Original article

Molecular & biochemical analysis of Pro12Ala variant of PPAR-γ2 gene in type 2 diabetes mellitus

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ABSTRACT

Diabetes has emerged as a major threat to human life globally. Genomic studies have found a significant link between the Pro12Ala polymorphism of the PPAR-γ2 gene with incidence as well as occurrence of the risk of metabolic syndrome. The present study was aimed at assessing the PPAR-γ2 variant in an Asian Indian cohort of type 2 diabetes patients and its correlation with metabolic parameters. The present case-control study involved 100 type 2 diabetic patients and 100 asymptomatic healthy volunteers enrolled in random. Assessment of demographic factors and biochemical parameters were done for all enrolled. In addition, genotyping for the Pro12Ala (CCA to GCA) polymorphism was done by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technology. The genotyping study detected the frequency of the CC genotype (Pro12Pro) to be higher in frequency in comparison to the heterozygous CG genotype in both, cases and controls. The homozygous GG genotype (Ala12Ala) was not detected in any of the cases or controls assessed. Biochemical analysis of the levels of malondialdehyde (MDA) detected a significant increase (p < 0.0001). Additionally, increase in levels of fasting and postprandial glucose, total cholesterol, triglycerides, and parameters of the liver and renal function tests were detected. This study detected the PPAR-γ2 to be a significant biomarker for type 2 diabetes mellitus.
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1. Introduction

Type 2 diabetes mellitus (T2DM) is a complex disorder which accounts for over 90% of the diabetes cases mainly characterized by hyperglycemia, due to defects either in insulin secretion or its activity and the signaling. The prevalence of this condition is likely to be double by the year 2040 in India and the cause is majorly linked to indirect causes of death due to diabetes-associated comorbidities. These include macrovascular complications like coronary artery disease (CAD) apart from disabilities like diabetic nephropathy, retinopathy and neuropathy. A projection by

International Diabetes Federation (IDF) for India predicted that T2DM may reach upto 87 million by 2030 (Sicree et al., 2009). Population-based studies in India have shown the prevalence in urban areas to range between 15% − 19% (Mohan & Deepa, 2006; Ramachandran, 2005).

In the etiology of T2DM & metabolic syndrome, environmental factors play a key role in association with genetic susceptibility (Mayer et al., 1996; Hong et al., 1997). Molecular level polymorphisms have been documented to alter many factors around T2DM including: pathophysiology either by increasing or decreasing susceptibility, effect of medications and also outcome of various components in dietary interventions such as dietary fiber, different types of fats, etc. Genes popularly studied include *PPAR-γ2* gene (peroxisome proliferator activated receptor gamma2), *FTO* gene (fat mass and obesity associated), *KCNJ11* gene (potassium voltage-gated channel subfamily J member 11), and the *SLC30A 8* gene (solute carrier family 30 member 8) (Shu et al., 2010; Raza et al., 2012). Recent investigations have also identified

a sum of 1,874 exceptional markers from 421 genes related with T2DM (Lim et al., 2010). Candidate gene approach studies in T2DM have detected sequence variants in and near genes which are known to have vital physiological role. *PPARG* gene is considered as a vital candidate gene involved in insulin resistance, adipogenesis, & adipocyte gene expression in T2DM (Radha et al., 2011). PPAR-γ gene is known to be positioned at on chromosome 3p25 & spans about 100 kb inclusive of 9 exons (Fajas et al., 1997).

PPAR commonly exists in 3 isoforms *PPAR-α, PPAR-β/δ & PPAR-γ* expressed in adipose tissue and mainly in the liver & heart, which is functionally associated with diabetes (Desvergne & Wahli , 1999). Several studies investigated polymorphisms in the *PPAR-γ* gene with the variant Pro12Ala, which is well-defined by a change from CCA to CGA in 12th codon of the exon B (Yen C-J et al., 1997). Many studies detected the Ala allele known to reduce menace for T2DM among different ethnic populations (Engwa et al., 2018). Another cross-sectional study involving different population groups detected the common Pro12 allele to confer a 1.25 fold higher prevalence in T2DM (Altshuler et al., 2000).

The purpose and the main objective of this proposed study is to assess the presence and frequency of Pro12Ala polymorphism in this case-control study and to determine its relation with levels of relevant biochemical parameters in adult Indian population from Telangana.

2. Materials and methods

2.1. Study cohort

This case-control investigation comprised an overall of one hundred type 2 diabetic cases recruited based on clinical symptoms and history shared by treating physicians from the outpatient department of the Mahavir Hospital, Telangana. A specific case record form designed for this study was used to document demographic characteristics, clinical features, family history and other relevant predisposing factors for both the cases and controls. The control group included one hundred healthy volunteers free of positive family history for T2DM. Institutional Ethics Committee of the Bhagawan Mahavir Medical Research & Centre (BMMRC) approved our study and patient consent was taken from each participant who have been enrolled following the principles of the Helsenki declaration procedures.

2.2. Sample collection and analysis

K2 EDTA vacationer whole blood sample of 5 mL was collected from all the consented participants and analyzed in this study. The collected whole blood samples from cases and controls were utilized for both genetic analysis of Pro12Ala polymorphism of the gene PPAR-γ2 and biochemical analysis. Collected blood samples were shipped in ice box to the molecular laboratory of the hospital and were stored at refrigerator until further use.

Biochemical parameters assessed in both cases and controls for this study include: plasma glucose levels of both fasting and postprandial,triglycerides, total cholesterol, HDL, LDL & VLDL lipoproteins; liver function tests inclusive of bilirubin, serum SGOT/AST (aspartate transaminase), SGPT/ALT (alanine transaminase), ALP (alkaline phosphatase) & renal function tests inclusive of urea and creatinine. Biochemical analysis for all parameters was done using the Erba Auto Analyzer (ERBA Diagnostics, USA).

Estimation for the levels of serum malondialdehyde (MDA), a lipid peroxidation was done using 96 well microplate assay and lipid peroxidation (MDA) assay kit (Sigma-Aldrich, USA).

For genetic analysis, DNA isolation was done by using the K2 EDTA whole blood sample (HiPurA™, HI Media, USA). By using 2%

agarose, qualitative analysis of genomic DNA has been determined using gel electrophoresis, while quantity was analyzed using nanodrop technology. The prepared genomic DNA sample was refrigerated at −20 °C till further analysis. Pro12Ala variant of the PPAR-γ2 gene was analyzed by PCR technique using appropriate primer pairs followed by genotyping by RFLP (restriction-fragment length polymorphism) technique using an appropriate restriction enzyme, and incubation at 60 °C was done. Post-digestion, the fragments were studied by using gel electrophoresis using 2.5% agarose gel stained by ethidium bromide & examined using a transilluminator as shown in Fig.1.

2.3. Statistical analysis

The students *t*-test was utilized for statistical analysis. Differences in the clinico-pathological characteristics between cases and controls was done by the chi-square test for categorical data and the students *t*-test for numerical data. Odds ratio and the 95% confidence interval (95% CI) was used to compute the genotype - diabetes association. A two-sided p value of < 0.05 was considered as an indicator of statistical significance.

3. Results

The cohort study which consisted of a hundred diabetic patients representing the cases, and age matched controls was genetically assessed for the status of the Pro12Ala polymorphism in the PPAR-γ2 gene and also for biochemical parameters involving lipid peroxidation, lipids, and liver and renal function tests apart from plasma glucose levels.

The cohort characteristics and the outcomes of analyses of the biochemical parameters have been highlighted in Table 2.

Statistically significant difference (p < 0.0001), was detected for the levels of plasma glucose in both fasting and postprandial conditions, levels of urea in renal function tests and VLDL in lipid profile between cases and controls.

The genotyping study for the Pro12Ala polymorphism in the PPAR-γ2 gene detected the CC genotype (Pro12 allele) to be higher in frequency in comparison to the heterozygous CG genotype (Pro12Ala allele). No homozygous GG genotype (Ala12 allele) was detected in our study among both cases and controls. The PCR-RFLP outcome has been highlighted in Table 1. The frequency of different Pro12Ala genotypes detected in this study among
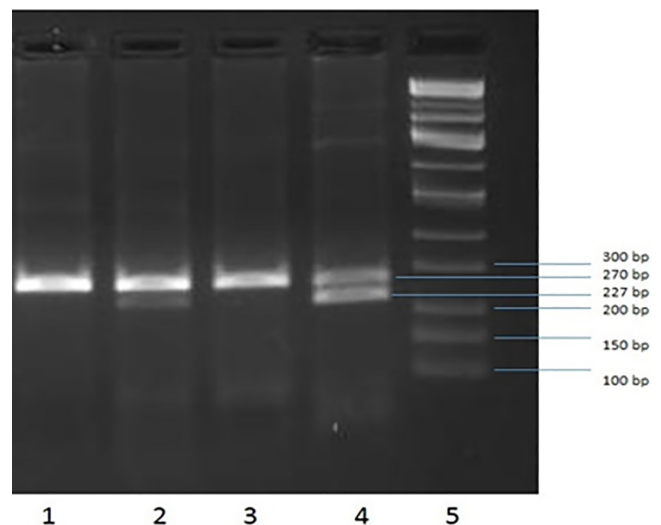

Fig. 1. PCR-RFLP results for the Pro12Ala polymorphism. Lane 1 for uncut DNA; lanes 2 and 4 for Pro12Ala heterozygotes; lane 3 for Pro12Pro homozygote; lane 5 for 100 bp marker.

Table 1
PCR and RFLP for the Pro12Ala polymorphism.

GENE	Forward & reverse Primers	PCR (bp)	Restriction Digestion enzyme	Restriction Products
PPAR γ Pro12Ala (Liao et al., 2006)	Forward -5′-GCC AAT TCA AGC CCA GTC-3′ Reverse -5′-GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC-3′	270 bp	*BstUI*	CC: 270 bp GG: 43 bp GC: 270 bp, 227 bp,43 bp

Table 2
Significant association of biochemical and clinical characteristics of the cohort studied population.

Parameters	Reference Values	Controls (n = 100)	Cases (n = 100)	*p* Value
1. Gender				
Male		54	55	
Female		46	45	
2. BMI		27 ± 5.1	28 ± 4.7	<0.0253
3. Plasma glucose levels				
FBS	65–110 (mg/dL)	99 ± 25	156 ± 57	<0.0001
PLBS	90–140 9 mg/dL)	142 ± 50	247 ± 89	<0.0001
4. Lipid Profile				
Triglycerides	25–160 mg/dL	137 ± 63	174 ± 89	0.0009
Cholesterol	50–200 mg/dL	192 ± 35	212 ± 48	0.0007
HDL	35–80 mg/dL	43 ± 12	40 ± 5.7	0.075
LDL	<100 mg/dL Optimal	121 ± 31	141 ± 43	0.0003
VLDL	</= 30 mg/dL	27 ± 9.4	32 ± 10.4	<0.0001
5. Liver function tests				
ALP	15–113 IU/L	84 ± 25	90 ± 27	0.1046
Bilirubin	0.1–1.2 mg/dL	0.7 ± 0.34	0.7 ± 0.35	0.999
ALT	0.5–40 IU/L	26 ± 12	25 ± 11	0.539
AST	0.5–40 IU/L	26 ± 13	25 ± 10	0.542
6. Renal function tests				
Urea	13 – 45 mg/dL	24 ± 5.3	28 ± 7.5	<0.0001
Creatinine	0.7–1.4 mg/dL	1.0 ± 0.15	1.0 ± 0.19	>0.999
7. MDA (nmol/μL)		1.34 ± 0.33	4.05 ± 1.45	0.001

significance = p value < 0.05

Table 3
Frequencies & variants of PPAR-γ2 Pro12Ala genotype in the studied population.

Gender		Cases (n = 100)	Controls (n = 100)	p-Value
Male	CC	52.2 ± 11.14	38.43 ± 11.99	<0.0001
	CG	53 ± 10.84	39.8 ± 11.66	<0.001
Female	CC	51.05 ± 7.40	36.30 ± 9.82	<0.001
	CG	52.42 ± 8.12	35.85 ± 2.35	<0.001
Age Distribution				
<50yrs Males	CC	40.84 ± 4.57	34 ± 8.52	<0.0001
	CG	42.16 ± 2.11	34.75 ± 6.35	<0.0001
<50yrs Females	CC	57.38 ± 4.17	34.47 ± 7.76	<0.0001
	CG	Not found	35.85 ± 2.35	
>50yrs Males	CC	59.43 ± 9.35	56.75 ± 4.60	0.0111
	CG	62.28 ± 5.25	60.3 ± 3	0.0002
>50yrs Females	CC	45.05 ± 4.03	58.33 ± 2.35	<0.0001
	CG	52.42 ± 8.12	Not found	

different age-groups for cases and controls as shown in the Table 3. The difference in frequency between cases and controls for both Pro12 and Pro12Ala allele was found to be statistically significant. The biochemical & clinical characteristics for each of the Pro12 and the Pro12Ala alleles has been summarized in Tables 4 and 5.

The levels of plasma glucose in fasting and postprandial condition between cases and controls of both the CC and CG genotype was detected to be significant at p < 0.0001. The difference in levels of VLDL and urea between cases and controls was detected to be significant only in the Pro12 allele carriers and not the heterozygous cohort. The difference in BMI among control & cases was shown to be significant (p < 0.005) in the CG genotype cohort and not the CC genotype carriers.

4. Discussion

Metabolic syndrome characterized by majorly glucose intolerance and abdominal obesity has been documented to be the major predisposing factors for diabetes and cardiovascular diseases, and the prevalence has been detected to be around 20% − 25% globally (Robitaille J et al., 2003; Sun K et al., 2012). When considering the genetic aspects of predisposing factors for T2DM, the PPAR-γ gene has been widely studied as it is involved in adipocyte differentiation and regulating action of insulin (Sundvoid H and Lien S, 2001; Zhang R et al., 2014). The Pro12Ala polymorphism in exon B of the PPAR-γ2 gene encodes the amino-terminal domain and the frequency of the

Table 4
Summarizing the biochemical & clinical characteristics of the Pro12 allele cohort.

Parameters	Reference Values	Controls	Cases	p Value
1. Gender				
Male		43	40	
Female		39	37	
2. BMI		27 ± 5.0	28 ± 5.0	0.1589
3. Glucose level				
FBS	65–110 (mg/dL)	100 ± 26	155 ± 59	<0.0001
PLBS	90–140 (mg/dL)	143 ± 52	240 ± 87	<0.0001
4. Lipid Profile				
Triglycerides	25–160 mg/dL	135 ± 63	174 ± 94	0.0007
Cholesterol	50–200 mg/dL	191 ± 38	209 ± 49	0.0032
HDL	35–80 mg/dL	43 ± 4.3	39 ± 6.6	0.015
LDL	<100 mg/dL Optimal	120 ± 32	137 ± 45	0.0024
VLDL	</= 30 mg/dL	26 ± 8.6	32 ± 10	<0.0001
5. Liver function tests				
ALP	15–113 IU/L	83 ± 24	90 ± 26	0.0493
Bilirubin	0.1–1.2 mg/dL	0.7 ± 0.3	0.7 ± 0.3	0.999
Urea	13 – 45 mg/dL	24 ± 4.9	28 ± 7.7	<0.0001
Creatinine	0.7–1.4 mg/dL	1.0 ± 0.1	1.0 ± 0.1	0.999

Note- BMI (Body Mass Index) : FBS (Fasting Blood Sugar) : PLBS (Postprandial plasma glucose) : HDL (High-density lipoproteins): LDL (Low-density lipoproteins):VLDL - Very low-density lipoprotein :AST (Aspartate aminotransferase): ALT (Alanine aminotransferase) :ALP (Alkaline phosphatase)

Table 5
Biochemical & Clinical aspects of the Pro12Ala allele cohort.

Parameters	Reference Values	Controls	Cases	p Value
1. Gender				
Male		11	15	
Female		07	08	
2. BMI		25 ± 5.1	29 ± 2.2	<0.001
3. Glucose level				
FBS	65–110 (mg/dL)	104 ± 33	156 ± 43	<0.0001
PLBS	90–140 (mg/dL))	138 ± 31	278 ± 96	<0.0001
4. Lipid Profile				
Triglycerides	25–160 mg/dL	145 ± 42	158 ± 31	0.219
Cholesterol	50–200 mg/dL	204 ± 38	226 ± 40	<0.0001
HDL	35–80 mg/dL	43 ± 4.3	39 ± 6.6	0.015
LDL	<100 mg/dL Optimal	129 ± 31	157 ± 30	0.0021
VLDL	</= 30 mg/dL	31 ± 11.7	30 ± 6.7	0.712
5. Liver function tests				
ALP	15–113 IU/L	87 ± 29	88 ± 33	0.909
Bilirubin	0.1–1.2 mg/dL	0.7 ± 0.2	0.6 ± 0.1	0.0005
Urea	13 – 45 mg/dL	24 ± 6.1	28 ± 6.6	0.015
Creatinine	0.7–1.4 mg/dL	1.0 ± 0.13	1.0 ± 0.2	0.031

Note- BMI (Body Mass Index) : FBS (Fasting Blood Sugar) : PLBS (Postprandial plasma glucose) : HDL (High-density lipoproteins): LDL (Low-density lipoproteins):VLDL - Very low-density lipoprotein :AST (Aspartate aminotransferase): ALT (Alanine aminotransferase) :ALP (Alkaline phosphatase)

minor allele among different ethnic groups range between 2% – 23% (Stumvoll and Haring , 2002).

Association of the Pro12Ala polymorphism with BMI, lipid profile, insulin resistance, and hypertension apart from T2D has been shown by many studies (Estivalet et al., 2011; Gao et al., 2010; Passaro et al., 2011). Studies have also shown significant interaction of the nutritional components with genetics in the BMI and T2DM at PPARγ gene in the general population (Lamri et al., 2012). The effect of Ala allele of the Pro12Ala polymorphism on the improvement of glucose and insulin metabolism in response to regular endurance training has also been demonstrated by certain studies [Ruchat SM et al., 2010].

Our study was focused at assessing the prevalence of the Pro12Ala polymorphism in T2DM in relation with many biochemical markers among a cohort of T2DM patients and age matched healthy controls. Our study detected the Pro12 allele and the Pro12Ala allele among both cases and controls. No Ala12 allele was detected in our study of the 100 cases and controls analyzed. The frequency of the heterozygous genotype detected in our study was 18% among the controls and 23% among the cases. The Pro12 allele was detected at frequencies of 82% and 77% among the con-

trols and cases respectively. This frequency is comparable with those reported in other population groups including the Chinese at 90%, Japanese at 95% and the French at 81% respectively (Dehwah et al., 2008; Ghoussaini et al., 2005; Mori et al., 2001). Among similar studies in different population groups in India, a report on Pro12Ala in a North Indian cohort detected the frequency of the CC genotype to be 31% and much lower in comparison to our findings. This frequency also detected the frequency of C allele to be 52% and 41% across the cases of T2DM and matched controls respectively (Raza ST et al., 2012). Another study on Pro12Ala carried among population from Chennai in South India detected the frequency to be 19% and no significant difference between cases and controls was detected. Furthermore, a haplotype analysis detected the Pro/Pro allele and the −1279G/A showed increased susceptibility to T2DM (Radha V et al., 2011).

Apart from genotyping for the Pro12Ala polymorphism, biochemical analysis was also done to identify the correlation if any. In case of the Pro12Ala heterozygotes, significant difference in BMI, fasting and postprandial glucose levels, levels of cholesterol, HDL and LDL, bilirubin, urea and creatinine was detected (p < 0.05) between cases and controls. In case of the Pro/Pro allele,

significant difference was detected for levels of plasma glucose in both fasting and postprandial condition, biomarkers of the lipid profile, levels of ALP and urea at p < 0.05 between cases and controls. Studies have identified significant differences in circulating levels of triglyceride, glucose, LDL, high-sensitive c-reactive protein, interleukin-6, tumor necrosis factor-alpha and urinary 8-epiprostaglandin $F_{2\alpha}$ between diabetic and non-diabetics (Ha et al., 2012). A study which reported on association between BMI and the Pro12Ala polymorphism as a *meta*-analysis of 49,092 subjects, detected Pro12 allele to be associated with low BMI (Galbete et al., 2013). Another study among the Nigerian population detected prevalence of lipid abnormalities like hypercholesterolaemia (TC > 200 mg/dL), hypertriglyceridaemia (TG > 150 mg/dL), high HDL (>100 mg/dL), and low LDL (<50 mg/dL) to be significantly higher (p < 0.001) among the T2DM patients in comparison to non-diabetic controls [Engwa GA et al., 2018].

5. Conclusion

Many genetic studies as well as nurtigenomic approaches have identified inconsistent results pertaining to definite markers and expressed phenotype. This indicates the need to have further research and studies in different population groups to neutralize the confounding findings in T2DM. The Ala12 allele was not detected in this study, and the significant association between Pro12 allele and biomarkers were detected in our study. The need for further large-scale population studies encompassing different ethnic groups is a need to identify genetic markers of great relevance for risks of metabolic syndrome.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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