

Association between PRO12ALA polymorphism of the PPAR- γ 2 gene and type 2 diabetes mellitus in Iranian patients

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BACKGROUND: Peroxisome proliferator-activated receptor (PPARs) have been identified as ligand-activated transcription factors that belong to the nuclear receptor superfamily. It has been shown that an association exists between Proline 12 alanine (Pro12Ala) polymorphism of PPAR-GAMMA2 (PPAR- γ 2) gene and increased risk of type 2 diabetes mellitus (T2DM) in different populations. Therefore, the present study was designed to investigate the association between Pro12Ala polymorphism of PPAR- γ 2 gene and T2DM in an Iranian population.

MATERIALS AND METHODS: Two hundred unrelated people, including 100 healthy controls and 100 diabetic patients were recruited diagnosed based on American Diabetes Association criteria. Blood samples were used for isolation of genomic deoxyribonucleic acid (DNA). Having extracted the genomic DNA from human blood leukocytes by means of High Pure polymerase chain reaction (PCR) Template preparation kit, we carried out polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on each blood sample. Then, Genomic DNA was digested by BstU-I restriction enzyme. Thereafter, restriction products were analyzed by means of Polyacrylamide gel electrophoresis and stained by Ethidium Bromide.

RESULTS: We found that the frequency of Ala allele in healthy subjects was significantly higher than in diabetic subjects ($P = 0003$). Moreover, the genotype frequency of Ala/Ala in healthy subjects was significantly higher than

in diabetic subjects ($P < 0.001$). However, the genotype frequency of Ala/Pro in diabetic subjects was significantly higher than in healthy subjects ($P < 0.001$).

CONCLUSION: The present study suggests that polymorphism of PPAR- γ 2 gene is associated with T2DM. Furthermore, Ala allele is significantly found in non-diabetic individual's Iranian population.

Key words: Polymorphism, peroxisome proliferator-activated receptor-GAMMA 2 gene, type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes. It is characterized by a cluster of metabolic dysfunctions and cardiovascular risk factors, such as obesity, insulin resistance, dyslipidemia, atherosclerosis, hypertension, prothrombotic state, and endothelial dysfunction collectively known as the metabolic syndrome.^[1] Environmental factors (e.g., obesity and sedentary lifestyles) give rise to T2DM.^[2] Moreover, the association between lean first-degree family members of diabetic patients with insulin resistance shows the prominent role of genetic factors in this disease.^[3] In spite of intensive research, no specific genes causing T2DM have not yet been conclusively identified. Recent findings have shown that polymorphism of some genes can influence the risk of T2DM. To illustrate, one of these is Peroxisome proliferator-activated receptor-GAMMA (PPAR- γ) gene polymorphism. PPARs

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have been identified to be ligand-activated transcription factors that belong to the nuclear receptor superfamily.^[4] There is evidence that PPAR-γ receptor plays a part in control of blood pressure, insulin sensitivity, and glucose homeostasis.^[5] PPAR-α and PPAR-β have shown distinct tissue distribution.^[11] There are some data confirming the expression of PPAR-γ in human peripheral blood mononuclear cells.^[6,7] However, PPAR-γ, which has dominantly been found in adipose tissue, serves a significant role in glucose homeostasis, regulation of lipid, and adipocyte differentiation.^[8] In spite of the fact that the association between Pro12Al polymorphism of the PPAR-γ2 gene with insulin sensitivity in diabetic patients has been reported in some populations,^[9,10] a number of conflicting results have heightened the need for further investigation.^[11-13] Thus, this study was designed to examine whether the association between polymorphism of the PPAR-γ2 gene and T2DM exists in an Iranian population.

Materials and Methods

Two hundred unrelated people with the average age of 51, including 100 healthy controls and 100 patients with T2DM were recruited from Sedighe Tahere research center. T2DM was clinically diagnosed (polyuria, polydipsia and polyphagia were present) and was confirmed by American Diabetes Association criteria. To clarify, T2DM was determined by^[1] a fasting plasma glucose level of more than 7.0 mmol/l after a minimum fast of 12 h or^[2] a 2-h post-glucose level (2-h oral glucose tolerance test [OGTT]) of more than 11.1 mmol/l. Impaired glucose tolerance (IGT) was determined by a fasting plasma glucose level of between 5.6 and 7.0 mmol/l or a 2-h

OGTT of between 7.8 and 11.1 mmol/l. Normo-glycemic subjects were diagnosed by a fasting glycemia of less than 5.6 mmol/l or a 2-h glucose of less than 7.8 mmol/l. More clinical and biochemical characteristics of the participants are summarized in Table 1. Cholesterol, triglycerides, High-density lipoprotein and plasma glucose were measured by standard enzymatic assays. Low-density lipoprotein cholesterol was derived using Friedewald equation.^[14] Hemoglobin A1c was measured by means of ion-exchange high performance liquid chromatography with the normal reference range of 4.1-6.4%. Additionally, we applied exclusion criteria as follows: Individuals with IGT and positive family history of T2DM were excluded from the study due to the production of misleading results in analyses of the control group (CG). Individuals with previous diagnosis of blood disease, impaired renal and hepatic function and those receiving pharmacological treatment for T2DM, hypercholesterolemia, or hypertension were also excluded from the study in order to eliminate interference in biological variables. All participants gave a written consent before their enrolment for the study. The study protocol was approved by the Ethics Committee of Isfahan Medical University Research Center.

Obtained blood samples were used for isolation of genomic deoxyribonucleic acid (DNA). Having extracted the genomic DNA from human blood leukocytes by means of High Pure polymerase chain reaction (PCR) Template preparation kit (Roche, Germany), we carried out PCR-RFLP on each sample containing genomic DNA of the patients or controls for detecting PPAR-γ2 gene polymorphism.

Forward primer 5'-CCAATTCAAGCCCAGTCCTTTC-3', and reverse primer 5'-CAGTGAAGGAATCGCTTTC-3'.

Table 1: Clinical and biochemical characteristics of type 2 diabetic and non-diabetic subjects

Characteristics	Type 2 diabetic subjects (n=100)	Non-diabetic subjects (n=100)	P value
Sex (men/women)	66/34	72/28	
Age (year)	51.98±5.5	51.12±8.5	0.551
Body mass index (kg/m ²)	28.4±1.1	26.4±1.1	<0.001
Systolic blood pressure (mmHg)	140.8±10.3	122.8±9.9	<0.001
Diastolic blood pressure (mmHg)	89.6±10.2	78.7±6.5	<0.001
Fasting glucose (mmol/l)	8.03±0.14	5.1±0.2	<0.001
2-h plasma glucose (mmol/l)	12.6±0.2	6.7±0.2	<0.001
HbA1c (%)	7.5±0.1	5.2±0.1	<0.001
Total cholesterol (mmol/l)	4.96±0.1	4.83±0.1	0.410
HDL cholesterol (mmol/l)	1.3±0.1	1.2±0.1	0.003
LDL cholesterol (mmol/l)	3.4±0.1	3.2±0.8	0.689

Values are mean±SD, P values by student's t-test, HbA1c: Hemoglobin A1c, HDL: High-density lipoprotein, LDL: Low-density lipoprotein

Using BioRad thermal cycler (My Cycler, Germany), each PCR was performed in a volume of 50 μ l, including 100 ng of genomic DNA, 10 pmol of each primer, 0.8 mM of deoxynucleotide triphosphates (BIORON), and 0.5 U of Taq DNA polymerase in the reaction buffer (50 mmol/l of KCl, 2.8 mmol/l of MgCl₂, 10 mmol/l of Tris-HCl, pH 9.0 (BIORON)). For warming up, initial denaturation at 94°C for 3 min was carried out. Thereafter, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed for 30 cycles. Then it was followed by a post-extension at 72°C for 10 min. In order to confirm the PCR amplification, gel electrophoresis (8% polyacrylamide gel and Ethidium bromide staining) was carried out [Figure 1]. Then, Amplified products (244 bp) were digested by restriction endonuclease BstU-I (Fermentas, Poland) at 37°C for 1 h. In order to analyze the restriction products, electrophoresis was performed using an 8% polyacrylamide gel and Ethidium bromide staining [Figure 2].

Statistical analyses were performed using the SPSS statistical software package (Version 17.0). More precisely, data were analyzed by Student's *t*-test and Chi-square test. *P* < 0.05 was considered significant. Using the χ^2 results, the test for Hardy-Weinberg equilibrium and comparison of genotype and allele frequencies in the diabetic and non-diabetic subjects was carried out.

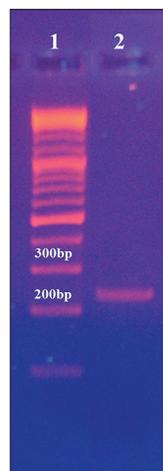


Figure 1: Confirmation of polymerase chain reaction amplification using gel electrophoresis. Lane 1: 100 bp deoxyribonucleic acid ladder; Lane 2: PCR products from peroxisome proliferator-activated receptor-GAMMA2 gene using the forward and reverse primers mentioned in the method section. Extracted DNA from peripheral blood was used as template

Results

PCR amplification of PPAR- γ 2 gene produced a 244 bp DNA band [Figure 1]. Digestion of this product with BstU-I gave different patterns as follows: Pro12 homozygotes, Ala12 homozygotes, and heterozygotes were shown by one band (244 bp), two fragments (223 and 21 bp), and three fragments (244, 223 and 21 bp), respectively [Figure 2]. What should be noted is that 21 bp fragments were not visualized in the gel.

Table 2 illustrates the genotype frequency of PPAR- γ 2 gene. As can be shown, the genotype frequency of Ala/Ala in healthy subjects was significantly higher than in diabetic subjects (*P* < 0.001). However, the genotype frequency of Ala/Pro genotype in diabetic subjects was significantly higher than in healthy subjects (*P* < 0.001). As an aside, in healthy subjects, the genotype frequency of Ala/Ala (0.52) was significantly higher than that of Ala/Pro (0.36) and Pro/Pro (0.12) (*P* < 0.001). In diabetic subjects, however, the genotype frequency of Ala/Pro (0.56) was significantly higher than that of Ala/Ala (0.2) and Pro/Pro (0.24) (*P* < 0.001). The frequency of Ala12 carriers (Pro12Ala and Ala12Ala genotypes) in T2DM group (0.76) and CG (0.8) was almost equal.

Table 3 provides a breakdown pertaining to gene

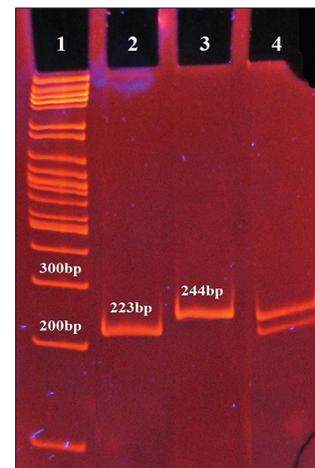


Figure 2: Polymerase chain reaction-RFLP detection of the Pro12Ala polymorphism of peroxisome proliferator-activated receptor-GAMMA2 gene using gel electrophoresis. Lane 1: 100 bp deoxyribonucleic acid ladder; lane 2: Ala12 homozygotes; lane 3: Pro12 homozygotes; lane 4: Pro12Ala heterozygotes. Extracted DNA from peripheral blood was used as template

Table 2: Genotype frequency of PPAR- γ 2 gene (Pro/Pro (homozygous for Pro12 allele), Pro/Ala (heterozygous for Pro12 and Ala12 alleles), Ala/Ala, (homozygous for Ala12 allele)) in type 2 diabetic and non-diabetic subjects

Genotype subject	Ala/Pro	Ala/Ala	Pro/Pro
Non-diabetic subjects (Total number of subjects=100)	0.36 (n=36)	0.52*(n=52)	0.12 (n=12)
Type 2 diabetic subjects (Total number of subjects=100)	0.56*(n=56)	0.2 (n=20)	0.24 (n=24)

* $P < 0.05$ by χ^2 test, PPAR- γ 2: Peroxisome proliferator-activated receptor-GAMMA2

Table 3: Gene frequency of the PPAR γ 2 in type 2 diabetic and non-diabetic subjects note

Allele subject	Pro	Ala
Non-diabetic subjects (Total number of alleles=200)	0.3 (n=60)	0.7* (n=140)
Type 2 diabetic subjects (Total number of alleles=200)	0.52 (n=104)	0.48 (n=96)

* $P < 0.05$ by χ^2 test, PPAR- γ 2: Peroxisome proliferator-activated receptor-GAMMA2

frequency of PPAR- γ 2 gene. As can be decidedly noted, the frequency of Ala allele in healthy subjects was significantly higher than in diabetic subjects ($P = 0.003$). As an apart, the frequency of Ala allele in healthy subjects was significantly high, in comparison with that of Pro Allele ($P = 0.002$). However, in diabetic subjects, the difference between the frequency of Ala allele and Pro allele was not statistically significant.

Finally, observed genotype frequencies of the Pro12Ala polymorphism of PPAR- γ 2 in diabetic and non-diabetic subjects were in accordance with the Hardy-Weinberg equilibrium (data not shown).

Discussion

A large and growing body of literature has investigated the relationship between Pro12Ala polymorphism of PPAR- γ 2 gene and various disorders such as T2DM,^[15-17] insulin sensitivity,^[18] obesity,^[19] cancer,^[20] cardiovascular disease^[21] and Alzheimer's disease.^[22] In as much as there are a number of disagreements on association between Pro12Ala polymorphism of the PPAR- γ 2 gene and T2DM, this study set out to determine aforesaid association in an Iranian population.

Our findings were consistent with those of Scacchi *et al.* who found a protective role of Ala frequency against T2DM prevalence in an Italian population.^[8] Tavares

et al. showed that carriers of Ala12 allele of the PPAR- γ 2 gene were more sensitive to insulin resistance when compared to Pro12 carriers in a Brazilian population.^[10] The protection against diabetic nephropathy in Brazilian diabetics caused by presence of Ala allele was reported elsewhere.^[23] It was demonstrated that Ala allele brought about lower development of T2DM in Caucasians.^[24] Furthermore, our findings were in accord with those of Mori *et al.* in which they found Ala variant of PPAR- γ was associated with a reduced risk for the development of diabetes in Japanese subjects.^[16] Nevertheless, Maciej *et al.* did not confirm the influence of Pro allele upon increased risk of the development of T2DM.^[25] An association was also reported between increased risk of T2DM and Pro allele of PPAR- γ 2 gene in a Russian population.^[26]

Moreover, present study showed a significant association between Pro12Ala polymorphism of PPAR- γ 2 gene with T2DM. We also found that Ala/Ala genotype was significantly present in non-diabetic subjects. Present study produced results, which corroborate the findings of Hara *et al.* showing the association between Pro12Ala polymorphism of PPAR- γ gene and T2DM in a Japanese population.^[27] Another study on Korean subjects showed that PPAR- γ 2 Pro12Ala polymorphism was protective against ischemic stroke with T2DM.^[28] Horiki *et al.* demonstrated a correlation between Pro12Ala polymorphism of PPAR- γ and genetic susceptibility to T2DM and hypertension in a Japanese population.^[9] The association between PPAR- γ and T2DM in Chinese diabetics was reported elsewhere.^[29] A strong relationship between Pro12Ala polymorphism of PPAR- γ 2 and T2DM was demonstrated in Sikhs living in northern states of India.^[30] However, on the contrary, lack of association between Pro12Ala variant and T2DM was shown in a south Indian population.^[31] Furthermore, the study carried out by Stefanski *et al.* did not detect any evidence for association of Pro12Ala PPAR- γ 2 variant with insulin resistance in Caucasian population.^[11] It should be mentioned that our findings did not support a preceding Iranian study, which reported no significant association between Pro12Ala polymorphism of PPAR- γ 2 and T2DM.^[32] Finally, it is somewhat surprising that our study was the first one in Iran that

demonstrated an association between polymorphism of PPAR- γ 2 and T2DM. It should, however, be noted that lack of association between polymorphism of PPAR- γ 2 and T2DM in Northern provinces of Iran was reported in a preceding Iranian study. Taking into an account of these conflicting results, some caveats need to be noted. A small sample was chosen in this study owing to the expected difficulty of obtaining blood samples. In fact, the samples were regionally representative of center of Iran and would naturally tend to miss people who were in marginal regions. Additionally, it appears that controversial findings may be due to population admixture of studied participants. Hence, a cross-national research with larger sample size would help us to better understand the precise effect of Pro12Ala polymorphism of PPAR- γ 2 gene upon T2DM risk.

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