Genetic Polymorphism of PPARG2 Gene And Its Association With Diabetes Type 2: A Case-Control Study

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Abstract- *Pro12Ala polymorphism is a missense mutation at codon 12 in peroxisome proliferator-activated receptor γ gene (PPARG). This polymorphism is known to be associated with increased insulin sensitivity. Pioglitazone, a thiazolidinedione, is an anti-diabetic drug which acts as an agonist at PPAR γ receptor. The genetic as well as environmental risk factors have been established which may increase the risk of diabetes type 2. A very complex gene environment association has been found as the causing factor behind the diabetes type 2.*

Overall distribution of PPARG2 Pro[12]Ala genotypes was significantly different in HC group as compared to disease group (χ^2=7.253, P=0.0266). HC group showed an increase of mutant 'GG' genotype as compared to Patients of diabetes type 2 (3.9% vs. 0.52%). Similarly, wild type 'AA' genotype was present in significantly low frequency in HC as compared to Diabetes type 2 patients group (73.3% vs. 82.11%). An odds ratio of 0.1336 in Diabetes group respectively for 'GG' genotype indicated a protective effect of this mutant type genotype in our population whereas an odds ratio of 1.668 of Diabetic patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is not significantly different but may be protective because of odds ratio of 0.7094. Overall allele 'G' was found to be in significantly low frequency in disease group as compared to HC group whereas allele 'A' was present in significantly high frequency in the disease group (χ^2 = 6.684, P= 0.0097**;). Overall G allele shows an odds ratio of 0.56 which indicates its protective association. Carriage rate of allele 'G' was high in HC group whereas carriage rate of allele 'A' was high in disease group (χ^2 =3.281, P= 0.0701) but the values were not significant. .*

Keywords- PPARG2 gene, Diabetes, Genotype, Allele frequency.

I. INTRODUCTION

Diabetes is a metabolic disorder characterized by hyperglycemia (excessive amount of glucose) and associated with abnormal lipid and protein metabolism. It has now become global health problem and now–a-days it has largest prevalence world wide and it is world's sixth leading cause of death. Although cause of diabetes are not very much clear but many habits (life style factors) as well as genetic susceptibility is now known to cause this disease. Type 2 diabetes (T2D) is a complex metabolic disorder resulting from the interplay of both genetic and environmental factors like lifestyle and food habits (McCarthy MI *et al*., 2002). In past two decades the genetic analysis with documentation of life style data collection has suggested the possible involvement of the genetic base as well as life style factors.

It has become the most frequently encountered metabolic disorder worldwide and the incidence continues to grow, especially among developing countries. Mortality of diabetes is due to organ dysfunction and patients of diabetes are strongly susceptible for cardiovascular disease. Many life style as well as genetic factors has been found to be associated with diabetes type 2 but results are not consistent in different race of different ethnic origins. The prevalence of diabetes is rapidly rising all over the globe at an alarming rate, it is important to note that the rise in prevalence is seen in all six inhabited continents of the globe. According to the World Health Organization, over 180 billion people now have diabetes worldwide, and this number is expected to double by the year 2030 (WHO report 2006).

T2D is mainly characterized as a state of hyperglycemia resulting from defects in insulin action and β-cell dysfunction. Normal levels of blood glucose are maintained through integrated mechanisms of glucose sensing, insulin production and glucose utilization through insulin secreted by β-cells (Reaven G, 1998). Hence, β-cells occupy central role in maintaining glucose homeostasis, therefore defects in its development and function might lead to T2D. However defects in the carbohydrate metabolism, lipid metabolism and inflammatory pathways have been also shown to elevate the risk of T2D, there by manifesting its complexity (Abate N and Chandalia M, 2003).

The genetic as well as environmental risk factors have been established which may increase the risk of diabetes type 2. A very complex gene environment association has been found as the causing factor behind the diabetes type 2. In determining the risk of developing diabetes, environmental factors such as food intake and exercise play an important role. The majority of individuals with type 2 diabetes are either overweight or obese. Inherited factors are also important, but the genes involved remain poorly defined. A major problem limiting our understanding of the genetic basis of type 2 diabetes is that many environmental and genetically based factors influence insulin sensitivity and insulin secretion: these include age, gender, ethnicity, physical fitness, diet, smoking (Targher G *et al*., 1997), obesity, and fat distribution (Yki-Jarvinen H *et al*., 1995), Although many of these may be under genetic control (Bouchard C, 1995). Genetic susceptibility plays a crucial role in the etiology and manifestation of type 2 diabetes, with concordance in monozygotic twins approaching 100%. Genetic factors may have to be modified by environmental factors for diabetes mellitus to become overt. An individual with a susceptible gene may become diabetic if environmental factors modify the expression of these genes. Since there is an increase in the trend at which diabetes prevail, it is evident that environmental factors are playing a more increasing role in the cause of diabetes mellitus. The complexity of type 2 diabetes is related to factors such as genetic heterogeneity, interactions between genes, and the modulating role played by the environment. This crucial role of environmental factors make it necessary to study about these factor. In this investigation we included physical activity and smoking as environmental factors which are previously established to cause diabetes type 2.

insulin resistance associated gene selected for this study is PPARG2. Peroxisome Proliferator–activated receptor was shown to cause type 2 diabetes with severe insulin resistance, indicating that PPARG2 is essential for insulin action and glucose homeostasis. A polymorphism in 12 exon of PPARG2 gene has been found to be associated with decreased risk of diabetes means less common allele is associated with protective effect. Many case control studies has been done in this field and shows the significant protective effect of less common allele.

The peroxisome proliferator–activated receptor γ gene (PPARG) encodes the PPARγ receptor, a transcription factor that belongs to the family of nuclear receptors and it regulates the carbohydrate and lipid metabolism. Among the two isoforms, PPAR-γ2 is specific for adipose tissue, where it plays a pivotal role in adipogenesis and is an important mediator of insulin sensitivity. Pro12Ala polymorphism is the result of a CCA-to-GCA missense mutation in the codon12 of exon B of the PPARG gene. There is substantial evidence from various genetic studies that the Pro12Ala polymorphism improves insulin sensitivity in humans . It is possible that alterations in transcriptional activity of the polymorphic gene in adipocytes primarily enhance insulin's action. Pioglitazone is a thiazolidinedione, the pharmacological ligand for PPARγ receptor. The thiazolidinediones act by decreasing the insulin resistance in muscle, liver and adipose tissue. The effect of Pro12Ala polymorphism (rs1801282) on the therapeutic response to pioglitazone has not yet been studied in the South Indian population. Hence our study was designed to investigate the association of PPARG gene Pro12Ala polymorphism and the therapeutic response to pioglitazone therapy in type 2 diabetes mellitus patients in a South Indian population. The secondary objectives were to determine the influence of patient characteristics like body weight, BMI, waist-hip ratio on glycaemic response to pioglitazone and also to identify the association between Pro12Ala polymorphism and these patient characteristics in the population studied (Carlsson S, et. al. 2004).

II. MATERIALS AND METHODS

Study population

The study population consisted of 400 unrelated subjects comprising of 190 T2D patients and 210 ethnically matched controls of central Indian population were included in this study. In this region Hindu, Muslim and some Sikh peoples are mainly living but most peoples belong to Hindu religion in this region.

Inclusion and Exclusion criteria for Cases

Cases included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Type 2 diabetes was diagnosed in accordance with World Health Organization (WHO Expert committee 2003) criteria. Pregnant women, children under age of 18 years and any patients with type 1 diabetes were excluded from the study.

Inclusion and Exclusion criteria for Controls

Control group composed of non-diabetic healthy individuals that were collected during "Diabetes Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the

regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India. (Indian Genome Variation Consortium 2008) The inclusion criteria for control group were as follows:-

1) ≥40 years of age
2) HbA1c level ≤6.0
3) Fasting glucose level<110mg/dl
4) No family history of diabetes in first and/or second degree relatives.

All the participants were asked to fill a detailed questionnaire at the time of recruitment, seeking information regarding individual's age, sex, ethnicity, dietary habits, physical activity, and life style, personal and family medical history.

Anthropometric and Biochemical Measurements

Anthropometry

Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 minute using a standard sphygmomanometer and the average of the two reading was used.

Biochemical Analysis

Biochemical parameters related to type 2 diabetes were estimated for both cases and controls subjects. Measurement of Serum levels of Total cholesterol (TC), Triglycerides (TG), HbA1c, High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C), Urea, Uric acid, C-reactive protein (CRP) and Creatinine were measured based on spectrophotometric method using automated clinical chemistry analyzer Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

Blood collection and plasma/serum separation

Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C.

Method for DNA isolation

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

Determination of quality and quantity of isolated DNA

The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

Quantitation by UV spectrophotometry

The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

Agarose Gel Electrophoresis

Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* III double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening

In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest.

All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called “star activity” which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO).

The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5 µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of PPARG2 Single Nucleotide Polymorphism via PCR-RFLP

The P12A (substitution of A base to C at 12 Exon) polymorphism of PPARG2 gene has been amplified by PCR. This polymorphism is a functional polymorphism causing change of Amino acid from proline to alanine. Primer sequences The oligonucleotide sequence (primers) were designed to amplify the gene wild type gene is lack of restriction site for BstU1 enzyme but Alanine allele contains a restriction site and cleaved in to 227 and 43bp fragment.

Restriction site for BSTU1 **CG^CG**

GC^GC

Primer sequence

Forward primer (5'-GCCAATTCAAGCCCAGTC-3')
Reverse Primer
5'GATATGTTTGCAGACAGTGTATCAGTGAAGGAATC GCTTTCCG3'

PCR Mix

The PCR was carried out in a final volume of 25 µl, containing 50-100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

PCR Thermal Program

After an initial denaturation of 5 min at 95˚C, the samples were subjected to 35 cycles at 95˚C for 1 min, at 58˚C for 45 s, and 72˚C for 45 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 2.5 % agarose gel electrophoresis. 238bp product will be generated after PCR.

Restriction digestion

The amplified product size of 270 base pairs (bp) was digested by the specific restriction enzyme, BstU1. for 16 h at 37˚C. The wild-type genotype was not digested, whereas the mutated homozygous genotype was cut as a doublet of 227 and 43 bp. The heterozygous genotype (KQ) was represented as 3 fragments of 270, 227, and 43 bp. Samples were analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene. When alanine was present at residue 12 of PPARγ2, the 270 bp DNA fragment was split into 227 and 43-bp fragments (heterozygous allele). The digestion products were then separated by electrophoresis on a 2.5% agarose gel. The results were documented by digital camera and further saved by gel documentation system.

III. RESULTS

Anthropometric results

The descriptive data and comparison of anthropometric and biochemical parameters of diabetic patients versus controls are presented in Table 2(A). The age, sex, BMI, WHR were the parameters. As expected the diabetic patients had markedly higher levels of weight of women (P=0.0024), Men (P=0.0157) and BMI of Women (P=0.0388), Waist circumference in women (P<0.0001), WHR in Women (P<0.0001) and WHR in Men (P=0.0147). Other results were not found significantly different between case and control group. **(See Table 2A)**

Biochemical and clinical findings

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using students t test and p value obtained suggest the level of significant changes here. The descriptive data and comparison of biochemical parameters of diabetic patients versus controls are presented in Table 3. As expected the diabetic patients had markedly higher levels of fasting plasma glucose (P<0.0001) and HbA1c (P<0.0001) and Post prandial glucose (P<0.0001) compared to that of control subject. Nominal difference was also observed for LDL-C (P=0.0462), triglyceride (P=0.0024), systolic blood pressure (P=0.0447). creatine value, blood urea level, HDL-C level and diastolic pressure was not significantly different between two groups.

TABLE No-1 Comparison of anthropometric parameters of diabetic patients and controls

Characteristics	*Cases*	*Controls*	*P-value*
n(Men/Women)	190(126/64)	210(114/96)	
Age(years)	52.5±12.5	53.0±14.2	0.7100
Height(m)	160.50±13.40	162.2±12.000	0.1815
Weight (Kg)			
Women	62.5 ±5.70	60 ± 4.50	0.0024 **
Men	68±5.60	66.0±7.1	0.0157*
BMI (kg/m²)			
Women	26.4±3.1	25.1 ± 4.3	0.0388*
Men	24.6±4.7	24.1± 5.1	0.4301
Waist circumference (cm)			
Women	92.5±6.2	84.5±6.7	P<0.0001***
Men	90.0±7.0	89.0±6.0	0.2383
Hip (cm)			
Women	95.0±5.0	96.5±6.0	0.178
Men	91.0±4.0	90.5±5.5	0.4183
WHR			
Women	0.97±0.05	0.88±0.08	P<0.0001***
Men	0.99±0.05	1.00±0.03	0.0147*

* denotes level of significant change between case and control

TABLE No-2 Comparison of Biochemical and clinical findings of diabetic patients and controls

Characteristics	*Cases*	*Controls*	*P-value*
FPG(mg/dL)	117.4±17.6	92.1±7.5	P<0.0001***
Post-Prandial Glucose (mg/Dl)	151.7±22.4	119.5±12.1	P<0.0001***
HbA1C(%)	6.9±0.8	5.3±0.6	P<0.0001***
HDL-C(mmol/L)	112.2±14.8	109.8±11.6	0.0705
LDL-C (mg/dL)	42.1±4.3	41.3±3.7	0.0462*
TG(mg/dL)	131.1±13.2	126.9±14.2	0.0024**
Systolic BP (mmHg)	130.20±8.1	128.8±5.7	0.0447*
Diastolic BP (mmHg)	87.1±5.8	86.5±6.0	0.3109
Blood Urea(mg/dL)	9.1±1.6	8.8±1.8	0.0801
Creatinine(mg/dL)	1.08±0.14	1.06±0.10	0.0986

(* denotes the level of significant change between case and control)

DETECTION OF GENETIC POLYMORPHISM IN PPARG2

The nucleotide position Pro 12 Ala polymorphism in PPARG2 gene create restriction site for BstU1. The PCR products when digested by restriction enzyme and wild type allele 270bp segment which were generated by PCR but the mutant allele shows 227 and 43 bp segments (figure 8).

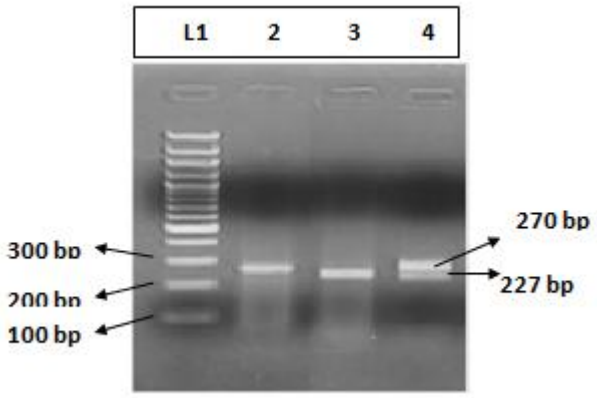


Figure No. 1; Representative gel picture of PPARG2 Pro 12 Ala polymorphism

The expected product sizes are: normal homozygote, 270 bp; Pro^{12}Ala homozygote, 227 and 43 bp; and Pro^{12}Ala heterozygote, 270, 227, and 43 bp, respectively. **Lane 1,** marker**,; lane 2** Pro homozygote; **lane 3,** Ala homozygote; **lane 4**, heterozygote. The 43-bp fragments are not visualized.
The distribution of polymorphic genotype were strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for PPARG2 Pro^{12}Ala polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of PPARG2 Pro^{12}Ala genotypes was significantly different in HC group as compared to disease group (χ^2=7.253, P=0.0266*). HC group showed an increase of mutant 'GG' genotype as compared to Patients of diabetes type 2 (3.9% *vs. 0.52%*). Similarly, wild type '*AA*' genotype was present in significantly low frequency in HC as compared to Diabetes type 2 patients group (73.3% *vs.* 82.11%). An odds ratio of 0.1336 in Diabetes group respectively for 'GG*'* genotype indicated a protective effect of this mutant type genotype in our population whereas an odds ratio of 1.668 of Diabetic patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is not significantly different but may be protective because of odds ratio of 0.7094. Overall allele 'G' was found to be in significantly low frequency in disease group as compared to HC group whereas allele '*A'* was present in significantly high frequency in the disease group (χ^2 = 6.684, P= 0.0097**;). Overall G allele shows an odds ratio of 0.56 which indicates its protective association. Carriage rate of allele 'G' was high in HC group whereas carriage rate of allele 'A' was high in disease group (χ^2 =3.281, P= 0.0701) but the values were not significant. .The pattern of genotype and allele distribution in disease and control group suggested a significant association of PPARG2 Pro^{12}Ala wild type allele 'A' carriage (carriage of 'AA') in Susceptibility to diabetes type 2 and also shows the protective effect of less common mutant allele G.

TABLE No-3 Frequency distribution and association of Genotype, allele frequency and carriage rate of PPARG2 P12A polymorphism in population of Vindhyan region using Chi Square Test

PPARG2	CASE N=190 N	%	CONTROL N=210 N	%	CHI SQUARE VALUE χ^2 (P Value)
GENOTYPE					
AA	156.0	82.11	154.0	73.3	7.253, (0.0266*)
AG	33.0	17.37	48.0	22.9	
GG	1.0	0.52	8.0	3.9	
ALLELES					
A	345	90.79	356	84.8	6.684, (0.0097**)
G	35	09.21	64	15.2	
CARRIGE RATE					
A	189	99.4	202	96.2	3.281, (0.0701)
G	34	17.89	56	26.7	

* denotes the level of significant association between case and control

N – Number of individuals in study group
%- Genotype allele frequency and carriage rate expressed in percentage

TABLE No-4

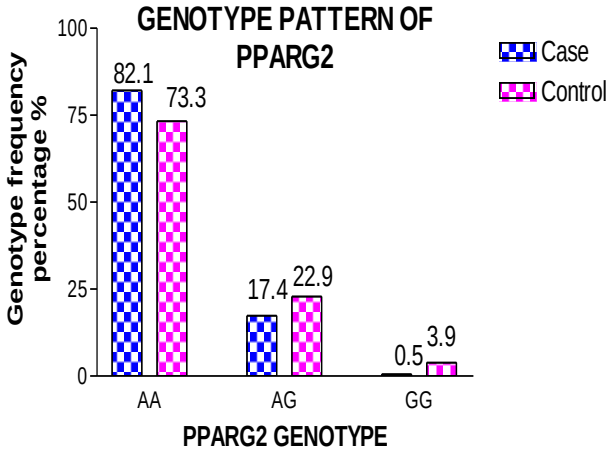
Fisher Exact Test values of PPARG2 P12A polymorphism

PPARG2 GENOTYPE	CASE N=190 n	%	CONTROL N=210 N	%	P Value	ODDS RATIO AND CI
AA	156.0	82.11	154.0	73.3	0.0415*	1.668 (1.031 to 2.699)
AG	33.0	17.37	48.0	22.9	0.2127	0.7094 (0.4326 to 1.163)
GG	1.0	0.52	8.0	3.9	0.0390*	0.1336 (0.01654 - 1.079)
ALLELES						
A	345	90.79	356	84.5	0.01**	1.772, (1.144 - 2.746)
G	35	09.21	64	15.5		0.5643, (0.3642-0.8744)
CARRIGE RATE						
A	189	99.4%	202	96.2	0.0789	1.541 (0.9630 - 2.466)
G	34	17.89	56	26.7		0.6489, (0.4055 -1.038)

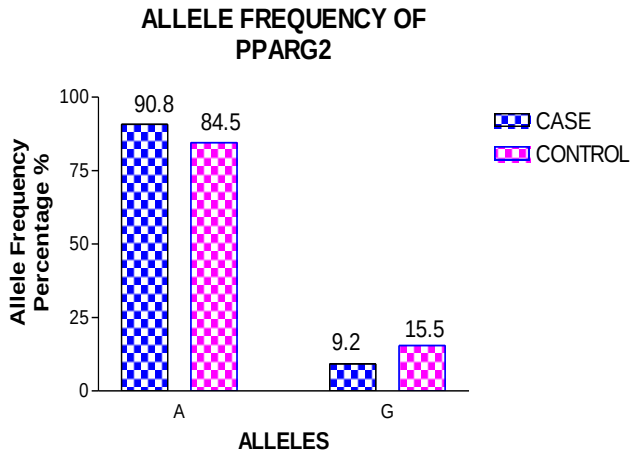
* denotes the level of significant association between case and control

N – Number of individuals in study group

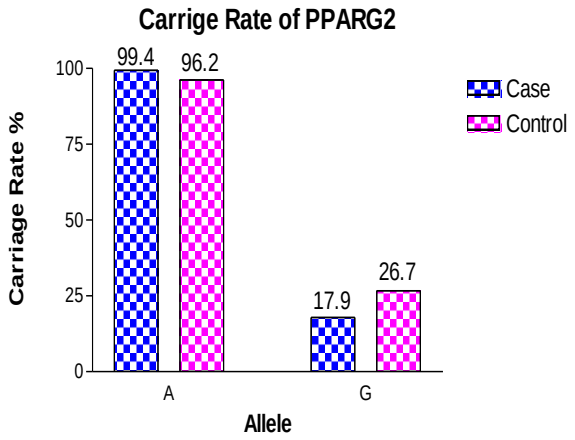
%- Genotype allele frequency and carriage rate expressed in percentage



Graph No. 1: Genotype distribution of PPARG2



Graph No. 2: Allele Frequency of PPARG2



Graph No. 3: Carriage Rate of PPARG2

IV. DISCUSSION

Type 2 DM is a disease that is increasing rapidly in a global environment of obesity and sedentary lifestyles. There is a systemic impact, both from a clinical and economic perspective on individuals and society. Diabetes type 2 is well established multifactorial disorder which could be associated with genetic as well as life style and environmental factors. It means to say that neither genetic nor environmental factors alone could be sufficient to cause diabetes type 2, but both of them are needed. Although etiology of type 2 diabetes is not very clear but many studies previously reported the inheritance in the progeny, supports its strong genetic link. Type 2 diabetes has reached epidemic proportions in the India and has become a major health problem worldwide. Escalating rates of type 2 diabetes is attributable to changes in the environment, favoring the onset of obesity, which is a major risk factor for type 2 diabetes. However, not all obese persons develop diabetes. Furthermore, a large variation in the occurrence of type 2 diabetes is observed among different ethnic groups, even when they are exposed to similar environmental conditions (Abate N *et al*., 2003).

In our study we had taken samples from both urban and rural population living in Vindhyan region. During the sample collection we prepared a questionnaire to gather the information about environmental and life style factors such as physical activity level and smoking habits. These factors have been previously found to be associated with increased diabetes risk in different other populations. BMI indexing is a tool used for documentation of obesity. In our present investigation we found that BMI was significantly higher in females. BMI of diabetic females were as compared to healthy females (P=0.0388). Weight Height Ratio (WHR) was also shown to be higher in male and female both. Sedentary lifestyle is strong factor behind the surprising rise in the prevalence of both obesity and diabetes (Steven N. Blair *et al*., 2003). In the

past decade, we have witnessed an epidemic of both type 2 diabetes and obesity. 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/m^2) or overweight (BMI 25–29.9 kg/m^2). The recent increase in the prevalence of obesity is closely paralleled by the increase in the prevalence of diabetes. Indeed, this new unprecedented phenomenon has been referred to as "diabesity." There is a clear strong relationship between obesity and the risk for diabetes (Sonia Caprio, 2003). In India our data also suggests that obesity and higher BMI can be an important factor which can affect the susceptibility to diabetes type 2.

In diabetic case lower number of physical active persons was seen as compared to control (41.57% Vs 53.33%). The significance level was sufficiently strong to reveal the protective association of physical activity (χ^2 =5.524, P Value 0.0188). An odds ratio of 0.62 clearly indicates the positive association of physical activity with prevention of diabetes and active life style could be concluded as a very important factor which can prevent pathophysiology of diabetes type. Exercise has been shown to increase insulin-stimulated glycogen synthesis through an increased rate of insulin-stimulated glucose transport by GLUT4 glucose transporters and increased glycogen synthase activity (Perseghin G *et al*., 1996) and this may be a important effect of active life style which may decrease the hyperglycemia by stimulating the glucose transport and glycogen synthesis. Many other protective role of active life style has been suggested and widely accepted worldwide.

Smoking is an established modifiable risk factor which is associated with many diseases such as CVD (Burke AP *et al*., 1997; Greenland P *et al*., 2003) and cancer. To some extent, the effects in physical conditions of smoking and diabetes are similar, which brings question if there is any association between smoking and diabetes. Many studies evidenced that chronic smokers have a higher risk for insulin resistance, and to develop type 2 diabetes mellitus (DM2) (Carlsson S *et al*., 2004; Willi C *et al*., 2007; Eliasson B. *et al*., 2003 and Facchini FS *et al*., 1992). Our result shows that percentage of smokers in case and control is not more different and there is lack of statistically significant association but an odds ratio of 1.205 shows a little higher risk of diabetes type 2 in smokers as compared to nonsmokers. Our results indicate that smoking may increase the risk of diabetes type 2 but relation with disease susceptibility was not established. It was previously reported in a metaanalysis that heavy smokers (at least 20 cigarettes daily) had a 61% higher risk, while less than 20 cigarettes daily were correlated to a 29% increase of the risk (Willi C *et al*., 2007).

Many other studies also show that current smokers have a 1.2- to 2.6-times higher risk of type 2 diabetes than nonsmokers (Rimm EB *et al*., 1993, 1995; Manson JE *et al*., 2000; Persson PG *et al*., 2000; Wannamethee SG *et al*., 2001; Will JC *et al*., 2001). Our results are consistent with this finding obtained by this metaanalysis but risk of diabetes is lower with smoking in our result as compare to results of this metaanalysis. The reason which could clarify our results is that we did not discriminated smokers in light and heavy smokers because the people included in this study were not sure about their daily smoking level. Probably due to this region our results shows a lower relative risk of smoking habit in diabetes type 2 in present investigation. Many other studies have been also published and shows, current smokers have a 1.2 to 2.6-times higher risk of type 2 diabetes than nonsmokers (Rimm EB *et al*. 1993; 1995; Manson JE *et al*. 2000; Persson PG *et al*. 2000; Wannamethee SG *et al*, 2001; Will JC *et al*, 2001). Our study strongly support these all studies and show that smoking may be a life style factor may be responsible in diabetes type 2 pathophysiology and if people start to quit smoking they can prevent themselves from being diabetic .

When we come to the results obtained by laboratory analysis we found that genes selected in our study may influence the susceptibility to diabetes type 2 by their polymorphic alleles. The PPARG2 gene abundantly expressed in adipose tissue has several variants, one of the most common (minor allele frequency of 10% in Caucasians) is the Pro12Ala substitution at codon 12 in *PPARG2*. This polymorphism has been shown to be associated with reduced ability to trans-activate responsive promoters and thus with lower PPARG2 transcriptional activity (Deeb SS *et al*., 1998). The importance of PPARG2 in lipid, glucose and energy metabolism is well established. Since PPARG2 promotes adipocyte differentiation, it is an attractive candidate gene for states of altered triglyceride storage, such as obesity or conditions associated with underweight. Since the Pro12 allele is present in at least 80% of humans, the population attributable to risk of type 2 diabetes associated with this polymorphism is as high as 25% (Altshuler D *et al*., 2000). More consistently, the Pro12Ala polymorphism has been associated with a lower risk of type 2 diabetes in a metaanalysis of genome-wide association studies. (Zeggini E *et al*., 2008) This gene is a confirmed type 2 diabetes susceptibility locus and is one of most 20 type 2 diabetes susceptibility loci identified over the last few years (Hebe N. Gouda *et al*., 2010).

In our results we found that rare ala allele 'G' was found to be in significantly low frequency in disease group as compared to Control group, whereas wild type allele '*A'* was present in significantly high frequency in the disease group.

These finding suggests that G allele may have a protective effect against pathophysiology of diabetes type 2. This study was done in small sample size but despite the sample size we found a strong relation with protection against diabetes type 2. In our study the ala allele is found significantly protective and was in healthy control in higher percentage as compared to Case (15.2 Vs 9.21%). In our study population, odds ratio of mutant AA genotype was 0.13 and heterozygous AG was 0.70 these results suggest that PPARG2 Pro 12 Ala polymorphism could be protective against diabetes type 2. The overall Odds ratio of less common 'A' allele was 0.56 which clearly indicates the possible protective role of PPARG2 Ala Allele.

Our study supports various studies already done before in different human race. Sanghera D.K. *et al*., (2008) demonstrated the protective effect of less common allele Ala is present in lesser frequency in case as compare to control in Punjabi population of India. The ala frequency is little higher in our population as compared to Punjabi Sikh population and can be concluded as more protective. In other studies such as in Japanese population (Kawasaki I *et al*., 2002), in Finnish population (Julie A. Douglas *et al.,* 2001), the results are consistent. A large sample study in Caucasian population and meta-analysis shows OR of 0.81 for ala allele which shows a significant protective association of ala allele. Another metaanalysis after 8 studies in Japanese population shows exactly similar odds ratio of 0.81 for the same allele (Hara *et al*., 2003). When only the largest studies (>500 cases) were considered, the association remained stable worldwide with odds ratio 0.84, 95% CI: 0.79, 0.90 (Hebe N. Gouda *et al*., 2010). Ludovico *et al*., (2007) postulated OR of 0.65 of less common ala allele in East Asian population. In present investigation our odds ratio for Ala allele is 0.56 which found to be more protective then in other populations. This work further confirms the association between the PPARG Pro12Ala polymorphism and type 2 diabetes, but results are not consistent in all other studies. Several studies suggest that Ala allele is associated with higher risk of diabetes including canadian ozi cree population (Robert A**,** Hegele *et al***.,** 2000**).** While a south Indian population study indicates that Ala allele don't have any protective effects (Radha v *et al*., 2006)**.** It is controversial to understand that north Indian and south Indian population shows difference in the association of PPARG2 polymorphism and susceptibility to diabetes type 2, but these findings may be due to diversity of Indian population and their different origins (Papiha SS, 1996). Our results are strongly supports the result obtained from north Indian population this indicate the similarity between north and central Indian population. In present study we found a protective significant association of Ala allele and our findings match with most population of the world including Caucasians, north Indian, Japanese and others.

Recently it has been observed that inflammation in the pancreas in diabetes type 2 patients and it may be associated with pathophysiology and etiology of diabetes type 2. Inflammatory biomarkers and their association with diabetes type 2 have been established. But there role in susceptibility to diabetes is still not very clear. Previously it was a hypothesis that inflammation is a characteristic feature of type 1 diabetes and was classified under autoimmune disorder but many studies in last few years clearly suggest the possible role of immune response and inflammation in insulin resistance, apoptosis of pancreatic cells and diabetes type 2. Our study can establish polymorphism of PPARG2, polymorphism as markers for diabetes susceptibility. we can conclude that this polymorphism don't have contribution in diabetes susceptibility in vindhyan region population. The replication of this study with larger sample size will be expected in future. Despite those genes many other genes can have strong effect in diabetes pathophysiology so we can use genome wide association study to discover some novel genes which have strong association with diabetes type 2 susceptibility.

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