The Pro115Gln Missense Mutation of Peroxisome Proliferator Activated Receptor γ (*PPARγ*) Gene in Diabetes in the Pakistani Population

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Abstract

Background: Diabetes is an alarmingly increasing public health concern. According to a World Health Organization (WHO) report, diabetes is highly prevalent in almost all regions of Pakistan, with an overall prevalence of 22.04% in urban and 17.15% in rural areas. In addition to lifestyle factors, genetics plays an important part in the progression to diabetes, of which peroxisome proliferator activated receptors (PPARs), a super-family of transcriptional regulators, are considered to be very critical.

Objectives: The aim of the current study was to investigate the prevalence of a missense mutation Pro115Gln in the PPARγ; gene in Pakistan and search for its effects on certain serum biochemical parameters.

Materials and Methods: A total of 1015 subjects (490 diabetics and 525 healthy controls) were genotyped by PCR-RFLP and serum profiling was performed.

Results: We could not detect the mutation in the study subjects, but the anthropometric and biochemical parameters were significantly different between the cases and controls.

Conclusions: In conclusion, as this mutation is present in a highly conserved region and any change in this region would adversely affect the protein structure and function, so the prevalence of this mutation is very low.

Keywords: Diabetes, Peroxisome Proliferator Activated Receptors, Pro115Gln, Pakistan

1. Background

Diabetes is characterized by abnormally high levels of glucose in the blood (1). The international diabetes federation reported 6.9 million cases of diabetes and 87,548 deaths due to diabetes in Pakistan in 2014. It is a metabolic disorder, associated with many specific and non-specific microvascular complications.

As the amount of glucose increases in the blood after meals, it triggers the release of a hormone, insulin, from the pancreas; this hormone stimulates the muscle and fat cells to remove the glucose from the blood and stimulates the liver to metabolize the glucose (2). Other functions of insulin include stimulation of the increased use of glucose in the synthesis of protein; increased gluconeogenesis; increased glucose metabolism, releasing energy in the form of ATP, and conversion of glucose to fats and so on (3). Glucagon is the hormone which has the opposite function to insulin. Insulin triggers the conversion of glucose into glycogen (anabolism of glucose) in the opposite way. Glucagon triggers the conversion of glycogen into glucose (catabolism of glucose) (4). The extent of the rise in glucose levels depends on various factors such as digestion rate, absorption rate, type, and amount of carbohydrate

(5). The cells that make insulin in the body are β-cells and they are present in the pancreas (6). Clumps of these cells are known as "islets of Langerhans" after their discoverer (7).

Peroxisomes are the cellular organelles present in all eukaryotic cells. These are involved in several cellular functions, including the beta oxidation of fatty acids (8). The isolation and characterization of one of the peroxisomes proliferators was a major breakthrough in scientific research and they act by binding to the peroxisomes proliferators activated receptors (PPAR) (9). They were reported in 1990 by Issemann and Green as the new member of the super-family of nuclear receptors (also regulators) which also includes steroid receptors, thyroid hormone receptors and retinoic acid receptors (10).

PPAR has three iso-forms: PPAR`, PPARβ and PPARγ (11). The gene encoding for PPARγ (PPARG) is located on the short arm of chromosome 3 (3p25). The gene is of approximately 100 Kb, containing nine exons (12). The PPARγ contains three different promoters that on transcription yields three further iso-forms of PPARγ - namely PPARγ1, PPARγ2 and PPARγ3 (13). PPARγ1 is the iso-form which has a broad tissue range in the body, while, on the other hand, PPARγ2 is only restricted to the adipose tissue of the body.

PPARγ3 expresses abundantly in macrophages, in the large intestine, and in white adipose tissue (14).

There are many causes of diabetes, although the underlying cause remains to be established. Among genetic causes, mutations and variants in PPARγ have been proposed to be a strong candidate. A missense mutation in the human PPARγ gene, Pro115Gln, causes the exchange of proline at the place of glutamine at position 115 (15). In a wild type state, the gene PPARγ can be inactivated by the enzyme MAP kinase dependent phosphorylation at serine that is present at position 114 (16). The Pro115Gln mutation prevents this phosphorylation at 114 serine and, in this way, it inhibits the deactivation of the receptor, resulting in continuous expression of the gene (17, 18).

2. Objectives

The purpose of the current study was to investigate the prevalence of the human PPARγ gene mutation Pro115Gln in subjects with diabetes and healthy controls collected from different regions of Lahore Punjab, Pakistan.

3. Materials and Methods

3.1. Study Subjects

For the present study, we collected samples from 490 randomly selected diabetic patients and 525 healthy subjects. Inclusion criteria for diabetic subjects were i) diabetes diagnosed according to etiologic classification of diabetes by the international diabetes federation (IDF) and ii) confirmation that all the grandparents of the subjects were of Pakistani origin. The exclusion criteria consisted of the presence of any infectious disease; conditions where phlebotomy is contraindicated; age below 10 years; body mass index (BMI) $\leq$ 18.5 kg/m², pregnancy; handicapped or mentally disturbed individuals; obesity; cancer, and being of an ethnicity other than Pakistani. The recruitment was done between July 2014 and April 2015. The controls were healthy subjects from the general population with normal blood sugar levels (< 126 mg/dL random or < 99 mg/dL fasting). All the subjects were genetically unrelated and gave written informed consent. The procedures employed were according to the Helsinki declaration and an ethical approval was obtained from the institutional ethics board.

3.2. Blood Sampling and Serum Isolation

5 mL blood was drawn from the subjects in a fasting state. The 2.5 mL blood was poured into disodium EDTA vials for isolation of DNA. The rest of the 2.5 mL of blood was poured into gel clot activator-containing vials for isolation of serum for biochemical analysis. The vials were centrifuged at maximum speed 14,000 rpm for 4 - 5 minutes.

3.3. Extraction of DNA

1000 μL of the red cell lysis buffer (0.01 M tris base, 320 mM sucrose, 5 mM magnesium chloride, 1%triton-X 100) was added in a clean, sterile and autoclaved 1.5 mL Eppendorf. We then added 500 μL of fresh whole blood to the Eppendorf. The Eppendorf was inverted about 4-5 times to homogenize the buffer and blood. Centrifugation was performed for 2 minutes at 7000 rpm at room temperature. The supernatant was discarded and the washing step was repeated 3 - 4 times. 400 μL of white cell lysis buffer (0.01 m tris HCl, 11.4 mM sodium citrate, 10% SDS, 1 mM EDTA) was added and then the pellet was re-suspended by pipetting. 100 μL of 5M solution of the NaCl and then 600 μL of ice-chilled chloroform were immediately added and mixed by inversion. Centrifugation was performed at 7000 rpm for 2 minutes. After this step, two layers were obtained. The uppermost layer was supernatant which was transparent. 400 μL of this transparent supernatant was transferred into a new 1.5 mL Eppendorf and 800 μL of chilled absolute ethanol was added and gently mixed by inversion. The reaction tube was centrifuged for one minute at 12,000 rpm to precipitate the DNA, then the supernatant was carefully discarded and the pellet was allowed to completely dry. After drying the pellet, 100 μl of TE buffer (Tris EDTA buffer) was added to the Eppendorf to rehydrate the DNA. The isolated DNA was stored at 4°C or at -20°C for further use.

3.4. Determination of Blood Glucose

Fasting blood glucose was measured using a digital glucometer (Accu-Chek®). The subject's finger was sterilized with spirit and a skin puncture made by a sterile lancet, so a blood drop could be taken on a glucometer strip and the reading noted.

3.5. Lipid Profiling

Serum total cholesterol was determined by the enzymatic colorimetric method (CHOD-PAP test) using a commercially available kit (Spectrum Diagnostics, Egypt). The assay was performed according to the manufacturer's instructions.

Triglyceride content was measured by the commercially available kit based on enzymatic colorimetric method (Spectrum Diagnostics, Egypt). The principle of the kit is as follows; triglycerides present in the plasma samples are hydrolyzed by an enzyme lipoprotein lipase (LPL), a reaction that gives glycerol and free fatty acids. Glycerol is then phosphorylated by the action of

glycerol kinase in the presence of ATP and magnesium ions. Phosphorylated glycerol is then further oxidized by glycerol-3-phosphate oxidase in the presence of O_2 and dihydroxyacetone phosphate and this reaction gives H_2O_2. H_2O_2, then reacts with 4-aminoantipyrine in the presence of phenol and peroxidase and produces a red quinone product that gives absorbance at 546 nm.

For the estimation of high density lipoprotein cholesterol, precipitation of chylomicrons, VLDL-C and LDL-C was performed using the commercially available kit (Spectrum Diagnostics, Egypt), according to manufacturer's instruction.

Low density lipoprotein fraction (LDL-C) and very low density lipoprotein (VLDL-C) were estimated using the commercially available kit (Spectrum Diagnostics, Egypt).

3.6. Leptin Determination

By using an ELISA (LDN Nordhorn leptin ELISA kit, serum leptin concentration was measured in the following way: wash buffer and working solutions of streptavidin-HRP conjugate were prepared. 20 μL of control, each calibrator and diluted serum sample were dispensed into the duplicate corresponding wells and 80 μL of monoclonal leptin biotin conjugate was also added to each well. After that, each plate was incubated on a shaker at room temperature for one hour. Then the wells were washed three times with wash buffer, and dried properly against blotting paper. 100 μL of streptavidin-HRP-conjugate was dispensed into each well and we kept the plate on the shaker for a second incubation at room temperature for 30 minutes. Again, we washed it three times with wash buffer and dried it properly. After washing, 150 μL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated for a third time on the shaker for 10 - 15 minutes at room temperature. After completion of the incubation period, stop solution was added into each well and OD was taken at 450nm within the time period of 20 minutes. A calibrator curve was drawn on semi-log paper with calibrator concentrations on the X-axis and optical densities on the Y-axis. At the end, the mean optical densities for all duplicate samples were calculated and the concentration of leptin was determined directly off the curve (19).

3.7. Genetic Analysis

After isolation, all DNA samples were quantified before amplification using an Epoch Biotek micro-plate reader (Biotek Instruments, Highlands Park, USA). This quantification was done to equalize the final concentration of DNA samples up to 10 ng/μL. PCR was done in an advanced primus 96 (Peqlab) thermal cycler to amplify the isolated genomic DNA. One strand PCR was performed for the SNP. We used published primers for the amplification of PPARprimers for the gene (15). The primer sequence was forward 5'-TGCAATCAAAGTGGAGCC-3' and reverse 5'-CAGAAGCTTTATCTCCACAGAC-3'. The PCR product size was 129bp. PCR reaction conditions consisted of initial denaturation at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds; annealing at 58 for 30 seconds; extension at 72 for 45 seconds, and final extension at 72 for 10 minutes. The PCR product contains the restriction site for HindII in mutation state, so the 5 μL of product of 129 bp was used for digestion with the help of the HindII restriction enzyme for the polymorphism, Pro115Gln. The optimum time for incubation was 8 hours. The 129 bp product would be cut into two fragments of 104bp and 25bp in the presence of mutation. The genomic DNA and PCR/restriction products were run on 1 and 2% agarose gel electrophoresis and visualized under a Gel Doc system/U.V transilluminator.

3.8. Statistical Analysis

Microsoft excel and the statistical package for social sciences (SPSS, IBM statistics, version 22) software were used for statistical analysis. The quantitative data was analyzed for mean and standard deviation. The study population was tested for Hardy Weinberg Equilibrium (HWE). The Chi-square test was used to test the significance of the differences of allele and genotype frequencies among the cases and controls. t-test and logistical regression were used to test the association of the SNP with diabetes, while one way ANOVA was employed to check the association of the polymorphism with anthropometric and biochemical parameters. The p-value of 0.05 was used as the significance cut off for all analyses.

4. Results

4.1. Characteristics of Subjects

This study included 1015 individuals from different areas of the Lahore, Pakistan. Among them, 490 were diabetic and 525 were non-diabetic. Individuals included in this study had a mean age of 41.47 $\pm$ 9.13years. The minimum, maximum and mean weight of the subjects was 26 kg, 110 kg and 64.4 kg, respectively. The overall gender-based proportion of subjects included 53.5% females and 46.4% male. Controls included 52% females and 48% males. On the other hand, the cases included 55% females and 45% males. The study results show that diabetes is more common in females than in males. The prevalence of comorbidities was higher in the diabetic group as compared to the controls. Four anthropometric parameters, including

age, weight, height and BMI were selected. The subjects' characteristics have been summarized in Table 1.

Table 1. Anthropometric Traits of the Study Population[a]

Parameters	Cases	Controls	P Value
Age, y	47.663 ± 1.2	35.02 ± 1.4	< 0.01
Weight, kg	64.25 ± 1.3	64.52 ± 1.3	< 0.01
Height, m	5.49 ± 0.3	5.45 ± 0.6	< 0.05
BMI, kg/m2	22.38 ± 5.3	22.53 ± 3.4	< 0.01

Abbreviation: BMI, body mass index.
[a]Values shown are in mean ±SE.

4.2. Biochemical Analysis in Subjects

An important aspect in diabetes is biochemical profiling of blood samples. For this purpose, biochemical parameters, such as levels of glucose, triglycerides, LDLC, HDLC, total cholesterol, and leptin were estimated. The biochemical parameters are given in Table 2.

Table 2. Biochemical Traits of the Study Population[a]

Biochemical Parameters	Cases	Controls	P Value
Glucose, mg/dL	197 ± 6.3	95 ± 2.84	0.042
Triglycerides, mg/dL	105.6 ± 6.27	90.3 ± 3.61	0.053
Total cholesterol, mg/dL	170 ± 6.4	164.5 ± 5.3	0.0453
LDLC, mg/dL	243.7 ± 6.5	218.5 ± 4.8	0.0527
HDLC, mg/dL	85.5 ± 5.4	55.5 ± 3.9	< 0.01
Leptin, ng/μL	15.4 ± 0.3	15.4 ± 0.8	0.099

Abbreviation: LDLC, low density lipoprotein cholesterol; HDLC, high density lipoprotein cholesterol.
[a]Values shown are in mean ±SE.

4.3. Genotyping

Following the amplification of genomic DNA, the restriction digestion was done for 129bp amplified products using restriction enzymes HindII. No restriction digestion was seen in any amplified product, indicating the absence of the mutation. Random samples were sequenced to confirm the absence of the mutation (Figure 1).

The amplification of DNA was followed by restriction enzyme digestion with HindII. The fragment amplified by PCR was not cleaved by the restriction enzyme, resulting in a single band. M is 50 bp ladder (Thermo Scientific, SM#1103).
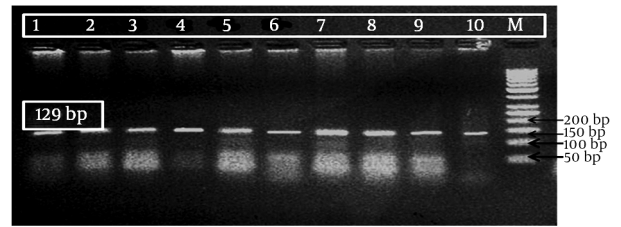

Figure 1. Visualization of Restriction Digestion

5. Discussion

Diabetes is a major health problem in Pakistan, as well as the rest of the world (2). According to a report published by world health organization (WHO) in 2011, the trend of diabetes in Pakistan is also increasing with the passage of time (20). Missense mutation Pro115Gln and Ser114 constitute negative effects on activity of protein and cause insulin resistance. The mutation causes the substitution of proline to glutamine at position 115. We tried to find out how the presence of this polymorphism affects certain biochemical parameters, such as fasting plasma glucose level, LDLC, HDLC, total cholesterol, triglyceride, and leptin in study subjects. According to the current study, the prevalence of diabetes is higher in females as compared to males in the local population. These results are in accordance with many previously published studies on the Pakistani population. The results of the current study showed the prevalence of diabetes in females to be 53.5% and 46.40% in males, compared to a previous study conducted in Pakistan which reported the disease's prevalence in females was 53.61% and 46.1% in males (21).

Four parameters, including age, weight, height, and BMI were selected. Among the cases, the mean age is high as compared to the mean age of the controls. The association of age with the disease is highly significant as the P value is < 0.01. The mean value of age in the cases is 47.66 ± 1.2 and this is congruent with a previous local study in which the mean age was 45 ± 15 (22). There is no significant difference in the mean weight of the cases and controls (p-value 0.09). The same is the case with height: there is no significant difference (P value 0.10). The P value is < 0.01 that also shows the significance of BMI to diabetes. Another study reported the BMI value in diabetics was 23 kg/m² (23).

An important aspect in diabetes is biochemical profiling. For this purpose, biochemical parameters, such as levels of fasting blood glucose, triglycerides, LDLC, HDLC, total cholesterol, and leptin were also estimated. For fasting blood glucose, the mean value for the cases is higher as compared to the controls, indicating the importance of

glucose metabolism in diabetes. The mean glucose concentration in the cases was 197 ± 73.7, while among the controls, it was 95 ± 2.84. For LDLC, high mean values were observed in the cases as to the controls. The results support the previously reported study which also showed a significant association of LDLC with diabetes (24). In the case of HDLC, the mean values in the controls are higher as compared to the controls. For total cholesterol, high mean values were observed in the cases as compared to the controls. The mean values of total cholesterol showed a significant difference between the cases and the controls (P value 0.045). A previous study reported the significance of total cholesterol to diabetes (25). For leptin, there was no difference between the mean values of the cases and controls. No significant difference in concentration was noted between the cases and controls (P value 0.099). According to the latest reported study, the mean value of leptin in the controls was 8.11 ± 2.39 (19).

We observed a high prevalence of cardiac disorders in the cases as compared to the controls. As the sampling of the cases was done mainly from the Punjab institute of cardiology, this could be the possible reason behind the high prevalence of cardiac disorders in the cases. In addition, it has been reported that cardiac disorders are associated with diabetes (26).

The selected mutation is well-studied in Caucasians, but has not been investigated in the Pakistani population. Some of the studies have reported its association with diabetes and others with obesity. We selected this polymorphism due to a lack of information on whether it has some role in the predisposition to and development of diabetes in the Pakistani population and hence added it to association patterns in a unique ethnic group. In Pro115Gln, serine replaces proline - serine is a polar amino acid and usually participates in hydrogen bonding, while, on the hand, proline is a hydrophobic amino acid. The replacement of proline by serine can cause drastic changes in structural and functional characteristics. We could not detect any mutation in the study subjects, but the anthropometric and biochemical parameters are significantly different between the cases and controls, so there must be other genetic factors contributing to the development of diabetes. This mutation is present in a highly conserved region that is involved in phosphorylation, so the prevalence of this mutation is very low.

We could not detect any mutation in the study subjects, but the anthropometric and biochemical parameters were significantly different between the cases and controls. In conclusion, mutations disrupting the splice sites are very rare due to their crucial function.

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