J. Bangladesh Acad. Sci. 44(2); 179-189: December 2020 DOI:10.3329/jbas.v44i2.51461

Journal of Bangladesh Academy of Sciences

Journal homepage: http://www.bas.org.bd/publications/jbas.html

**Research Article**

# Pro12Ala polymorphism in PPAR-γ gene of impaired glucose tolerance subjects in Bangladeshi population

Md. Omar Faruque*, Ayan Saha[1], Imran Khan[2], Zahid Hassan[2], Liaquat Ali[3]

*Department of Nutrition and Food Technology, Jashore University of Science and Technology, Jashore, Bangladesh*

**ARTICLE INFO**

**Article History**
Received: 15 October 2020
Revised: 03 December 2020
Accepted: 15 December 2020

**Keywords:** PPAR-γ gene polymor-phism, Impaired glucose tolerance.

**ABSTRACT**

Pro12Ala polymorphism in PPAR-gamma (PPAR-γ) gene is associated obesity and hyperglycemia in some races, but controversy exists for other races. In this study, 113 impaired glucose tolerance (IGT) and 113 healthy control subjects were recruited. Wild type (CC), heterozygous (CG), and homozygous (GG) variants of Pro16Ala polymorphism of PPAR-γ (rs1801282) were analyzed using Chi-square test, and the polymorphism was found significantly ($p=0.05$, $X^2=5.875$) associated with IGT subjects. Allele frequency also shows significant association with IGT subjects in the Chi-square test ($p=0.026$, $X^2=2.248$). When the waist-hip ratio was reanalyzed according to genotype variation, the waist-hip ratio was found significantly higher among subjects with heterozygous and homozygous variants of pro16Ala polymorphism. Therefore, our study concluded that PPAR-γ P12A variants might be associated with impaired glucose tolerance. It is also documented that higher waist to hip ratio in IGT subjects may associate with heterozygous (CG) and homozygous (GG) variants of Pro12Ala mutation in the PPAR-γgene.

## Introduction

Type 2 diabetes mellitus is a heterogeneous disorder both phenotypically and genotypically, and it is associated with defects in insulin secretion or insulin action, and sometimes both appear simultaneously (Defronzo, 1997). Mutations in several genes linked to monogenic forms of type 2 diabetes mellitus have been identified (Yamagata et al.,1996). However, in most type2 diabetic patients, the disease's genetic mechanisms and pathogenesis are still not clear.

Asian Indians, 25% of the global population, make up the most significant proportion of diabetics in the world (Basnayat and

*Corresponding author:<faruque.nft.just@gmail.com; faruquenft@just.edu.bd>
[1]Department of Biotechnology and Genetic Engineering, Chittagong University, Chittagong
[2]Department of Physiology and Molecular Biology, Bangladesh University of Health Sciences, Dhaka, Bangladesh
[3]Pothikrit Institute of Health Studies (PIHS), Dhaka, Bangladesh

Rajapaksa, 2004). The underlying reasons for the high prevalence of type 2 diabetes (T2D) and cardiovascular disease in Asian Indians are not well understood given the absence of conventional risk factors, like high smoking, diets rich in meat, or high body mass index (BMI).

People from India, indeed the entire Indian sub-continent, have a high prevalence of a characteristic metabolic syndrome, including elevated plasma triglycerides, low levels of high-density lipoprotein cholesterol (HDL-C), high prevalence of insulin resistance, and a tendency towards central obesity and premature atherosclerosis (Mather and Keen, 1985; McKeigue et al., 1992; Barrosos et al., 1999). Family and migrant studies point to strong genetic and ethnic predisposition in response to certain environmental factors (McKeigue et al., 1989; Kooner et al., 1998).

The peroxisome proliferator-activated receptor -gamma (PPAR-γ) is a transcription factor involved in adipogenesis and adipocyte gene expression regulation. PPAR-γ maps to chromosome 3p24 and has been implicated in several genome-wide linkage scans for T2D (McKeigue et al., 1989) and is widely studied for its role in insulin resistance, central obesity, T2D, and other related phenotypes in different populations (Kooner et al., 1998). The most widely reproduced association between genetic variation and population risk in diabetes is the Pro12Ala (rs1801282) polymorphism in the PPAR-γ2 that has been confirmed in several other genome-wide association studies (GWAS) in Caucasians (Zimmet, 1992; Parikh and Groop, 2004; Saxena et al., 2007; Scott et al., 2007; Bantley et al., 2007), except in the French GWAS (Sladek et al., 2007). Another study of obese and non-obese Europeans from France and Switzerland suggests the positive association of PPAR-γ2 (Pro12Ala) (rs1801282) on T2D in obese individuals (BMI $\geq$30 kg/m$^2$) (Cauchi et al., 2008). PPAR-γ exists in three different isoforms. Two mutations in the ligand-binding domain of PPAR-γ, Pro467 Leu, and Val290 Met, were found in three Caucasian subjects with severe insulin resistance and type 2 diabetes with normal body weight (Fajas et al., 1997). Within a unique domain of PPAR-γ2 that enhances ligand-independent activation, a prevalent Pro12Ala polymorphism has been identified. The polymorphism was shown to be involved in the pathogenesis of obesity, which is linked to diabetes (Ek et al., 1999). On the contrary, a study using a family-based design to control population stratification reported that the Ala-allele of the codon 12 polymorphism was associated with a decreased risk of type 2 diabetes (Alshuler et al., 2000). A recent study on the Egyptian population (Hasan et al., 2017) documented that the PPAR-γ Pro12Ala polymorphism plays a role in developing coronary artery disease in obese T2DM. The mechanism of these effects of the Ala-allele in the PPAR-γ gene is not fully understood.

Therefore, we examined whether the Ala-allele of the codon 12 polymorphism of the PPAR-γ2 gene is related to the progression of type 2 diabetes mellitus in Bangladeshi population and hence selected impaired glucose tolerant subjects as the study population.

## Subjects and Methods

In this case-control study, 226 subjects were recruited, where 113 were impaired glucose tolerant (IGT) subjects, and another 113 were healthy control subjects. IGT subjects were

purposively recruited in the study irrespective of race, religion, and socio-economic status from the Out-Patient Department (OPD) of BIRDEM Hospital, Shahbag, Dhaka. IGT was diagnosed following WHO criteria (WHO, 1999). Control subjects were collected from the friend circles of the IGT subjects assuming that they are from the same socio-economic status. Purposes and methods of the study were briefed to each individual, and informed consent was obtained. A predesigned case record form was used to record relevant clinical, medical, demographic, and socio-economic data such as age, gender, educational status, and occupational status of the subjects. They were requested to come on a prescheduled morning after 8-10 hr fasting.

Subjects with co-morbid diseases (infection, stroke, myocardial infarction, major surgery, essential hypertension, malabsorption, etc.), pregnant women, and subjects with a history of medication, which may significantly affect glucose metabolism (glucocorticoids, oral contraceptives containing levonorgestrel or high-dose estrogen, phenytoin, high-dose thiazide diuretics, etc.) were excluded from the study.

### *Anthropometric indices and blood pressure measurements*

Anthropometric measurements like height, weight, waist, and hip circumference were measured using standard technique. Blood pressure was measured using Barometric Sphygmomanometer.

### *Collection and storage of blood samples*

Venous blood (10 mL) was drawn in overnight fasted condition by venipuncture following standard procedure. Subjects were then given glucose to drink (75 g in 250 mL of water). They were requested not to take any food and be rested for two hours. The second blood sample was then obtained. A portion of blood (5 mL) sample was taken into a tube containing EDTA (1 mg/mL), mixed thoroughly, and preserved at -30 $^{o}$C for future DNA extraction and subsequent experimentation. Fasting and postprandial blood samples were also taken into another EDTA containing tube for plasma glucose, and plasma insulin measurement, and a portion of blood was taken without EDTA for serum lipid profile and GPT measurement. After 30 minutes, plasma and serum were separated by centrifugation for 10 min at 3000 rpm using a refrigerated centrifuge and preserved at -20 $^{o}$C for further biochemical analyses.

### *Biochemical analyses*

Plasma glucose was estimated by enzymatic colorimetric (GOD-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK. Total serum cholesterol, triglyceride and high-density lipoprotein-cholesterol (HDL-C) were measured by enzymatic colorimetric method using a reagent of RANDOX laboratories Ltd., UK. The LDL-cholesterol level in serum was calculated by using Friedewald formula (Friedewald et al., 1972), LDL-cholesterol = {Total cholesterol (HDL-cholesterol + $^{1}/_{5}$ Triglyceride)}. Serum glutamate-pyruvate transaminase (GPT) or alanine aminotransferase (ALT) was estimated by UV method using ALT (GPT) opt. kit (RANDOX, UK). Plasma insulin was measured by enzyme- linked immunosorbent assay (ELISA) method using a kit from Linco Research Inc., USA. Serum creatinine was estimated by standard laboratory method.

***Genetic analyses***

PPAR-γ genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Extraction of DNA was performed by using Favor Prep™ Genomic DNA Extraction kit (FAVORGEN®, Taiwan). The kit uses the principle of silica gel DNA isolation from whole blood adapted in a spin column. PCR was carried out using the following primer set: F5'-TCTGGGAGATTCTCCTATTGGC-3' and R5'-CTGGAAGACAAACTACAAGAG-3'. PCR was carried out using Hot Start Taq polymerase and PCR condition was−initial step of denaturation at 95 $^{o}$C for 5 minutes followed by 40 cycles of denaturation at 94 $^{o}$C for 30 seconds, annealing at 52 $^{o}$C for 30 seconds, elongation at 72 $^{o}$C for 30 seconds and final elongation at 72 $^{o}$C for 10 minutes.

PPARG Pro12Ala variant polymorphism was analyzed using HaeIII restriction enzyme digestion for 4 h at 37 $^{0}$C in a water bath. The digestion products were loaded on 8% polyacrylamide gel for genotyping and run for 4 h in a vertical electrophoresis system. The digested product was visualized using a gel documentation system following ethidium bromide staining (Fig. 1).

**Statistical Analysis**

Statistical analysis was performed using Statistical Package for Social Science (SPSS 20) software 20 for Windows version. Data were expressed as mean±SD, frequency (number) as appropriate. The statistical difference between the two groups was assessed by the unpaired Students 't' test. A Chi-square test was also performed in an appropriate situation. A two-tailed p value of $<0.05$ was considered statistically significant.

**Results and Discussion**

A total number of 226 subjects were included in the study, where 113 were healthy controls and the rest 113 were Impaired Glucose Tolerant (IGT) subjects.

Age (yrs) and BMI (kg/m$^2$) of the control (40±9 and 25.0±4.4, respectively) and IGT subjects (43±9 and 25.4±4.2, respectively) were similar. The mean waist-hip ratio (WHR) of the IGT subjects (0.93±0.06) was significantly higher compared to controls (0.91±0.01). Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the controls and IGT subjects were not significantly different. (Table 1). Mogre et al. (2014) documented that type 2 DM patients attending an outpatient clinic show a high prevalence of central obesity, waist-hip ratio (WHR) and a relatively low prevalence of elevated blood pressure. Abdominal obesity/central obesity measured by either waist circumference (WC) or waist-to-hip ratio (WHR) has been shown to be strongly associated with diabetes and other chronic diseases than general obesity measured by BMI. It is established that abdominal obesity is associated with decreased glucose tolerance, alterations in glucose-insulin homeostasis, reduced metabolic clearance of insulin, and decreased insulin-stimulated glucose disposal (Vazquez et al., 2007).

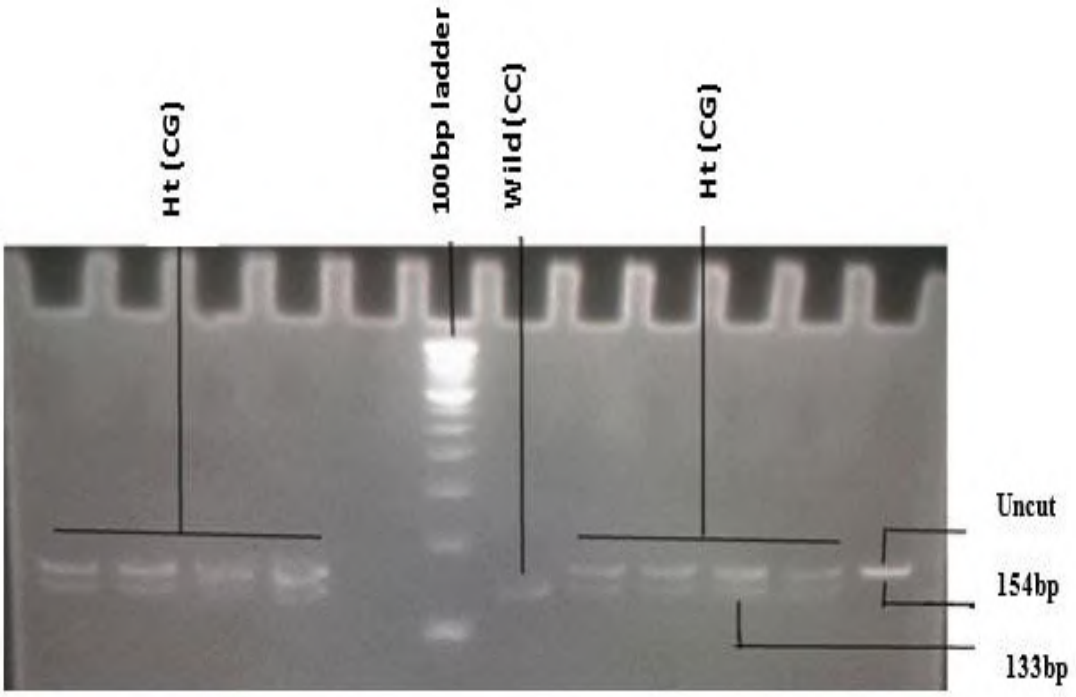


**Fig. 1. Polyacrylamide gel image of PPAR-r gene Pro12Ala marker analysis by Hae III restriction enzyme digestion.** Fragments produced by the Hae III restriction enzyme digestion of the PCR products were as follows: Homozygous wild genotype 133 bp and 21 bp; heterozygous (Ht) variant genotype 154 bp, 133 bp and 21 bp; and homozygous (Hz) variant genotype 154 bp. 21 bp is too small and cannot be resolved in 8% polyacrylamide gel.

**Table 1. Clinical characteristics of the study subjects.**

| Variables | Control (n=113) | IGT (n=113) |
|---|---|---|
| **Age, yrs** | 40 ±9 | 43 ±9 |
| **BMI, kg/m$^2$** | 25.0 ±4.4 | 25.4 ±4.2 |
| **WHR** | 0.91±0.01 | 0.93 ±0.06* |
| **SBP, mm-Hg** | 113 ±13 | 115 ±14 |
| **DBP, mm-Hg** | 75 ±8 | 76 ±10 |

Values are in Mean±SD. BMI= Body mass index, WHR= Waist-to-hip ratio, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, *p<0.05.

Table 2 describes the comparison of the lipid levels between the control and IGT subjects. The mean TG of the IGT subjects was significantly higher compared to control subjects; other indicators of lipid profile such as like total cholesterol, LDL- and HDL-cholesterol were not different in IGT subjects compared to control subjects. Liver function test (GPT) and kidney function test (creatinine) were also presented in table 2 and it shows similarity between IGT and control subjects.

**Table 2. Biochemical Characteristics of the study subjects**

| Variables | Control (n=113) | IGT (n=113) |
|---|---|---|
| **Fasting glucose, mmol/l** | 5.0 ±1.5 | 5.6 ±0.7* |
| **Postprandial glucose, mmol/l** | 6.0 ±2.6 | 8.2 ±1.6** |
| **Triglyceride, mg/dl** | 139 ±63 | 182 ±98** |
| **Total Cholesterol, mg/dl** | 190 ±39 | 198 ±40 |
| **LDL-cholesterol, mg/dl** | 126 ±36 | 126 ±35 |
| **HDL-cholesterol, mg/dl** | 36 ±9 | 35 ±8 |
| **Creatinine, mg/dl** | 0.97 ±0.16 | 0.99 ±0.16 |
| **GPT, mg/dl** | 28.9 ±16.8 | 31.9 ±21.5 |
| **Insulin, µI U/l** | 11.6 ±5.4 | 17.1 ±10.5** |

*p<0.05, **p<0.01 when compared between control and IGT subjects using student's 't" test.

Plasma insulin levels in IGT subjects (17.1±10.5) was significantly (p<0.01) higher compared to control subjects (11.6±5.4). Increment of serum triglyceride is a normal phenomenon in hyperglycemic subjects if not especially cared for, but the pattern of the different lipids may vary between ethnic groups, economic levels, and access to health care (Joshi et al., 2014). Significantly higher values of insulin in IGT subjects indicate that insulin resistance is the principal problem in this group of population. Our results are also supported by another study on the Bangladeshi population (Rahman et al., 2010). A study on Mexican-American descent has also documented higher insulin levels in IGT subjects compared to normoglycemic and impaired fasting glucose (IFG) subjects (Abdul-Ghani et al., 2006).

Table 3 shows the genotypic variation frequency in control and IGT subjects. Wild type (CC), heterozygous (CG) and homozygous (GG) variants of Pro16Ala polymerphism were analyzed and the polymorphism was found significantly associated with IGT subjects in Chi-square test (p=0.05, $X^2$=5.875). Allele frequency also shows significant association with IGT subjects in the Chi-square test (p=0.026, $X^2$=2.248). When heterozygous and homozygous genotypes were considered together as variants, it also shows strong association with IGT subjects in the Chi-square test (p=0.026, $X^2$=4.986).

The association between the substitution of alanine for proline at codon 12 of PPARG and the risk for type 2 diabetes mellitus has been

**Table 3. Genotypic variation of Pro12Ala polymorphism among the study subjects**

| Variables | Control | IGT | $X^2$ / p |
|---|---|---|---|
| **Genotype** | | | |
| Wild Type CC, frequency (n) | 0.903 (102) | 0.796 (90) | |
| Heterozygous CG, frequency (n) | 0.097 (11) | 0.186 (21) | **5.875 / 0.05** |
| Homozygous GG, frequency (n) | 0 | 0.018 (2) | |
| **Allele Frequency** | | | |
| C | 0.952 | 0.889 | **2.248 / 0.026** |
| G | 0.048 | 0.111 | |
| **Genotype** | | | |
| Wild Type CC | 0.903 (102) | 0.796 (90) | **4.986 / 0.026** |
| Variants CG & GG | 0.097 (11) | 0.204 (23) | |

widely studied since Yen et al., first reported this polymorphism in 1997. Nevertheless, the results of these studies vary considerably. In most studies, it was found that carriers of the Ala-12 allele had a lower risk for type 2 diabetes and insulin resistance (Alshuler et al., 2000; Fredriksen et al., 2002). Indeed, some studies have even showed an increased risk for type 2 diabetes mellitus in subjects with the Ala-12 variant (Lindi et al., 2002). Still, no association has so far been reported between the polymerphism and greater insulin resistance. Similar discrepancies have also been noted for the association between the Pro12Ala polymerphism of PPAR-γ and BMI (Masud and Ye, 2003). Contradictory results have been reported within the same study elsewhere, as was the case of Mori et al. (2001), who found that the Ala-12 variant was associated with a reduced risk for the development of diabetes in the general population, but that it may also be a risk factor for insulin deficiency and disease severity in individuals with type 2 diabetes mellitus. This paradox is similar to that found in our study. However, we cannot explain why the mechanisms of PPAR-γ are diverse and may produce the same effect through two completely opposing pathways. This occurs with insulin sensitivity, where PPAR-γ2 acts both via activation by agonists and by reduction of its activity. Florez et al. (2007) showed that the proline allele at PPAR-γ P12A increases the risk for diabetes in persons with impaired glucose tolerance, an effect modified by body mass index.

In a study on the north Indian population, the ProAla+AlaAla genotypes of PPAR-γ Pro12Ala were significantly associated with a higher risk of obesity, while C1431T polymor-

phism did not show any significant association. None of the haplotypes showed association with morbid obesity. However, a strong association of variant genotypes was observed with higher levels of insulin, HOMA-IR, and lower serum adiponectin concentrations (Prakash et al., 2012). When the waist-hip ratio

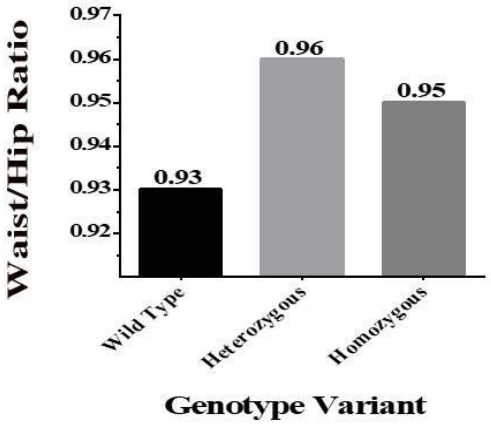


**Fig. 2. Waist/Hip ratio according to genotype variants of Pro12Ala polymorphism.**

was reanalyzed according to genotype variation, the waist-hip ratio was significantly higher among the subject's heterozygous and homozygous variants of pro16Ala polymorphism (Fig. 2). Therefore, our study concluded that PPAR-γ P12A variants might be associated with impaired glucose tolerance and it is also documented that higher waist to hip ratio in IGT subjects may be associated with heterozygous (CG) or homozygous (GG) variants of Pro12Ala mutation in the PPAR-γ gene.

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