Original article:

Association between PRO12ALA polymorphism at the PPARγ2 gene and insulin sensitivity in South Indian population with type 2 diabetes mellitus

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Abstract

Peroxisome Proliferator Activated Receptors (PPAR) are members of the nuclear receptor superfamily of ligand activated transcription factor consisting of 3 subtypes PPARα , PPARβ and PPARγ. PPARγ plays an important role in adipocyte differentiation and it regulates lipid metabolism as well as insulin sensitivity. Of the 3 isoforms of PPARγ, PPARγ2 are expressed in adipose tissue. Genomic DNA was obtained from 75 Type II Diabetes Mellitus cases and 75 controls. Biochemical parameters included fasting plasma glucose and fasting plasma insulin which were analyzed correspondingly. Screening for mutation of PPARγ2 gene was performed by polymerase chain reaction and Pro12Ala polymorphism was analyzed using Restriction Fragment Length Polymorphism. Among controls, 89.3% were Pro/Pro homozygotes and 10.7% Ala/Ala homozygotes. InT2DM 82.7% were Pro/Pro homozygotes and17.3% Ala/Ala homozygotes. No statistically significant association of Pro12Ala with Type 2 diabetes and insulin sensitivity using HOMA β and HOMA -IR was found. However, the role of Pro12Ala polymorphism may not be excluded by a negative association study.

Key words: diabetes, HOMA-IR, insulin sensitivity, PPARγ , polymorphism

Introduction

Type 2 diabetes mellitus (T2DM) is a heterogeneous metabolic disorder characterised by insulin resistance and reduced insulin secretion.The rising prevalence of diabetes in developing countries is associated with industrialization, socio-economic development and change in life style. As the data suggests; India (31.7 million) topped the world with the highest number of people with diabetes mellitus followed by China (20.8 million) with the United States (17.7 million) in second and third place respectively [1].We understand the advantages of tight control of blood glucose levels, but the knowledge about genetic susceptibility to the disease and interaction between potential therapies and individual genetic signature remains incomplete. The difference in response to medication may be the result of heterogeneity at the genetic level. An improved understanding of genetic mechanism should allow us to test whether behavioral and pharmacologic therapies can be tailored, thus alleviating the burden inflicted by the disease.Variants in many candidate genes for diabetes have been extensively studied over the past few decades. Evidences generated from metaanalysis of multiple published studies results in determination of susceptibility genes from among the candidate genes. Definition of the genetic factor that predispose to diabetes should enhance the ability to predict who in the population

may be at risk. Such improved prediction may facilitate the development of preventive therapies. Gene association studies have identified several common variants implicated in T2DM. One of it is Peroxisome Proliferator Activated Receptor γ2 (PPARγ2). A Pro12Ala polymorphism at extreme amino terminus of PPAR-γ2 gene has been studied but its effect on weight gain and insulin sensitivity is unclear [2].

PPARs are members of the nuclear receptor superfamily of ligand activated transcription factor that consists of 3 subtypes PPARα , PPARβ and PPARγ. PPARγ plays an important role in adipocyte differentiation and it regulates lipid metabolism as well as insulin sensitivity. There are 3 isoforms of PPARγ resulting from alternative splicing. They are PPARγ1 which is expressed in various tissues, PPARγ2 expressed in adipose tissue and PPARγ3 which is restricted to macrophages and large intestine. Expression ofPPARγ2 in adipose tissue makes it a candidate gene to be involved in regulation of adipogenesis and lipid storage, insulin and glucose metabolism[3].

Several groups have reported nucleotide sequence polymorphisms within the coding exons of the PPARγ gene [4,5]. The Ala12 allele frequency varies from 0.03 to 0.12 in several populations and was initially shown to be associated with increasing degrees of obesity [6]. In several additional studies, the Ala12 allele was associated with lower BMI, improved insulin sensitivity, and reduced incidence of type 2 diabetes [4]. In one large study, the more common Pro12 allele was associated with a 1.25-fold increase in risk of type 2 diabetes [7]. In contrast, other groups failed to detect an association of Ala12 with altered metabolic parameters [8,9]. Since this variant is relatively prevalent, it may contribute to altered physiology of fat metabolism in humans.A cytosine to guanine nucleotide

polymorphism at codon 12 ofPPARγ gene resulting in a proline to alanine substitution at 12th amino acid has been found to modulate the transcriptional activity of the genes involved in adipocyte differentiation and metabolism and associated with altered insulin sensitivity [10]. Although most studies have shown a statistically significant Type 2 Diabetes reduction given by Ala variant [11,12], some others have not [13,14], suggesting variability in the contribution of this variant to the risk of T2DM. Its association have been inconsistent among Asian population [15].The Indian population forms a heterogeneous population. We have studied the effect of Pro12Ala polymorphism at PPARγ2in South Indian population with Type 2 diabetes mellitus.

Methods

Study Population : Upon clearance from the institutional ethical committee**,** we studied a total of unrelated 150 subjects of South Indian population in the age group 40 – 50 yrs who visited Sri Ramachandra Hospital, Chennai for routine checkup. Out of this 75 were individuals with T2DM and 75 were controls. Inclusion criteria for controls which included 37 males and 38 females were non diabetic with fasting plasma glucose <126 mg/dl , no family history of diabetes and no other known illness. Out of 75 T2DM 36 were males and 39 were females. The cases were known diabetics on oral hypoglycaemic agents.Exclusion criteria for both cases and control were any kind of acute illness, malignancy or any systemic disorders. The data were included through a standard questionnaire.All patients were interviewed and examined regarding age, sex, duration of illness, mode and duration of treatment, past medical and surgical history, personal habits and family history of diabetes. All study participants gave their consent to participate in the study after being informed of its nature.

Biochemical analysis

About 5 ml of peripheral blood was drawn from each subject. Samples were collected in vacutainer with di-potassium EDTA as anticoagulant for genotype analysis. The samples were stored at -70°C till further analysis.Fasting Plasma glucose was measured by standard enzymatic method. Fasting serum insulin was measured with Chemi Luminescent Immune Assay technique (CLIA). Insulin resistance was calculated using the homeostasis model assessment score, which uses the formula:

HOMA-IR = Insulin(mU/L) x Glucose(mmol/dl) / 22.5.

Homeostasis model assessment of beta cell function (HOMA- β) = 20 x Insulin (mU/L)/ [Glucose(mmol/l)- 3.5]

DNA Analysis

The genomic DNA was isolated from peripheral blood of all study participants by means of salting out method optimised in our laboratory [16]. The DNA was subjected to qualitative and quantitative analysis. The quality of the DNA samples was checked in 0.8% agarose gel.The gel was electrophoresed at 2 volts/cm and was visualized in a gel documentation system (BioRad Laboratories). The Pro12Ala polymorphism was analysed with the help of PCR-RFLP. One base substitution was identified – a C to G substitution in exon B of the PPARγ2 gene.Amplification of the gene of interest was performed using specific primers under appropriate cycling conditions of initial denaturation at 98˚C for 3 minutes, denaturation at 94˚ C for 1 minute followed by annealing at 62˚C for one minute and extension for 72˚Cfor 1 minute in a Thermocycler (Master Cycler Gradient, Eppendorf).A segment of the PPARγ2 gene encompassing the Pro12Ala site was amplified with the help of primers obtained from Metebion International AG, Germany.

Table 1 : Sequence of primers

Primers	Primer sequence
Forward Primer	5´- CCAATTCAAGCCCAGTCCTTTC-3´
Reverse Primer	5´- CAGTGAAGGAATCGCTTTCCG-3´

Polymerase chain reaction (PCR) amplification was confirmed by 2% agarose gel electrophoresis. 100 base pair molecular weight marker was used to confirm the amplicon size. Electrophoresis was carried out and the gel was visualized in the gel documentation system. The purified PCR amplicon was subjected to restriction digestion with the BstU1 restriction enzyme procured from Fermentas Life Sciences, Germany **(Figure 1,2).**
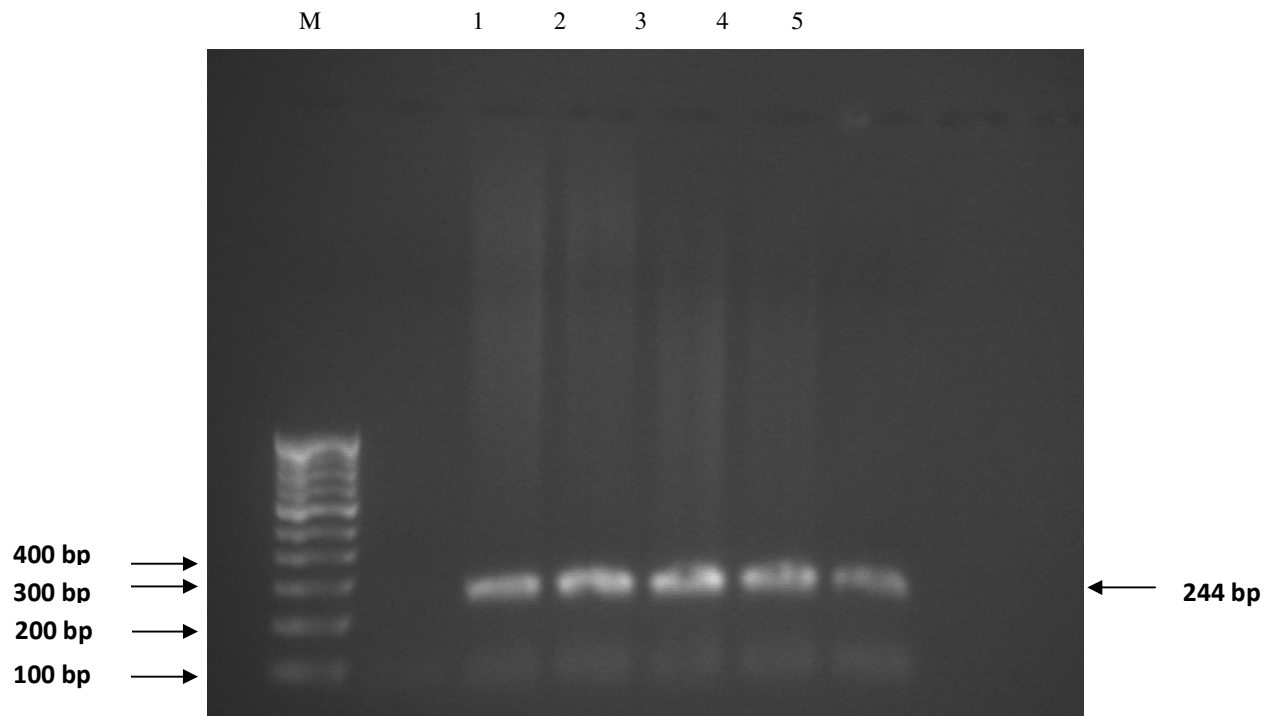
Figure 1: Amplified PCR product from whole genomic DNA

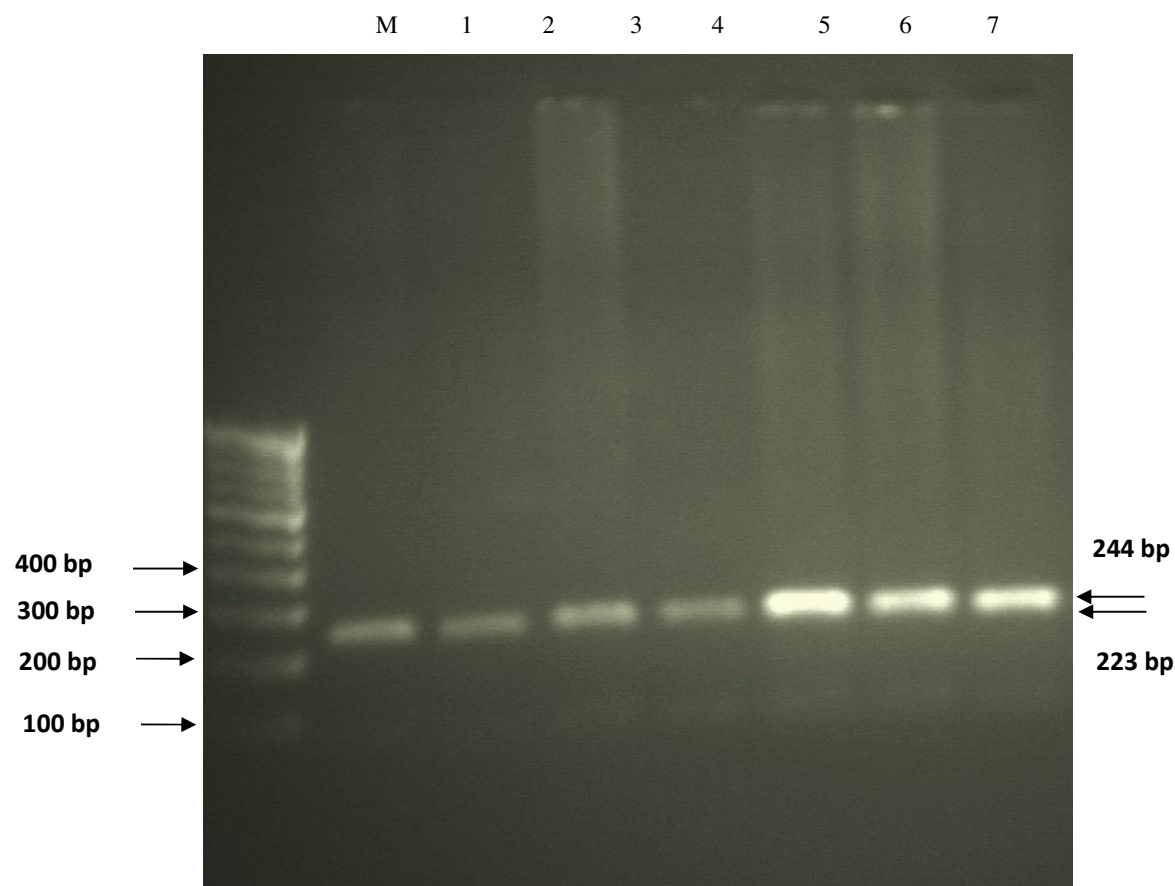Lane M: 100bp DNA Ladder, Lanes 1-5: 244bp amplified product

Figure 2: PCR-RFLP of Pro12Ala SNP using BstU1 restriction enzyme.

Lane M: 100 bp Marker, lanes 1 and 2 show 223bp products indicating Ala/Ala homozygotic condition, lanes 3-7 show 244 bp products indicating Pro/Pro homozygotic condition.

Statistical Analysis

The analysis was performed by means of Statistical Package for the Social Sciences (SPSS) for windows (version 15.0) software. The clinical and laboratory characteristics of controls and cases were compared by means of unpaired student´s t-test or chi-square test as appropriate. Genotype distribution in the cases and controls were compared by means of chi-square test.

Results

Distribution of Gene Polymorphism

DNA samples from all subjects 75 cases and 75 controls were amplified. The PCR products were visualized under UV light. The expected size of PCR products after digestion with BstU1 were 244bp Pro12 homozygotes, 223 and 21bp for Ala12 homozygotes and 244, 223 and 21 bp for heterozygotes. In this study no heterozygotes were identified. The distribution of polymorphism in controls and cases were studied. The association of this polymorphism with various biochemical parameters were also studied.On genotype analysis, the Pro/Pro homozygotes were 62 (82%) in cases and 67 (87%) in controls. The number of Ala/Ala homozygotes were 13 (17.3%) in cases and 8 (10.7%) in controls.Pearson´s Chi square test was applied to see the association of the two genotypes with Type 2 Diabetes and was not found to be significant.

Table 2: Biochemical parameters of patients with Type 2 diabetes mellitus (T2DM) and controls(CG) according to group and PPARγ2 Pro12Ala genotype

Parameters	Groups			T2DM Cases*			Control *		
	T2DM* (n =75)	CG* (n=75)	P	Pro/Pro (n=62)	Ala/Ala (n=13)	P	Pro/Pro (n=67)	Ala/Ala (n=8)	P
Fasting glucose (mmol/l)	10.17 ± 2.9	5.58 ± 0.57	< 0.001	10.33 ±2.96	9.37 ± 2.56	0.283	5.56 ± 0.52	5.82 ± 0.35	0.182
Fasting insulin (µU/ml)	10.03 ± 5.23	7.97 ± 3.77	< 0.01	10.29 ±5.6	8.8 ± 2.7	0.352	8.22 ± 3.8	5.8 ± 2.2	0.08
HOMA-IR	4.62 ± 3.22	2.0 ± 1.0	< 0.001	4.83 ± 3.4	3.6 ± 1.35	0.238	2.06 ± 1.03	1.4 ± 0.50	0.123
HOMA β	34.9 ± 21.34	78.89 ± 37.95	< 0.001	34.99 ± 22.43	34.49±15.8	0.726	81.93±38.05	53.44±27.26	<0.05

*Values are mean± S.D. ,P values for Student´st-test; HOMA- IR,Homeostasis model assessment for insulin resistance; HOMA-β, Homeostasis model assessment of beta cell function

Table 2 represents various parameters from 150 unrelated individuals. On comparison of fasting glucose, fas-ting insulin, HOMA-IR and HOMA-β of cases and controls the difference was found to be significant indicating T2DM cases to be more insulin resistant than control and a better β cell function in controls. Biochemical para-meters for 2 genotypes were compared, there was no significant difference found between Pro12 and Ala12 car-riers in cases and controls. However, HOMAβ in Pro/Pro homozygotes was higher than among Ala homozy-gotes.This was statistically significant but no such significance was found in HOMAβ with respect to cases.

Discussion

The present study aimed to study the Pro12Ala polymorphism in PPARγ2 gene in South Indian population. Various biochemical parameters were analyzed in controls and Type 2 diabetics.The association of this polymorphism with T2DM was studied. The relationship of this polymorphism with insulin and other biochemical parameters were also studied. In this study, Pro12Ala polymorphism was examined in DNA samples of 75 T2DM cases and 75 controls. The PCR-RFLP method has been applied to identify Pro/Pro homozygotes (244 bp fragment), Ala/Ala homozygotes (223 and 21 bp fragments) and Pro/Ala heterozygotes (244, 223 and 21 bp fragments). In this study no heterozygotes were identified.

Association of Pro12Ala and Type 2 Diabetes mellitus

On genotype analysis, Pro/Pro homozygotes were 62 (89.3%) in controls and 67(82.7%)in cases. The number of Ala/Ala homozygotes were 8(10.7%) in controls and 13(17.3%) in cases . These results did not reveal any statistically significant association of the Pro12Ala SNP with T2DM. This result corroborates the findings of study done on South Indian population from Chennai by **Radha et al. 2006** [17]. Another study on South Indian population by **Vimleswaram et al. 2007** reported no association of Pro12Ala SNP with metabolic syndrome [18]. **S.Ereqat et al. 2009** were also unable to demonstrate a significant association of Pro12Ala variant and T2DM in Palestinians [19]. The finding of the present study is contradictory to the report of **Altshuler Det al. 2000** a study done on caucasians**,** where an association of this polymorphism was found with the insulin

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sensitivity. , **Ek J et al. 2001**,**Douglas J A et al 2001** and **Hara K et al 2000** have also reported an association between Ala12, increased insulin sensitivity and a reduced risk of T2DM [7,10,20,21]. Another study done by **Dharambir K Set al2008** on Asian Sikh community from North India revealed a significant association Pro12Ala with T2DM [22]. The observed difference of association within Asian population is important in view of extensive diversity present in Indian population.

Analysis of Insulin Resistance in Cases and Controls and Its Relationship with Pro12Ala Polymorphism

On the comparison of FPG among cases and controls (Table2), the level was higher in cases compared to controls and it was found to be highly significant (p<0.001).The mean level of fasting insulin was 7.97µU/L in controls and higher in cases 10.03µU/L. The difference was found to be significant. To measure insulin resistance HOMA-IR and HOMA –β were calculated. In controls HOMA –IR was calculated to be 2.0 which is less than that of Type 2 diabetics where it was found to be 4.62.The difference was found to be highly significant (p<0.001) showing T2DM cases to be more insulin resistant than controls. HOMA-β was calculated as a measure of β cell function and was found to be significantly higher in controls 78.89 than in cases where it was 34.9 (p<0.001), indicating a better β cell function in controls. A positive correlation was found between FPG and fasting insulin levels in controls as well as cases. In controls this correlation was statistically significant (p<0.05) where as in cases it was not. HOMA-IR also showed a significant positive correlation with FPG (p<0.01). HOMA-β had a negative correlation with FPG in controls and cases but it was highly significant in cases (p<0.001) indicating a fall in β cell function.

The FPG levels in the two genotypes were compared (Table2). In T2DM cases Pro/Pro homozygotes had a mean FPG of 10.33mmol/l which was higher than the Ala/Ala homozygotes 9.37mmol/l but this difference was not statistically significant. In controls the Pro/Pro homozygotes had FPG 5.56mmol/l which was marginally lower than the Ala/Ala homozygotes 5.82mmol/l. This difference was also not found to be significant. This result is similar to that of **E.Shyoung Tai et al.2004, V.Tavares et al.2005,Rhee EJ et al 2006** [23-26]where Pro/12Ala polymorphism did not affect FPG levels. However, **Ahluwalia M et al. 2002 and Raffaella Buzzetti et al.2004** reported that Pro12Ala polymorphism was associated with lower fasting plasma glucose[27,28].

The relationship of Pro12Ala polymorphism and insulin resistance was analyzed in controls and Type 2 diabetics (Table 2). The fasting insulin levels in Pro/Pro homozygotes in controls were 8.22µU/ml and in those of Ala/Ala homozygotes were 5.8µU/ml.This difference was not found to be significant. Similarly in the Type 2 diabetics Pro/Pro homozygotes had fasting insulin of 10.29µU/ml and the Ala/Ala homozygotes had 8.8µU/ml, which was also not significant. In the present study, there is no significant difference in the fasting insulin levels between the Pro12 and Ala 12 allele carriers in cases as well as controls. HOMA-IR was found to be lower in Ala/Ala homozygotes than in Pro/Pro homozygotes in controls but was not found to be statistically significant. A similar trend was observed in cases where HOMA-IR in Ala/Ala homozygotes was lower than those of Pro/Pro homozygotes. This may be related to the protective role of Ala allele. In controls HOMA-β in Pro/Pro homozygotes was higher than that among the Ala/Ala homozygotes. This was statistically significant but no such significance was found in HOMA-β with respect to

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cases. In the present study, no significant association of Pro12Ala polymorphism was seen with insulin sensitivity.

Ek J et al. 2001 and **Hara K et al. 2002** who studied on Japanese population reported increased insulin sensitivity in overweight obese group. **V.Taveres et al.2005 and H Grazeielle et al.2010**reported a similar finding in Brazilian population.[24,25]

Rhee E J et al. 2006 had a similar report in a study done on Korean population [26]. **Dharambir K Sanghera et al 2007** who studied on Asian Indian Sikh did not find this variant to influence insulin levels [22]**. Radha et al. 2006** who studied on South Indian population in Chennai, the same place where this present study has been done, did not observed any significant difference in insulin concentration in Pro12Ala polymorphism carriers and those with wild genotype [17].We need to exercise caution when interpreting the result of present study, as association studies require attention to their design. A study on larger population are needed to understand the mechanism of Pro12Ala polymorphism which will further help in better understanding of the role ofPPAR-γ2 in adipose tissue metabolism and insulin sensitivity. In conclusion, the result of our study showed no statistically significant association of Pro12Ala polymorphism with Type 2 diabetes mellitus.The difference in reports of various studies may suggest that the effect of genetic variation may be restricted to particular ethnic groups. This may also be due to the influence of other genetic variants in the candidate gene or the interaction of certain yet uncharacterized genetic factors with environmental factors.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest: Nil

References

1. Seema Abhijeet and John Cornwall. The current state of diabetes mellitus in India. Australas Med J. 2014; 7(1): 45–48.

2. Allen F Moore & C Florez Jose. Genetic Susceptibility to Type 2 Diabetes & implication for antidiabetic therapy. Ann. Rev.Med.2008;59:95-111

3. J Berger and David E Mullar. The mechanism of action PPARS. Ann.Rev.Med.2002; 53: 409-435

4. Deeb SS, Fajas L, Nemoto M, et al. A Pro12Ala substitution in PPARγ2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nat. Genet.1998; 20:284-7

5. Meirhaeghe A, Fajas L, Helbecque N et al. A genetic polymorphism of the PPAR γ gene influences plasma leptin levels in obese humans. Hum. Mol. Genet. 1998;7:435-40.

6. Beamer BA, Yen C-J, Anderson RE, et al. Association of the Pro I 2Ala variant in the PPARγ2 gene with obesity in two Caucasian populations. Diabetes 1998;47:1806-8.

7. Altshuler D, Hirschhorn SN, Klannemark M, et al.The common PPARγ Pro l2Ala polymorphism is associated with decreased risk of Type 2 diabetes. Nat. Genet. 2000; 26:76-80

8. Mori Y, Kim-Motoyama H, Katakura T, et al. Effect of the Pro12Ala variant of the human PPAR γ2 gene on adiposity, fat distribution, and insulin sensitivity in Japanese men. Bio. Cem. Biophys. Res. Commun.1998 ;251:195-98.

9. Ringel 3, Engeli S, Distler A, Sharma AM. Pro12Ala missense mutation of the PPARgamma and diabetes mellitus. Biochem. Biophys. Res. Commun.1999; 254:450-53.

10. Ek J et al. Studies Pro12Ala polymorphism of PPARγ2 gene in relation to insulin sensitivity in among glucose tolerant Caucasians.Diabetologia,2001; 44(9):1170-1176

11. E.Zeggini et al.Examining the relationship between the Pro12Alavariant in PPARG and Type 2 diabetes –related traits in UK sample.Diabetic Med.2005 ; 22(12) :1696-1700

12. M Ghoussaini et al. Implication of Pro12Ala polymorphism of PPARγ2 gene in type 2 diabetes and obesity in French population. BMC Medical Genetics,2005; 6 (1): 11

13. . R Badii et al. Lack of association between Pro12Ala polymorphism of PPARγ2 gene and type 2 diabetes mellitus in Qatari consanguineous population. Acta Diabetologia.2008;45(1):15

14. K Z Bouassida et al .The PPARγ2 Pro12Ala variant: lack of association with type 2 diabetes in obese and non obese Tunisian patients. Diabetes and metabolism. 2005; 31 (2) :119-123

15. Stumvoll M et al. The PPARγ2 Pro12Ala Polymorphism. Diabetes.2002 : 51;2341-2347

16. Salah M Aljanabi et al. Universal and rapid salt extraction of high quality genomic DNA for PCR based technique. Nucl. Acid Res.1997; 25(22): 4692-4693.

17. Radha V et al. Role of genetic polymorphism PPARgamma2 Pro12Ala on ethnic susceptibility to diabetes in South Asian and Caucasian subjects: evidence for heterogeneity. Diabetes Care, 2006 ; 29(6) :1046-1051

18. Vimleswaran K S etal.Absence of association of metabolic syndrome with PPARGC1A, PPARG and UCP1 gene polymorphism in Asian Indians. Metab. Syndr. Relat.Disord. 2007,5:153-162

19. . S Ereqat et al. Impact of the Pro12Ala polymorphism of the PPARγ2 gene on metabolic and clinical characteristic in the Palestinian type 2 diabetic patients.PPAR Research.2009.Art.874126: 5

20. Douglas J A et al. The PPARgamma 2 Pro12Ala variant :association with type 2 diabetes and trait difference. Diabetes,2001;50:886-890

21. Hara K et al. The Pro12Ala polymorphism inPPARγ2 may confer resistance to type 2 diabetes. Biochem. Biophy.Res. Commun. 2000; 271:212-216

22. Dharambir K S et al. Impact of nine common type 2 diabetes risk polymorphism in Asian Indian Sikhs: PPARG2(Pro12Ala), IGF2BP2, TCF7L2and FTO variants confer a significant risk. BMC Med.Genet.2008,9:59

23. E Shyoung Tai et al. Differential effects of the C1431T and Pro12Ala PPARγ2 gene variant on plasma lipids and diabetes risk in an Asian population. J Lipid Research.2004, 45:674-685

24. V Tavares et al. Association between Pro12Ala polymorphism of PPARγ2 gene and insulin senstivity in Brazilian patients with type 2 diabetes mellitus. Diabetes Obesity and Metabolism. 2005,7:605-611

25. H Grazielle et al. The Ala allelein the PPARgamma 2 gene associatedwith reduced risk of type 2 diabetes mellitus in Caucasian and improved insulin sensitivity in overweight subjects. Br J Nutr.2010; 104(4):488-97

26. Rhee E J et al. Effects of two common polymorphism of PPARγ2 gene on metabolic syndrome. Arch. Med. Res.2006;37(1):86-96

27. Ahluwalia M. et al. The influence of Pro12Ala mutation of PPARγreceptor gene on metabolic and clinical characteristics in treatment –naïve patients with type2 diabetes. Diabetes Obesity and Metabolism.2002;4(6):376-378

28. Raffaella Buzzetti et al. The common PPARγ2 Pro12Ala variant is associated with greater insulin sensitivity. Euorpian J of Hum. Genet.2004;12:1050-1054