual observation of ethidium bromide stained gels. When genomic DNA was electrophoresed through 0.8% agarose gels, a distinct bright DNA band migrated equivalent to or slower than the 21.1 kb band of Eco RI/ Hind III double digested lambda DNA. This showed that the isolated DNA was of high molecular weight and was also undegraded as inferred from the lack of or minimal smearing seen.

Status of anthropometric data

The descriptive data with comparison of anthropometric parameters of Type 2 diabetes cases versus controls are presented in (Table 2).The mean age ± SD of controls was 53.2 ± 13.7 and Type 2 diabetes cases was 52.6 ± 11.2. Comparative BMI of all subjects and that of women showed significant association (P<0.05) in Type 2 diabetes cases when compared to controls. Similarly, waist circumference of women showed significant association (P<0.0001) in Type 2 diabetes cases. Comparative age showed no association but WHR in women showed significant association (P<0.0001) in Type 2 diabetes cases when compared to controls (Table 2).

Table 2 : Comparison of anthropometric parameters of normal healthy controls and Type 2 diabetes cases.

Characteristics	Cases	Control	P-value
n(Men/Women)	450(279/171)	470(239/231)	
Age(years)	52.6.±11.2	53.2±13.7	0.4683
Height(m)	164.2±13.30	163.3±12.1	0.2829
Weight (Kg)			
Women	62.6±4.90	57.0 ±4.90	P<0.0001***
Men	67±6.50	62.8±8.2	P<0.0001***
BMI (kg/m^2)			
Women	25.3± 2.8	24.7±4.3	0.123
Men	22.2± 2.8	21.3±4.2	0.0038**
Waist circumference (cm)			
Women	95.6±6.3	83.4±6.6	P<0.0001***
Men	92.6±5.3	91.8±4.5	0.0671
Hip (cm)			
Women	96.8±5.8	97.6±5.9	0.1776
Men	92.7±4.3	91.9±5.8	0.0727
WHR			
Women	0.97±0.04	0.89±0.008	P<0.0001****
Men	0.101±0.04	1.30±0.03	P<0.0001***

SD = Standard deviation; WHR= Waist hip ratio; BMI = Body mass index, (denotes level of significant change between case and control) Status of biochemical parameters*

Biochemical profile of normal healthy controls and Type 2 diabetes cases has been shown in (Table 3). The fasting glucose levels of controls were 93.2±8.8 and cases were 119.5±18.4. Postprandial glucose in controls and cases was 122.2±16.2 and 156.7±26.5 respectively. The HbA1c level of controls was 5.8 ± 0.6 and cases were 7.2±0.8. TGL of controls and cases were 129.4±17.1 and 136.4±16.4 respectively. HDL-C and LDL-C in controls were 113.5 ± 14.2 and 43.4 ± 5.2 respectively. However, HDL-C and LDL-C, in cases where 116.4±17.5and 46.2±6.1 respectively. Systolic BP and Diastolic bp, in controls were 134.1 ± 6.3and 86.5 ± 6.3 respectively. However, Systolic BP and Diastolic bp, in cases were 135.3± 9.2and 89.3 ± 6.4 respectively. Blood urea level of controls was9.2 ±1.3and cases were 9.4±1.4. Serum Creatinine level of controls was 1.06 ±0.10 and cases were 1.08 ±0.18. Highly significant association was observed in some biochemical parameters ie. FPG(P<0.0001), Post Prandial Glucose (P<0.0001), HbA1C(P<0.0001), LDL-C (P<0.0001) and TGL (P<0.0001) as expected when compared between controls and cases showed (P<0.001) While HDL-C, Systolic BP, Diastolic BP, blood urea level and creatine value were not found significantly different between controls and cases.

Table 3: Comparison of Biochemical and clinical findings of diabetic patients and controls.

Characteristics	Cases (450)	Controls (470)	P-value
FPG(mg/dL)	119.5±18.4	93.2±8.8	P<0.0001****
Post-Prandial Glucose (mg/dL)	156.7±26.5	122.2±16.2	P<0.0001****
HbA1C (%)	7.2±0.8	5.8±0.6	P<0.0001****
HDL-C(mmol/L)	116.4±17.5	113.5±14.2	0.0058**
LDL-C (mg/dL)	46.2±6.1	43.4±5.2	P<0.0001****
TGL(mg/dL)	136.4±16.4	129.4±17.1	P<0.0001****
Systolic BP (mmHg)	135.3±9.2	134.1±6.3	0.0207*
Diastolic BP (mmHg)	89.3±6.4	86.5±6.3	P<0.0001***
Blood Urea(mg/dL)	9.9±1.5	9.6±1.6	0.035**
Creatinine(mg/dL)	1.08±0.18	1.06±0.10	0.0365

FPG=Fasting plasma glucose test; Hb1AC= Glycated Hemoglobin; HDL-C= High Density Lipoprotein Cholesterol; LDL-C=Low Density Lipoprotein Cholesterol; BP= Blood Pressure TGL=Triglyceride; (denotes the level of significant change between case and control)*

Status of Enzyme activity

To determine the impaired antioxidant activity in Type 2 diabetes cases, enzyme activity of antioxidant enzymes viz Superoxide dismutase (SOD) and Catalase (CAT) were evaluated in normal healthy subjects and Type 2 diabetes cases.(Table 4.; Figure 2)

SOD enzyme assay was carried out in 57 normal control subjects and 64 Type 2 diabetes cases. 29.98 % significant decrease (P<0.001) in enzyme activity was observed in Type 2 diabetes cases with respect of control.Similarly, CAT enzyme assay was carried out in 69 normal control subjects and 75 Type 2 diabetes cases. 23.64 % significant decrease (P<0.05) in enzyme activity was observed in Type 2 diabetes cases with respect of control.

Table 4. Comparison of antioxidant enzyme activities between normal healthy subjects and Type 2 diabetes cases.

Antioxidant enzymes	Normal healthy controls		Type 2 diabetes cases		Control vs Case (activity)	P-value
	Number of samples	Mean ± SE	Number of samples	Mean ± SE	Percentage change	
SOD (units/mg protein)	57	25.12±1.39	64	17.59±1.678	29.98 ↓	<0.001
CAT (units/mg protein)	69	16.96±1.434	75	12.95±1.21	23.64 ↓	<0.0001

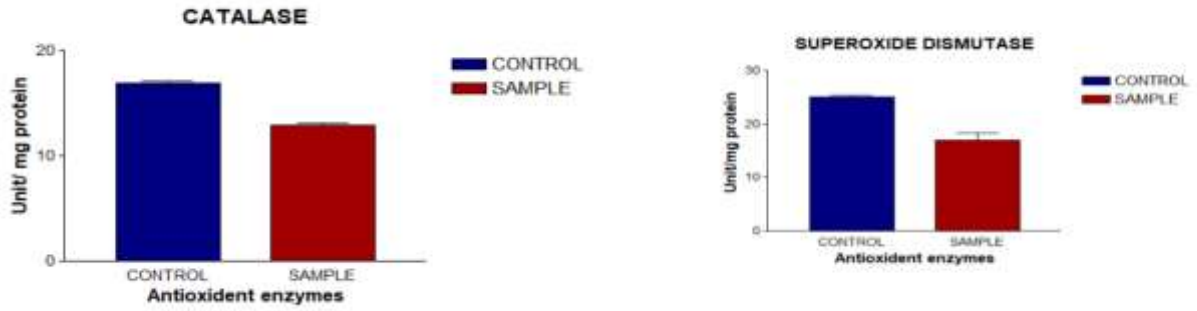

Fig. 1: Status of SOD and CAT Enzyme Activity in normal healthy control and Cases (Type 2 diabetes). Sample from each group were analyzed and were subjected to statistical analysis. Bars represent the standard Error. * P<0.05, ** P<0.01, *** P<0.001 with respect to control.

Status of CAT - C-262-T polymorphism

Out of 450 Type 2 diabetes cases, 43.75% (n=196) had CC, 45.42 % (n =204) had CT while only 10.83 % (n=48) showed TT genotype. Among 470 healthy control subjects, 46.82% (n=220), 43.64% (n=205) and 9.54% (n=44) carried the CC, CT and TT genotypes respectively (Table 5). Allele 'C' frequency in Type 2 diabetes cases was 66.46 % and 'T' was 33.54 %, and in healthy controls Allele 'C' frequency was 68.63% and 'T' was 31.37%. There was no association of CAT-C-262-T gene polymorphism was observed in Type 2 diabetes case. In addition, carriage rate of T allele was also found no significant (Table 5).

The representative gel picture showing CAT genotypes is shown in Figure 3.

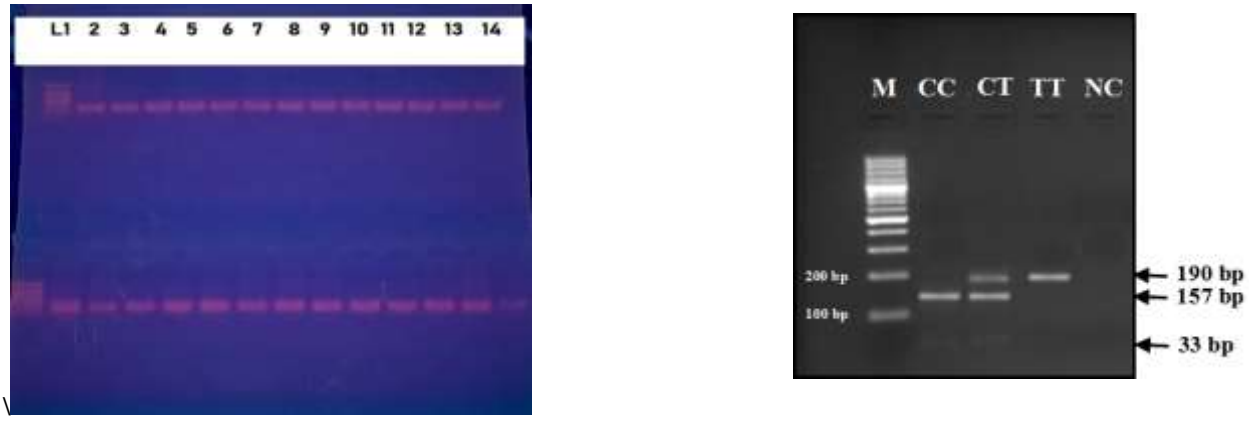

Fig 3: Representative gel showing CAT -C-262-T genotypes. Lane 1 100bp DNA ladder (Marker, M), Lane 2 CC (157 & 33 bp), Lanes 3 CT (190, 157 & 33 bp), Lane 4 TT UD Undigested (190bp).

Table 5: Genotype distribution and allele frequencies as well as carriage rate of CAT–C-262-T in controls and Type 2 diabetes cases.

Genotype frequencies						
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Controls (n=470)		**Cases (n=450)**			
CC	220	46.82	196	43.75	--	1 (Ref.)
CT	205	43.64	204	45.42	0.5826	1.136 (0.875-1.474)
TT	44	9.54	48	10.83		0.9288 (0.7157-1.205)
Allele frequencies						
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Controls (n=470)		**Cases (n=450)**			
C	645	68.63	596	66.46	0.3180	1 (Ref.)
T	293	31.37	300	33.54		1.108 (0.9110-1.348)
Carriage rate						
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Controls (n=470)		**Cases (n=450)**			
C (+)	425	90.45	400	89.16	0.6601	1 (Ref.)
T (+)	249	53.18	252	56.25		1.075 (0.8611-1.343)

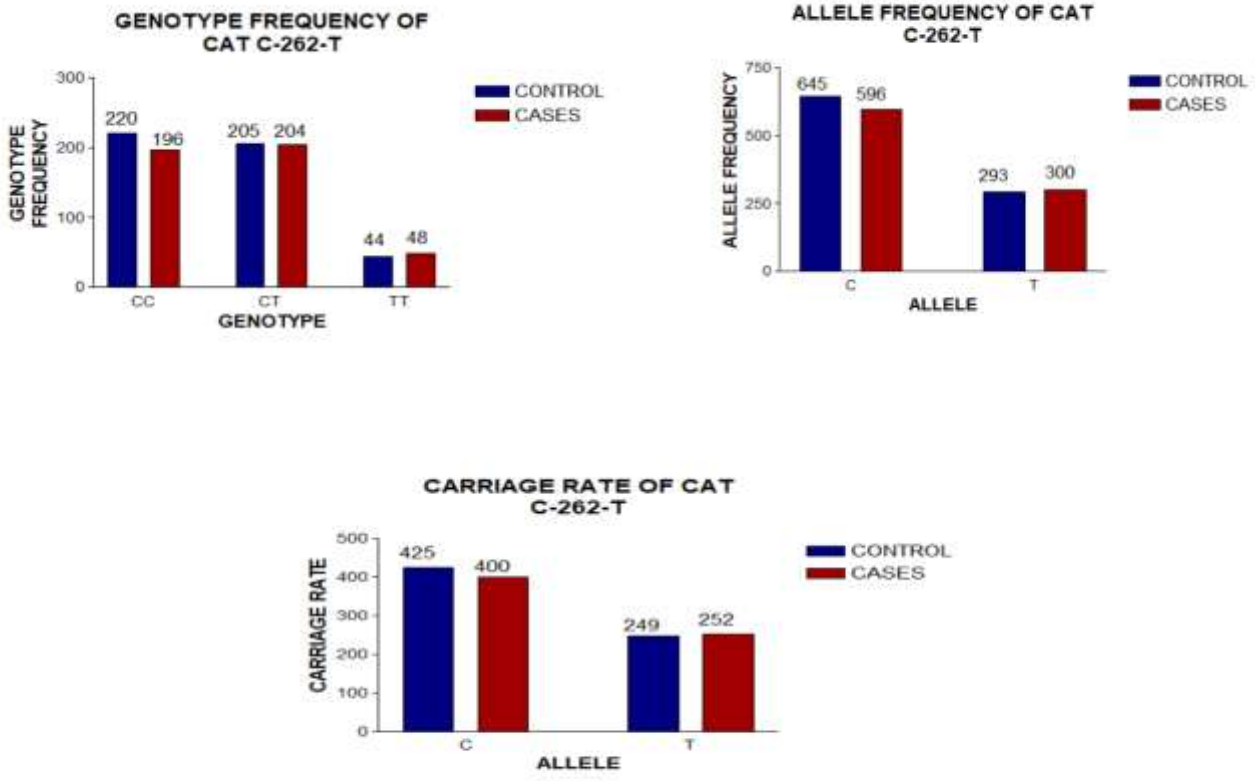

Fig 4: Status of CAT C-262-T Polymorphism**.** (A) Genotype, (B) Allele frequency and (C) Carriage pattern. Sample from Control and Cases (T2DM) were analyzed and were subjected to statistical analysis. All values are shown in percentage.* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Status of SOD1 -A-35-Cpolymorphism

Out of 450 Type 2 diabetes cases, 36.25% (n=163) had AA, 46.25 % (n =208) had AC while only 17.50 % (n=78) showed CC genotype. Among 470 healthy control subjects, 28.18% (n=132) ,49.10% (n=230) and 22.72% (n=106) carried the AA, AC and CC

genotypes respectively (Table 6). Allele 'A' frequency in Type 2 diabetes cases was 59.37% and 'C' was 40.63 %. and in healthy controls Allele 'A' frequency was 52.72% and 'C' was 47.28%. Although there was no association observed in SOD1 -A-35-C gene polymorphism and carriage rate of C allele in Type 2 diabetes cases.but allele frequency of C allele was slightly significant($P<0.05$) in Type 2 diabetes cases,

The representative gel picture showing SOD1genotype is shown in Figure 5

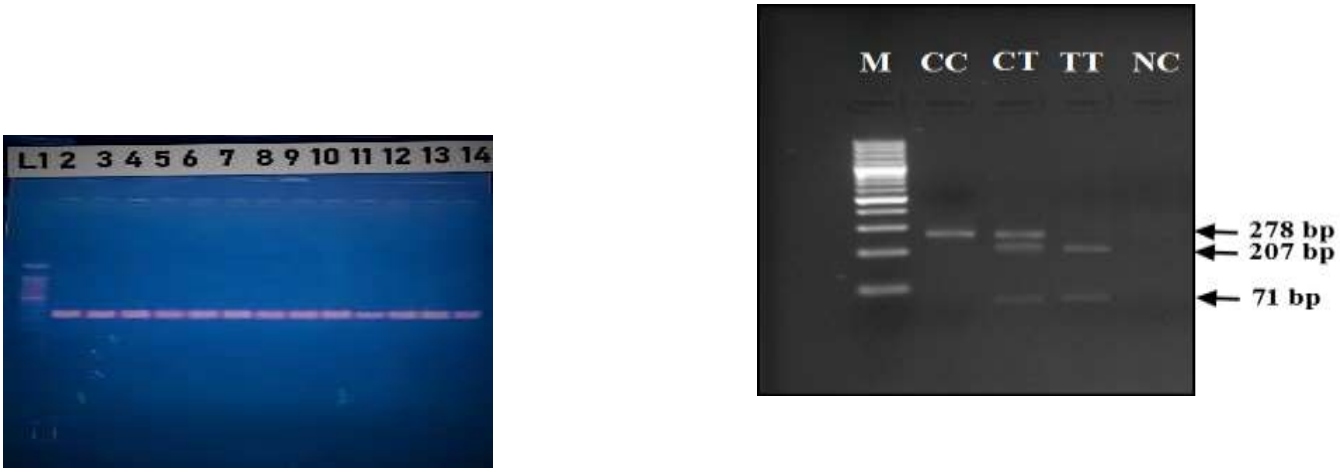

Fig 5: Representative gel showing SOD1 -A-35-C genotypes. Lane 1 100bp DNA ladder (Marker, M), Lane 2 CC UD Undigested (278bp), Lanes 3 CT (278, 207 & 71bp), Lane 4 TT (207&71bp).

Table 6: Genotype distribution and allele frequencies as well as carriage rate of *SOD1*-A-35-C in controls and Type 2 diabetes cases

Genotype frequencies						
	Controls (n=470)		**Cases (n=450)**		**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**		
AA	132	28.18	163	36.25	--	1 (Ref.)
AC	230	49.10	208	46.25	0.0163	0.6893 (0.5217-0.9107)
CC	106	22.72	78	17.50		1.120 (0.8639-1.451)
Allele frequencies						
	Controls (n=470)		**Cases(n=450)**		**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Bapista-Pike) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**		
A	494	52.72	534	59.37	**0.0041**	1 (Ref.)
C	442	47.28	364	40.63	**<0.05**	0.7618 (0.6332-0.9167)
Carriage rate						
	Controls (n=470)		**Cases(n=450)**		**P-value Chi ^{2} value**	**Reciprocal Odd's Ratio (Bapista-Pike) with 95% CI (Lower-Upper)**
	Count (n)		**Count (n)**			
A+	362	77.27	371	82.50	0.0938	1 (Ref.)
C+	336	71.82	286	64.09		0.8305 (0.6704-1.029)

Genotype frequencies						
	Controls (n=470)		**Cases (n=450)**		**P-value** ^{2} **Chi value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**		
AA	132	28.18	163	36.25	--	1 (Ref.)
AC	230	49.10	208	46.25	0.0163	0.6893 (0.5217-0.9107)
CC	106	22.72	78	17.50		1.120 (0.8639-1.451)
Allele frequencies						
	Controls (n=470)		**Cases(n=450)**		**P-value** ^{2} **Chi value**	**Reciprocal Odd's Ratio (Bapista-Pike) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**		
A	494	52.72	534	59.37	**0.0041**	1 (Ref.)
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Carriage rate						
	Controls (n=470)		**Cases(n=450)**		**P-value** ^{2} **Chi value**	**Reciprocal Odd's Ratio (Bapista-Pike) with 95% CI (Lower-Upper)**
	Count (n)		**Count (n)**			
A+	362	77.27	371	82.50	0.0938	1 (Ref.)
C+	336	71.82	286	64.09		0.8305 (0.6704-1.029)

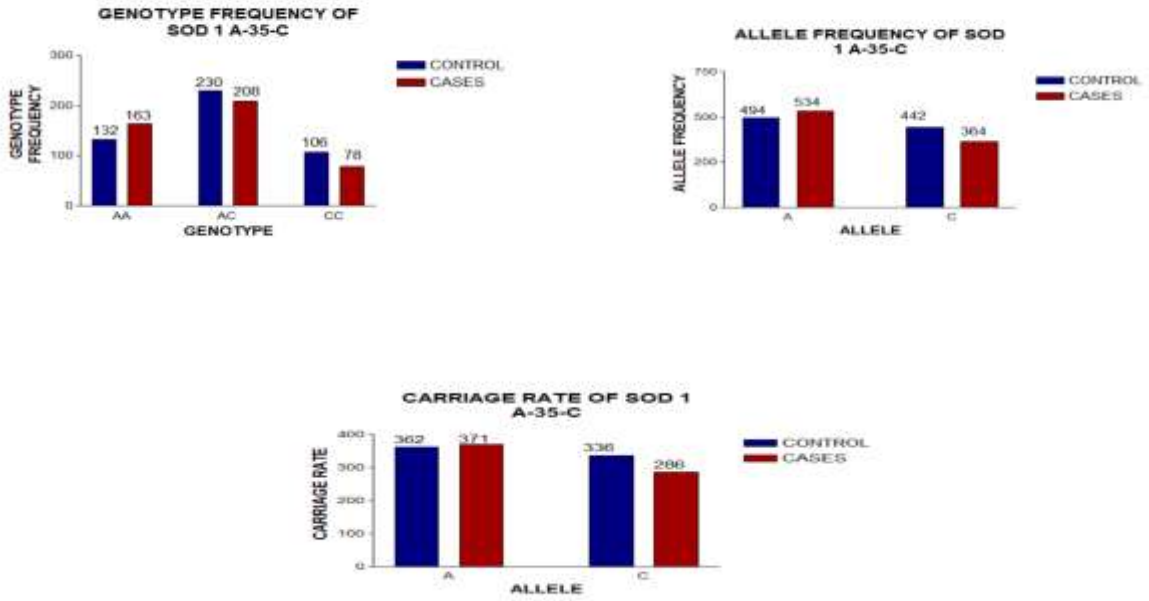
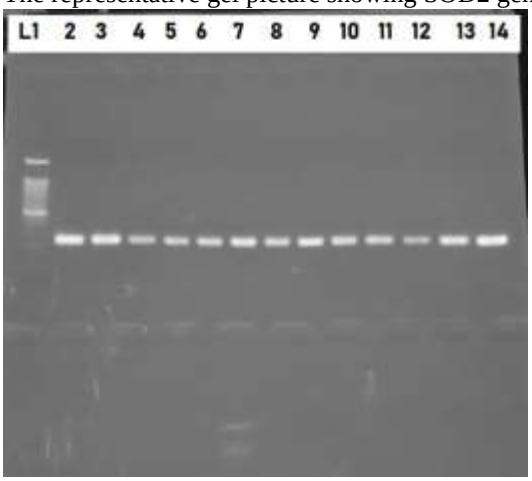


Fig 6: Status of SOD1 A-35-C Polymorphism. (A) Genotype, (B) Allele frequency and (C) Carriage pattern. Sample from Control and Cases (T2DM) were analyzed and were subjected to statistical analysis. All values are shown in percentage.* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Status of SOD2 -C-47-Tpolymorphism

Out of 450 Type 2 diabetes cases, 40.00% (n=180) had CC, 49.60 % (n =232) had CT while only 10.40 % (n=46) showed TT genotype. Among 470 healthy control subjects,32.27% (n=151) ,56.82% (n=267) and 10.91% (n=51) carried the CC, CT and TT genotypes respectively (Table 7). Allele 'C' frequency in Type 2 diabetes cases was 64.79% and 'T' was 35.21%. and in healthy controls Allele 'C' frequency was 60.68% and 'C' was 39.62%. Although there was no association observed in SOD2-C-47-T gene polymorphism and carriage rate of C allele in Type 2 diabetes cases.

The representative gel picture showing SOD2 genotypes is shown in Figure 7.



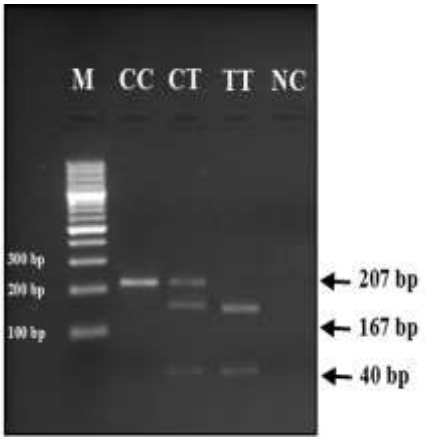


Fig 7: Representative gel showing SOD2 -C-47-T genotypes. Lane 1 100bp DNA ladder (Marker, M), Lane 2 CC UD Undigested (207bp), Lanes 3 CT (207, 167 & 40 bp), Lane 4 TT (167 & 40 bp).

Table 7: Genotype distribution and allele frequencies as well as carriage rate of SOD2-C-47-T in controls and Type 2 diabetes cases.

Genotype frequency						
	Controls (n=470)		**Cases (n=450)**		**P-value**	**Reciprocal Odd's Ratio (Fishar Exact) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**Chi ^{2} value**	
CC	151	**32.27**	180	40.00	--	1 (Ref.)
CT	267	**56.82**	232	49.60	0.0772	0.7334 (0.5600-0.9604)
TT	51	**10.91**	46	10.40		1.269 (0.9799-1.643)
Allele frequencies						
	Controls (n=470)		**Cases(n=450)**		**P-value**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**Chi^2 Value**	
C	569	60.68	592	64.79	**0.0775**	1 (Ref.)
T	369	39.32	324	35.21		0.8439 (0.6990-1.019)
Carriage rate						
	Count (n)	**Frequency (%)**	**Count (n)**	**Frequency (%)**	**P-value Chi^2**	**Reciprocal Odd's Ratio (Fisher Exact) with 95% CI (Lower-Upper)**
	Controls (n=470)		**Cases(n=450)**		**Value**	
C+	418	86.01	412	89.58	**0.2644**	1 (Ref.)
T+	318	32.27	278	40.00		0.8869 (0.7184-1.095)

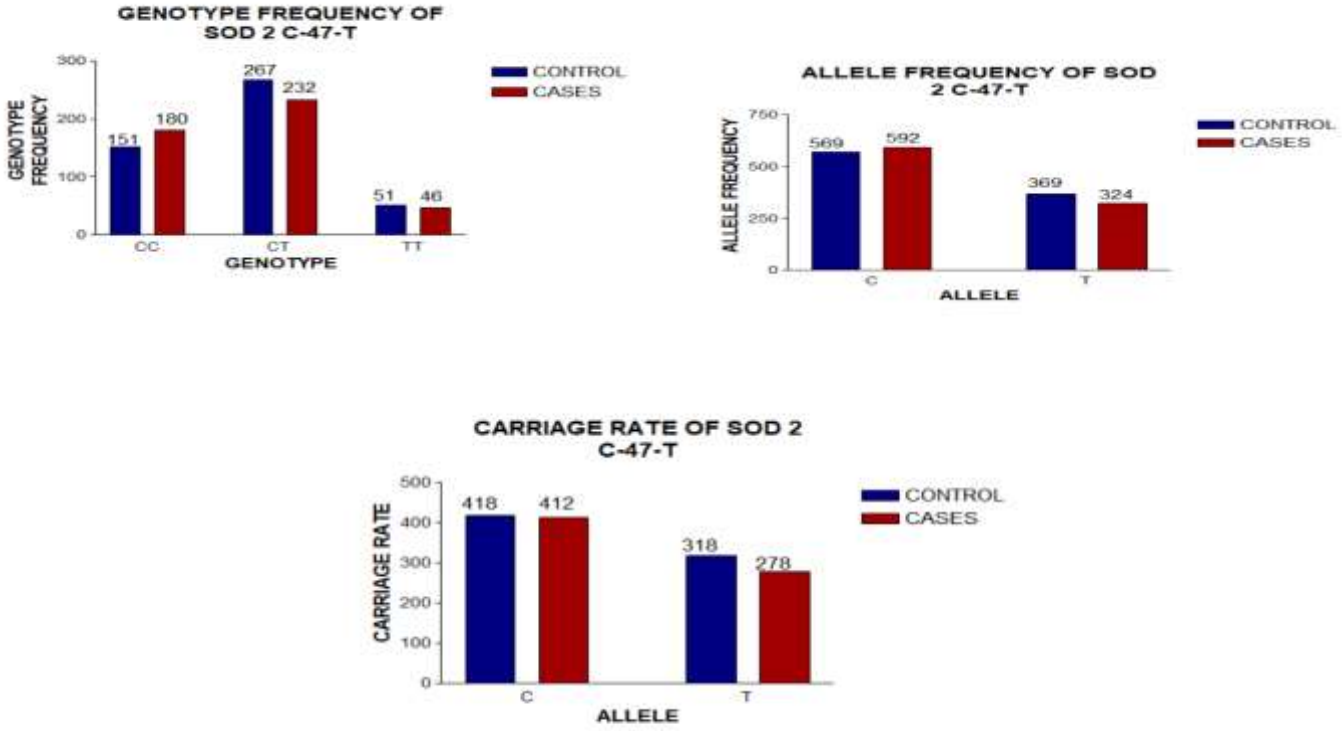


Fig 8: Status of SOD2 C-47-T Polymorphism.(A) Genotype, (B) Allele frequency and (C) Carriage pattern. Sample from Control and Cases (Type 2 diabetes) were analyzed and were subjected to statistical analysis. All values are shown in percentage.* P<0.05, ** P<0.01, *** P<0.001, **** P<0.00

Discussion

Type 2 diabetes mellitus (T2DM) is a common syndrome and a serious global health problem. The 6th edition of IDF (International Diabetes Federation) Diabetes Atlas, 2014, estimates show 382 million people have T2DM throughout the world between the age group of 20 – 79 years. This is expected to increase by 55% in the year 2035 with the figure rising to 592 million. The most dangerous fact about this particular syndrome is that about 50% individuals are undiagnosed. The epidemiological survey also states that the South-East Asian countries had a diabetic population (20– 7 9 years) of 72.1 million in 2013 with 49% undiagnosed diabetic subjects and a proposed increase of 71%, that is, 123 million till 2035 [23]. Different populations of the world vary considerably in their predisposition to diseases and in allele frequencies at pharmacogenetically important loci [23].In this investigation we had taken small sample size but this is the first molecular level study in vindhyan region (Central India) population and we endorse a Genome wide association study in this region to find out the another genes associated with type 2 diabetes. It is also anticipated in future if we will be suitable to discover some medicines which can affect the patient's physiology at basic cellular level then diabetes and another metabolic disorders could be rehabilitate. Genetic association study also proteomic studies has proposed a new dimension to understand the possible role of genetic polymorphisms in metabolic and life threatening disorders such as diabetes, cancer, many other autoimmune and life threatening diseases.Diabetes and associated metabolic syndromes are growing in Indian population with alarming rate and increasing risk of mortality. Genetics as well as proteomic studies can determine the causing factors and better drugs could be. Synthesized for the lowering risk of disease and associated risk factors. Genes have widely studied and found to be connected with massive range of metabolic disorders accompanied type2 diabetes and many type of cancers.

Anthropometric measures are used to estimate abdominal obesity and subsequent Type 2 diabetes disease risk [24,25,26,and 27].The risk of Type 2 diabetes increases with the obesity and weight gain. Body mass index (BMI) and waist hip ratio (WHR) are two commonly used anthropometric measures.BMI provides a diagnosis of common obesity. It was found that patients of metabolic diseases were some one overweight or obese. These clinical concepts have been well-accepted by clinicians and researchers. Relationship between BMI and metabolic disease is delicate toward a better understanding of Type 2 diabetes cases. High BMI has been shown to be a predictor of Type 2 diabetes in older persons [28].As a consequence of increased blood glucose level BMI increases and causes increased lipid biosynthesis and hence body weight [29].

In our analysis we observed that BMI was significantly higher diabetic women as compared to healthy women's. Based on comparative study of anthropometric parameters between men and women, we observed the association (P<0.05) of reduced BMI of women in Type 2 diabetes cases. Waist circumference of women exhibit significant association (P<0.0001) in Type 2 diabetes cases.

Sedentary lifestyle is strong factor behind the astounding enhancement in the prevalence of both obesity and diabetes [29,30]. In the past few decades it was observed that the prevalence of type 2 diabetes has increased by 33% in the United States, and 62% of Americans are classified as obese (BMI ≥ 30 kg/m2) or overweight (BMI 25–29.9 kg/m2). Being overweight (BMI of 25-29.9), or affected by obesity (BMI of 30-39.9) or severe obesity (BMI of 40 or greater), greatly increases your risk of developing type 2 diabetes. Indeed, this new unprecedented phenomenon is now been referred to as "diabesity." There is a clear strong relationship between obesity and the risk for diabetes [30]. Waist circumference, a measure of central obesity that is highly correlated with BMI, is associated with Type 2 diabetes in different populations. In our study comparative age showed no association but WHR in women showed significant association (P<0.0001) in Type 2 diabetes cases when compared to controls. Our study data indicate that obesity and higher BMI may be an important factor which can affect the susceptibility to diabetes type 2 in vindhyan region of Madhya Pradesh. FPG level has been expensively used in earlier studies for the preparatory screening of Type 2 diabetes cases. As per ADA recommendations (1997), FPG level can be an important determinant for diagnosis of T2DM[31]. In our study we also observed enhanced level of FPG in Type 2 diabetes cases and hereby showing significant change (P<0.0001) with increased level of FPG in Type 2 diabetes cases.

The glycosylated hemoglobin (HbA1c) is one of the essential determinants of blood sugar control progressively. The International Expert Committee (IECJuly 2009) recommended HbA1c as one of the additional diagnostic measures in Type 2 diabetes cases. In our study we found increased level of HbA1c in Type 2 diabetes cases and thus showing significant change (P<0.0001) with increased level of HbA1c in Type 2 diabetes cases. Low density lipoprotein-Cholesterol (LDL-C) factor is generally connected with cardiovascular disease risk but it is also an important measure in diabetic patients [32].
Although no any direct connection of LDL-C can be founded with Type 2 diabetes but in our study we observed significantly increased level of LDL-C in Type 2 diabetes cases (P<0.0001) , it may be due to dyslipidemia. Another study also propounds similar findings. [33].

As per our study finding we can suggest that the increased level of FPG,PPG,HbA1c,LDL-C and TGL are the main biochemical dissimilarity in pre and post-diabetic situations that can be targeted for the control and management of Type 2 diabetes cases.
Major antioxidant enzymes such as SOD and CAT play a important defensive role against high ROS and support to maintain proper functioning of erythrocytes throughout their 120 days of life span[34].
Erythrocytes, which are providing with a highly productive antioxidant defense system, can scavenge free radicals by the action of their antioxidant enzymes [33,34]. Free radicals play a crucial role in beta cell damage [35]. The decreased SOD activity in Type 2 diabetes could be the result of hyperglycemia which result in activation of different biochemical pathways such as non-enzymatic glycosylation of proteins, activation of protein kinase C and glucose auto oxidation results in overproduction of superoxide and hydroxyl radicals as well as H2O2. The elevated glycosylation of SOD commonly leads to inactivation of enzyme [34]. In our study we observed significant diminish (P<0.001) in SOD enzyme activity in Type 2 diabetes cases with respect of control in vindhyan region. These uncovering were found in accordance to several earlier studies which showed significant association between reduced SOD activity and Type 2 diabetes [35,36]. As per earlier studies on Type 2 diabetes with complication such as diabetic neuropathy, diabetic retinopathy and diabetic cardiovascular disease the SOD activity has been diminished in cases as compared to healthy control group [33.36].

Catalase is an antioxidant enzyme pervasive found in all known organisms, mostly in liver, kidney, and erythrocytes. In hepatocyte and mature RBC it is predominantly situated in peroxisomes, and cytoplasm respectively Catalase provides primary antioxidant defence against hydrogen peroxide [39,40].CAT enzymatic activity shows decreased in cases according to, [42,43]. But in our study we observed significant decrease (P<0.05) in CAT enzyme activity in Type 2 diabetes cases with respect of control in vindhyan region. These findings were found in conferring to several earlier studies which showed significant association between decreased CAT activity and Type 2 diabetes [44].
Several genetic polymorphisms play a crucial role in divergent expression and enzymatic activity of CAT and SOD. In circumstances of these studies we have investigated the status of various genetic polymorphisms which were connected with CAT and SOD.

There are various SNPs recognized in the CAT gene, of which the C-262-T polymorphism C-262-Twas the many widely studied (Su.et al.2015). The CAT C-262-T polymorphism is translated on the promoter region, influencing transcriptional and splicing regulation[37]. CAT deficiency was known to lead to the development of Type 2 diabetes [37.38].Therefore; in present study we estimate the possible association of CAT C-262-T polymorphism in the CAT gene with Type 2 diabetes in vindhyan region. But we could not detect any applicable association of CAT C-262-T gene polymorphism and carriage rate of T allele in Type 2 diabetes case when compared with normal healthy control in vindhyan region. CuZnSOD also called SOD1 is a prominent antioxidant enzyme. We have concentrated on SNPs for their probable functional role: SOD1 +35A/C which are located contiguous to the splice site (exon3/intron3 boundary). SOD1 activity with AA genotype exhibited the higher activity in comparison to AC (Flekac et al., 2008).
Therefore, in contemporary study we assessed the possible association of + 35A/C polymorphism in the SOD1 gene with Type 2 diabetes in vindhyan region. In our study we have not found any association of SOD1 -A-35-C gene polymorphism and carriage rate of C allele in Type 2 diabetes case. But in case of C allele frequency moderately significant (P<0.05) was observed in Type 2 diabetes cases. These evaluations indicate that although there is no association of SOD1 -A-35-C gene polymorphism with Type 2 diabetes case. MnSOD is encoded by a single gene accommodate five exons and it is located on chromosome 6q25 [38]. MnSOD also called

SOD 2 is present in the mitochondria. We have concentrated on SNPs for there to be expected known functional role: SOD2 C-47-T present in the mitochondria. C/T substitution (GCT/GTT) has been shown to variation the structural conformation of the mitochondrial targeting sequence (MTS) of the enzyme. Associations have been found between the Ala16Val SNP and neurodegenerative disorders [39].

This mutation may deliberate a functional polymorphism of mitochondrial transport of human MnSOD. It has been shown that the 16Ala variant permits efficient targeting of MnSOD to the mitochondria, as evidenced by Sutton et al., 2005 [40].
Therefore, in present study we assessed the feasible association of + 47C/T polymorphism in the SOD2 gene with Type 2 diabetes in vindhyan region. But we could not observe any consistent association of SOD2-C-47-T gene polymorphism and carriage rate of C allele in Type 2 diabetes case when compared with normal healthy control in vindhyan region. Fewer other studies have also shown no significant association of + 47C/T polymorphism in the SOD2 gene with diabetes risk [41]. Our study suggests that there is no significant role of SOD2-C-47-T with Type 2 diabetes cases in Vindhyan Region Population.

Conclusion

Antioxidant gene polymorphism studies are a extensive procedure of understanding the stress sensitive pathways. As evident from our present study as well as others, it is necessary to recognize the risk genotypes/haplotypes in respective populations. Such genetic studies may distribute prognostic markers for disease risk identification and help the clinicians to develop personalized regimens for treatment. Our study in the vindhyan region population showed that out of the two major antioxidant enzymes, Catalase and SOD levels were significantly lowered in T2DM patients as compared to normal healthy subjects. This means that the antioxidant enzyme generation is simulatedin T2DM subjects dominant to higher risk of cell organ damage. Therefore, detection of these antioxidant enzyme levels can be used as markers for monitoring T2DM in our population. This will allow the individuals at risk to take timely action and obstruct or delay the onset of disease.

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