Peroxisome proliferators-activated receptor γ 2 Pro12Ala variant is associated with body mass index in non-alcoholic fatty liver disease patients

A. C. Gupta · A. K. Chaudhory · Sukriti ·
C. Pande · P. Sakhuja · Y. Singh · S. F. Basir ·
S. K. Sarin

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Abstract

Background Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease globally and commonly associated with insulin resistance and metabolic syndrome (MS). Peroxisome proliferator-activated receptor- γ (PPAR γ) is a transcription factor abundantly expressed in adipocytes and plays a key role in the regulation of adipocyte differentiation, lipid and glucose homeostasis. Pro12Ala variant has been earlier associated with obesity, type 2 diabetes and MS.

Aim The present study aimed to determine the genotype frequencies of the Pro12Ala variant in NAFLD patients and any further association with other phenotype in the patients.

Patients and methods Ninety-eight NAFLD patients and 280 matched controls were genotyped for presence of the Pro12Ala variant. Genomic DNA was extracted and polymerase chain reaction–restriction fragment length

polymorphism using *Bst*-UI was performed for the detection of C–G change at codon 12 position of PPAR γ 2 gene. Genotype and allele frequencies were compared between patients and controls. The Hardy–Weinberg equilibrium was tested by comparing expected/observed genotype frequencies by χ^2 test.

Results The frequencies of Pro/Ala genotype were comparable between NAFLD patients and controls. In the controls, 213 (75.7%) were homozygous for the wild-type (Pro/Pro) genotype and 67 (23.9%) were heterozygous (Pro/Ala). In NAFLD patients, genotypic distribution of wild type, heterozygous and homozygous were 63 (64.3%), 34 (34.7%) and 1 (1%), respectively. Heterozygous genotype was found to be significantly higher in the patients ($P = 0.01$). We also analyzed related phenotypic association of the patients with Pro12Ala genotype. We observed that the Pro12Ala (heterozygous) genotype was significantly higher in the patients who had body mass index $>25 \text{ kg/m}^2$ ($P = 0.025$).

Conclusions Pro12Ala variation of the PPAR γ 2 gene is associated with NAFLD and might play a role in the pathogenesis of NAFLD.

Keywords Non-alcoholic fatty liver Disease (NAFLD) · Peroxisome proliferator-activated receptor γ (PPAR γ) · Body mass index (BMI)

A. K. Chaudhory · Sukriti · C. Pande · S. K. Sarin (✉)
Department of Gastroenterology, G.B. Pant Hospital,
New Delhi, India
e-mail: shivsarin@gmail.com

A. C. Gupta
G.B. Pant Hospital, Institute of Genomics & Integrative Biology
(IGIB), Jamia Millia Islamia University, Delhi, India

P. Sakhuja
Department of Pathology, G.B. Pant Hospital, New Delhi, India

Y. Singh
Allergy & Infectious diseases, Institute of Genomics &
Integrative Biology (IGIB), Delhi, India

S. F. Basir
Department of Biosciences, Jamia Millia Islamia University,
New Delhi, India

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the commonest forms of chronic liver disease, affecting nearly 16–20% of the general population globally and rising to 90% in obese patients in the developed countries [1]. NAFLD has been described by histological features

ranging from simple fatty liver, non-alcoholic steatohepatitis (NASH), progressive fibrosis to liver failure [2–4]. Obesity [as measured by body mass index (BMI)], type 2 diabetes mellitus and insulin resistance have been frequently associated with NAFLD patients and are considered to be the major risk factors [5, 6]. The prevalence of NAFLD becomes greater with higher BMI and it has been reported that progression of NASH is greater in obese than non-obese patients [5, 7]. Recent studies have suggested that genetic and environmental factors play an important role in pathogenesis of multifactorial diseases such as obesity, type 2 diabetes and metabolic syndrome (MS) [8]. Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors and consist of three subtypes: PPAR α , PPAR δ , and PPAR γ . PPAR γ is a transcriptional regulator, which is abundantly expressed in adipose tissues and regulates adipocyte differentiation, glucose and lipid metabolism [9, 10]. Molecular scanning studies of the PPAR2 gene have identified a missense mutation at nucleotide 34 position, which resulted in an amino acid exchange of proline to alanine at position 12 due to a C–G substitution [11]. This substitution possibly results in a conformational change in the protein structure and reduced function of the PPAR γ 2 protein. Pro12Ala variant of PPAR γ 2 gene has been earlier associated with obesity, type 2 diabetes, insulin sensitivity and MS [12–15]. Insulin sensitization has been observed as a promising therapy for NASH [16]. Human and animal studies showed significant improvement in biochemical and histological features in NASH patients on treatment with PPAR γ agonists (such as thiazolidinedione) [17]. This study aimed to analyze the genotype frequency of Pro12Ala variant and any association with other phenotypes in NAFLD patients.

Patients and methods

Patients enrolled in the Department of Gastroenterology, G. B. Pant Hospital, New Delhi, India between 2007 and 2009 were screened and those who fulfilled the criteria for NAFLD were included in the study. The diagnosis of NAFLD was considered with persistently raised AST/ALT (>1.2 times ULN) levels, evidence of fatty liver on ultrasound or other imaging modalities and absence of any other detectable cause of liver disease. An attempt was made to confirm the diagnosis by histopathology. Patients found to be positive for any other cause of liver disease such as HBV and HCV infection, autoimmune hepatitis, Wilson's disease, drug induced hepatitis and increased alcohol intake (>30 g/day for males, >20 mg/day for females) were excluded from the present study. Biochemical and clinical parameters were analyzed in the patients as per the

guidelines of Adult Treatment Panel III (ATPIII) at the time of enrollment.

Biochemical test

Each patient and few controls underwent biochemical testing for liver enzymes (transaminases), lipid profile, fasting glucose and insulin levels. The oral glucose tolerance test (OGTT) was performed in all patients with 75 g of glucose according to the WHO criteria. Insulin resistance was evaluated according to two different criteria: the homeostatic metabolic assessment (HOMA) insulin resistance index (fasting serum insulin μ IU/ml, fasting serum glucose mmol/L) and insulin level at 120 min after the OGTT [18, 19]. The formula for the HOMA model was as follows: $\text{HOMA-IR} = [\text{fasting insulin } (\mu\text{IU/ml}) \times \text{fasting glucose (mmol/l)}] / 22.5$.

Histological data

Liver biopsies were performed in 71 patients. Tissue sections were stained with hematoxylin–eosin, impregnated with silver for reticulin framework and stained with periodic acid Schiff for glycogen, periodic Schiff diastase for non-glycogen proteins, Perls' for iron and trichrome for collagen. The presence of NAFLD was assessed based on the presence of macrovesicular steatosis, parenchymal inflammation, ballooning and fibrosis [17]. Histological evaluation was assessed by experienced pathologists, blinded to the clinical and laboratory profile of the patients.

Controls

Two hundred and eighty unrelated healthy subjects, with no previous history of liver disease and negative for all HBV and HCV viral markers (HBsAg, anti-HBc, anti-HCV and HCV RNA) were considered as controls. Both patient and control groups were prospectively matched for the same ethnic origin. Clinical and biochemical assessment of the patients was performed according to the study protocol approved by the Institutional Ethical Committee. Informed written consent was obtained from all the patient and control subjects. Patients and controls were prospectively matched for age, sex and BMI.

PPAR γ Pro12Ala genotyping

Genomic DNA was extracted from EDTA preserved whole blood using sodium perchlorate method. Proline–Alanine (CCG–GCG) alleles were detected after DNA amplification by polymerase chain reaction (PCR) followed by restriction digestion with BstUI. Genotypes were determined by PCR–restriction fragment length polymorphism (RFLP) using previously published primers [20].

Sense primer (5'-GCCAATTCAAGCCCAGTC-3'); anti-sense primer (5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG-3'). For each reaction of total volume 25 μ l, 50 ng of genomic DNA, 0.3 units of *Taq* polymerase (Fermantas) and 2.5 μ l 10 $\times$ PCR buffer (1.5 mM MgCl₂) were used. The following PCR conditions were used: initial denaturation at 95°C for 5 min, denaturation at 95°C, annealing at 58°C and extension at 72°C followed by 35 cycles. Each of these steps lasted 45 s. This was followed by final extension at 72°C for 10 min. The amplified product size of 270 base pairs (bp) was digested by the specific restriction enzyme, BstUI. When alanine was present at residue 12 of PPAR γ 2, the 270 bp DNA fragment was split into 227 and 43-bp fragments (heterozygous allele). The digestion products were then separated by electrophoresis on a 2.5% agarose gel (Fig. 1). The results were documented by digital camera and stored as computer files in Biometra gel documentation system. To validate RFLP results, 10% randomly selected PCR product was sequenced and analyzed for the C to G change (Fig. 2).

Statistical analysis

Genotypes were in Hardy–Weinberg equilibrium for both NAFLD patients and controls when analyzed for each group separately. Frequencies were compared and *P* value calculated by wessa.net software. Multivariate analysis was performed to assess the association between Pro12Ala variant and several variables of phenotypic expression in NAFLD patients. Results are expressed as mean $\pm$ standard deviation and considered significant when *P* = 0.05 (two-tailed). Adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were calculated.

Results

The baseline characteristics of patients and controls are shown in Tables 1 and 2. Majority of the patients were male (68%). The mean BMI of the patients was 27 kg/m². 68% patients had BMI more than 25 kg/m². None of the patients showed manifest obesity (BMI > 30 kg/m²). 29%

Fig. 1 PCR–restriction fragment length polymorphism detection of the Pro12Ala PPAR γ 2 missense mutation. PCR followed by digestion with BstU-1–2.5% agarose gel electrophoresis followed by ethidium bromide staining and UV transillumination was performed. The expected product sizes are: normal homozygote, 270 bp; Pro12Ala

homozygote, 227 and 43 bp; and Pro12Ala heterozygote, 270, 227, and 43 bp, respectively. Lane 1 molecular marker, lane 2 normal homozygote, lane 3 Pro12Ala heterozygote, lane 7 Pro12Ala homozygote mutated. The 43-bp fragments are not visualized

Fig. 2 DNA sequence analysis showing C–G change at codon 12 of PPAR γ gene

Table 1 Demographic profile of patients and healthy controls

	Controls	Patients	<i>P</i>
<i>N</i>	280	98	–
Sex (M/F)	182:98	66:32	ns
Age (years)	32 ± 8	36 ± 7	0.7
BMI (kg/m ²)	23.6 ± 3.5	26.9 ± 4.5	0.001
Waist circumference (cm)	84 ± 6.6	94.6 ± 12	0.001
ALT (IU/L)	27 ± 4.6	65 ± 5.6	0.003

Results expressed as mean (standard deviation of the mean)

Table 2 Biochemical parameters in NAFLD patients

Parameters	NAFLD
Hypertension, <i>n</i> (%)	24 (25%)
Diabetes mellitus, <i>n</i> (%)	25 (27%)
FBS (mg/dl)	104 (±42)
Fasting insulin (mU/ml)	13 (2.6–80.3)
HOMA-IR	2.7 (0.51–3.6)
HDL-cholesterol (mg/dl)	38 (±6)
Median Triglycerides (mg/dl)	144 (58–980)

Results expressed as mean (standard deviation of the mean) and range
Controls had normal values

Table 3 Distribution of Pro12Ala genotype frequency in controls and NAFLD patients

Subjects (<i>n</i>)	Pro/Pro	*Pro/Ala	Ala/Ala	<i>P</i> value
Controls (280)	213 (76%)	67 (23.9%)	–	1.6
NAFLD (98)	63 (64.3%)	34 (34.7%)	1 (1%)	0.01*

Hardy–Weinberg and χ^2 test were performed to calculate allelic frequency. Significant *p*-values compared between the 2 groups have been highlighted in bold

* *P* < 0.05 significantly higher than controls

patients showed associated MS as defined by ATPIII criteria. The ultrasound examination of the abdomen revealed fatty liver in all the patients.

Distribution of Pro12Ala genotype in the study groups

Pro12Ala genotype was determined by the Hardy–Weinberg equilibrium in study groups. Observed Pro12Ala genotype frequencies are shown in Table 3. The frequency of Pro/Ala variant was comparable between patients and controls. Of the healthy controls, 213 (76%) were homozygous for the wild-type allele and 67 (23.9%) were heterozygous (*P* = 1.5). In NAFLD patients, genotype distribution of wild-type homozygous, heterozygous and homozygous allele was 63 (64.3%), 34 (34.7%) and 1 (1%) respectively. Pro12Ala genotype was significantly higher

Table 4 Pro12Ala genotype frequency distribution in BMI based subgroups of NAFLD patients

Subjects (<i>n</i> = 98)	Pro/Pro	*Pro/Ala	Ala/Ala	<i>P</i> value
BMI ≤ 25 kg/m ²	22 (73.3%)	8 (26.6%)	–	0.1
BMI ≥ 25 kg/m ²	40 (58.8%)	27 (39.7%)	1 (1.4%)	0.025*

Hardy–Weinberg and χ^2 test were performed for association. Significant *p*-values compared between the 2 groups have been highlighted in bold

* *P* < 0.05 significant

in NAFLD patients (34.7 vs. 23.9%, OR 2, 95% CI 1.3–2.0, *P* = 0.01).

Analysis of Pro12Ala with BMI and histological features

We observed 68% patients had BMI >25 kg/m². Further analysis was done for any association of Pro12Ala genotype and BMI in the patients. Subgroups were divided based on BMI [(lean <25 kg/m²) and (overweight/obese >25 kg/m²)] in controls and patients. Determined genotype frequencies are shown in Table 4. In the lean patients, 22 (73.3%) of 30 were homozygous for the wild type, while 8 (26.6%) were heterozygous with no homozygote mutant genotype. Forty (58.8%) of 68 obese patients were homozygous for the wild type, 27 (39.7%) of 68 were heterozygous and 1 (1.4%) had mutant genotype. The frequency of heterozygous genotype (Pro12Ala) in obese patients was significantly higher as compared to the lean NAFLD patients (39.7 vs. 26.6%; OR 2.2, 95% CI 1.2–2.4, *P* = 0.025). No significant distribution of heterozygous genotype was observed when analyzed based on BMI in controls. Although histological features were analyzed in 71 patients with varying grades of steatosis, ballooning degeneration, lobular inflammation and fibrosis (Table 5), we were unable to find any association of any histopathological features with the Pro12Ala genotype in the patients.

Discussion

In the present study, we observed that Pro12Ala genotype is associated with NAFLD and higher BMI in patients. The substitution of Ala12 has been identified with extremely variable frequencies in several populations such as Caucasian, Italian, Finnish, Danish, German and Japanese from different ethnic origins (8–20%) [20, 21]. The observed frequency of Pro12Ala variant was 24% as also reported in previous studies done in Indian population [21, 22]. Studies suggesting association between Pro12Ala polymorphism

Table 5 Histological features in patients with NASH ($n = 71$)

Histological parameter	Frequency N = 71(%)			
	Grade 1	Grade 2	Grade 3	
Steatosis (0–3)	17 (24)	23 (32)	31 (44)	
Ballooning (0–2)	53 (74)	18 (26)	–	
Lobular inflammation (0–3)	30 (42)	38 (54)	3 (4)	
Portal inflammation (0–3)	18 (25)	44 (62)	9 (13)	
Necroinflammation (Grade) (0–3)	21 (30)	26 (36)	24 (34)	
Fibrosis (Stage) (0–4)	Stage 0	Stage 1	Stage 2	Stage 3
	12 (17)	43 (60)	12 (17)	4 (6)

with obesity and other related metabolic disorders have yielded inconsistent findings [18, 23, 24]. In our study, we found an association of Pro12Ala genotype with higher BMI ($>25 \text{ kg/m}^2$) in patients. This finding suggests an important role of Pro12Ala variant in the obesity related NAFLD disease pathogenesis. Pro12Ala, a functional variant results from a non-conservative substitution of alanine to proline leading to a reduction in transcriptional activity (a decrease in binding affinity to the PPAR response element and other regulatory transcriptional factor in its target genes) and reduce the ability to regulate the expression of target genes [18]. Ala12 substitution has been associated with increased insulin sensitivity. PPAR γ agonists (thiazolidinediones) are used for therapeutic purposes in type 2 diabetes and obese patients [19, 25]. Data from animal models showed activation of PPAR γ receptors by thiazolidinediones, which promote adipocyte differentiation and regulation of a number of genes that regulate lipid metabolism [26]. Pro12Ala is a modulator for PPAR γ activity and has been reported to moderate reduction of the PPAR activity in heterozygous PPAR γ -deficient mice which prevented adipocyte hypertrophy, increased fatty acid consumption, decreased triglycerides and lipogenesis, resulting in an improvement of insulin sensitivity by glucose removal and glucose production [27, 28]. Recent studies indicated that PPAR γ agonist is able to reduce insulin resistance and improve liver histology in both animals and humans [29, 30]. A study by Kang and colleagues showed an effect of Pro12Ala polymorphism in type 2 diabetic patients during treatment with PPAR γ agonist [31]. This finding suggests a potential beneficiary role of Pro12Ala genotype in identification of the patients who are more likely to respond better during PPAR γ agonist treatment.

In summary, our results showed an association of Pro12Ala genotype of PPAR γ with NAFLD and BMI in the patients. The Pro12Ala may be used as a clinically relevant pharmacogenetic marker for NAFLD. Further studies are warranted to investigate the cellular and molecular mechanisms underlying these associations.

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