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PRO12ALA SUBSTITUTION IN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (*PPARY*) GENE AND NON-ALCOHOLIC FATTY LIVER DISEASE

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Abstract - The aim of this study was to analyze the relationship between Pro12Ala substitution in the peroxisome proliferator-activated receptor gamma (*PPARy*) gene and non-alcoholic fatty liver disease (NAFLD). Ninety-seven patients with NAFLD and 51 healthy subjects were included in the study. The height, weight, abdominal wall fat thickness, blood pressure, serum triglyceride, total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting glucose level, hip and waist circumference, and body fat percentage were measured. The PPARγ Pro12Ala genotypes were analyzed using oligonucleotide microarray. Among the NAFLD patients, 11.34% (11/97) had the GC genotype (Pro/Ala) and 88.66% (86/97) had the C genotype (Pro). Among the healthy control group, 5.88% (3/51) had the GC genotype and 94.12% (48/51) had the C genotype. There was no significant difference in the distribution of *PPARγ* Pro12Ala polymorphism between the NAFLD patients and control subjects. There was no significant difference between *PPARγ* Pro12Ala polymorphism distribution or blood pressure, weight, body mass index, hip circumference, waist circumference, waist-hip ratio, percentage of body fat, abdominal wall fat thickness, fasting serum glucose, triglyceride, or total cholesterol when compared between these genotypes. No association between *PPARγ* Pro12Ala substitution and non-alcoholic fatty liver disease was found in the study.

Key words: Non-alcoholic fatty liver disease, peroxisome proliferator-activated receptor gamma, polymorphism

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases. It can range in severity from simple fatty infiltration to chronic hepatitis (non-alcoholic steatohepatitis [NASH]), which can result in cirrhosis and organ failure. The prevalence of NAFLD is as high as 30% in developed countries and nearly 10% in developing nations (Smith, et al., 2011). Most NAFLD cases are associated with features of the recently characterized metabolic syndrome (MS), including obesity, diabetes, hypertension and dyslipidemia (McCullough, 2011), and NAFLD is now recognized to be the hepatic component of MS. NAFLD is an increasing global public health problem. As the number of patients with NAFLD increases, so will the number of patients with end-stage liver disease (cirrhosis), liver failure and hepatocellular carcinoma, as more than 30% of NAFLD patients have NASH that will likely progress (Torres, 2008). The development of hepatic insulin resistance has been ascribed to multiple causes, including inflammation, endoplasmic reticulum (ER) stress, and accumulation of hepatocellular lipids in NAFLD (Kumashiro et al., 2011).

Peroxisome proliferator-activated receptor-γ (PPARγ), a member of the nuclear hormone re-

ceptor superfamily, is secreted from adipose tissue. PPARγ plays an important role in the regulation of adipocyte-specific genes and lipids and glucose metabolism (Semple et al., 2006). The PPARγ gene is considered a potential candidate gene for the determination of insulin sensitivity (Day, 2004). PPARγ has been implicated in the etiology of type 2 diabetes mellitus (T2DM) and has been investigated in numerous epidemiologic studies (Gouda et al., 2010). PPARγ contains two protein isoforms, PPARγ1 and PPARγ2. PPARγ2 is primarily found in adipose tissue and the large intestine (Fajas et al., 1997). Several variants in the PPARγ gene have been identified, and the Pro12Ala variant resulting from a C to G substitution is the most extensively examined variant in epidemiologic studies (Tonjes et al., 2007). Several investigations have revealed that the Ala allele of PPARγ2 is associated with a significantly decreased development of T2DM in Caucasians and confers a greater sensitivity to insulin in overweight subjects (Huguenin et al., 2010). With regard to NAFLD, some investigations have examined the relationship between the Pro12Ala variant and NAFLD, although the results have been inconclusive. The aim of this work was to elucidate the relationship between the Pro12Ala polymorphism in the PPARγ gene and NAFLD, based on the polymorphism distribution of NAFLD patients and normal subjects.

MATERIALS AND METHODS

Subjects

Ninety-seven patients with NAFLD and a mean age of 49.72 ± 13.33 years, participated in the study. In addition, 51 healthy subjects with a mean age of 48.22 ± 14.24 years were evaluated. All the subjects were selected based on the guidelines for diagnosis and treatment of NAFLD created by the Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association (Fan et al., 2010). Informed consent was obtained from all subjects. NAFLD was confirmed via B-ultrasound of the liver on more than two occasions. The study protocol was approved by our institution and was conducted in accordance with ethnical and human principles of research.

The height, weight, hip and waist circumference, body fat, abdominal wall fat thickness and blood pressure of the patients were measured. The body mass index (BMI) was also calculated as weight/height^2 (kg/m^2). The waist-hip ratio (WHR) was calculated as waist circumference/hip circumference. Serum triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL) and fasting glucose levels were measured with an autoanalyzer.

Polymorphism genotyping
PCR amplification

Genomic DNA from the peripheral blood samples of the 148 subjects was isolated with a commercial kit (SK1252, Shanghai Sangon Biological Engineering Technology & Service Co. Ltd., China) and stored at -20°C. The DNA was subjected to the polymerase chain reaction (PCR) using the following primers: 5'-GGA TAT TGA ACA GTC TCT GCT C-3' (forward primer) and 5'-GTT TGC AGA CAG TGT ATC AGT G-3' (reverse primer). The length of the PCR product was 454 bp. The nucleotide sequence reported in this study has been deposited in GenBank under accession no. AB005520. The reverse primers were fluorescence cyan 3 (Cy3) labeled at the 5' end for signal detection. To generate fluorescence-labeled single-stranded target segments, asymmetric PCR was used, with the optimal ratio of the forward to the reverse primer of 1:10. The PCR products were amplified in a thermal cycler under the following condition: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were resolved on a 2% agarose gel and stained with ethidium bromide.

Oligonucleotide microarray preparation

To generate the optimal hybridization signal, we designed the maximized detection probe according to the Pro12Ala *PPARγ2* variant. The optimal probe

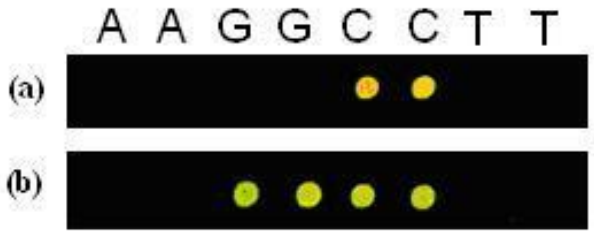

Fig. 1. Fluorescence signal from hybridization of oligonucleotide chip for detection of Pro12Ala substitution in the *PPARγ* gene. (a) C genotype (b) GC genotype.

sequence was (1) CTA TTG AC**A** CAG AAA G,(2) CTA TTG AC**G** CAG AAA G, (3) CTA TTG AC**C** CAG AAA G, and (4) CTA TTG AC**T** CAG AAA G. The polymorphic nucleotides for detection were located in the middle of the probe sequences (bold). The probes were diluted to a final concentration of 100 μmol/L. Five microliters of probe solution was mixed with 5 μL of spotting solution (5*SSC, 0.1% SDS, 1% Ficoll, and 5% glycerin) and was transferred into 96-well microtiter slides. The mixtures were spotted onto aldehyde-coated glass slides with a microarray printer (Cartisan), which deposits 0.5 nL at each spotting site, resulting in 200 μM diameter spots. Each probe was spotted in duplicate. The humidity during spotting was 90% and the temperature was maintained at 23°C. The microarrays were kept at room temperature for at least 24 h.

Hybridization and signal detection

The asymmetric Cy3-labeled target PCR products (4 μL) were mixed with 6 μL of hybridization solution (5*SSC, 0.1% SDS, 1*Denhart's ragents, and 0.1 μg/μL fragmented salmon DNA), and 10 μL of this mixed solution was transferred to the reaction chamber on the glass slides. The slides were hybridized at 42°C for 1h in an airtight humidified container and the hybridized slides were washed sequentially in washing solution A (1*SSC and 0.2% SDS), washing solution B (0.2*SSC), and washing solution C (0.1*SSC) for 1 min each and were dried by centrifugation. Fluorescence signals were detected and quantified with Genepix 4000B (Axon Instruments, Inc, USA), with excitation at 540 nm and emission at 570 nm (Cy3). Sixteen-bit TIFF images of at μM resolution were analyzed. (Fig. 1)

PCR product sequencing

The target PCR products from genomic DNA were purified and ligated to the CEQ™TDCS-vector (Beckman Coulter, Inc., USA) according to the manufacturer's instructions. Sequencing results were in concordance with the microarray-based methods (Fig. 2). All experiments were performed in triplicate.

Statistical analysis

The data were analyzed using SPSS 17.0 (SPSS Inc., USA) statistical software, with data represented as means ± standard deviation (SD). Chi-squared analysis and Fisher's exact test were applied to test for significant differences in the allele frequencies. Differences in continuous variables between groups were tested using independent t-tests when the variables were normally distributed.

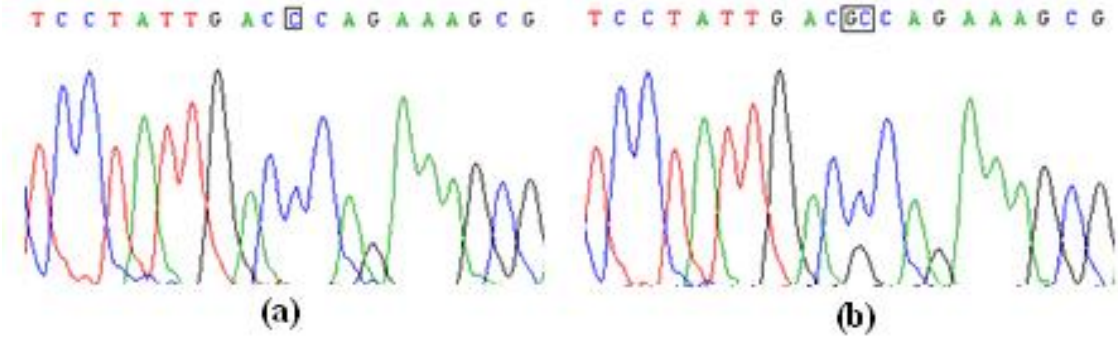

Fig. 2. Results of the *PPARγ* sequencing analysis. (a) C genotype (b) CG genotype. This process yielded the same results as the microarray-based methods shown in Fig. 1.

Table 1. Clinical characteristics of the subjects in the NAFLD and healthy groups

Clinical parameters	NAFLD (n=97)	Healthy (n=51)	T	P
Age (years)	49.72 ± 13.33	48.22 ± 14.24	0.638	0.524
Height (cm)	164.84 ± 7.37	163.86 ± 7.12	0.776	0.439
Weight (kg)	70.95 ± 9.65	60.02±10.48	6.355	0.000
Body mass index (kg/m^2)	26.03 ± 2.45	22.26 ± 2.91	8.340	0.000
Hip circumference (cm)	97.78 ± 4.77	92.88 ± 6.42	4.798	0.000
Waist circumference (cm)	88.25 ± 7.04	77.51 ± 8.9	7.481	0.000
Body fat percentage (%)	31.29 ± 5.68	24.77 ± 6.86	5.816	0.000
Abdominal wall fat thickness (cm)	2.95 ± 0.68	2.5 ± 0.78	3.690	0.000
Systolic blood pressure (mmHg)	133.16 ± 16.48	119.13 ± 17.01	4.681	0.000
Diastolic blood pressure (mmHg)	82.02 ± 7.91	75.46 ± 8.53	4.492	0.000
Total protein (g/L)	74.91 ± 3.37	73.31 ± 3.5	2.708	0.008
Albumin (g/L)	48.36 ± 3.1	45.32 ± 6.26	3.958	0.000
ALT (U/L)	46.57 ± 29.71	20.84 ± 9.76	7.766	0.000
Triglyceride (mmol/L)	2.34 ± 1.45	1.05 ± 0.47	8.000	0.000
Total cholesterol (mmol/L)	4.97 ± 0.8	4.34 ± 0.87	4.419	0.000
High-density lipoprotein (mmol/L)	1.22 ± 0.35	1.41 ± 0.24	-3.677	0.000
Glucose (mmol/L)	5.25 ± 0.79	4.59 ± 0.45	6.422	0.000

Table 2. *PPARγ* Pro12Ala genotype frequencies in the NAFLD and healthy groups

Group	Cases	Genotype (frequency)		Allele (frequency)	
		Pro/Pro	Pro/Ala	C	G
NAFLD	97	86 (88.66%)	11 (11.34%)	183 (94.33%)	11 (5.67%)
Healthy	51	48 (94.12%)	3 (5.88%)	99 (97.06%)	3 (2.94%)
χ^2		1.163		1.105	
p		0.281		0.293	

RESULTS

Clinical characteristics of NAFLD patients and healthy control individuals

The weight, BMI, hip and waist circumference, body fat percentage, abdominal wall fat thickness, blood pressure, albumin, alanine aminotransferase (ALT) and triglyceride, total cholesterol and glucose levels were significantly higher in the NAFLD group relative to the healthy subjects (Table 1).

PPARγ Pro12Ala genotype frequencies

In the NAFLD group, 11.34% (11/97) of the subjects were of the GC genotype (Pro/Ala) and 88.66% (86/97) were of the C genotype (Pro). In the healthy control group, 5.88% (3/51) of subjects were of the GC genotype and 94.12% (48/51) of subjects had the C genotype. There was no significant difference in the distribution of the *PPARγ* Pro12Ala polymorphism between the NAFLD and control groups (Table 2).

There was no significant difference between *PPARγ* Pro12Ala polymorphism distribution and blood pressure, weight, BMI, hip and waist circumference, waist-hip ratio, body fat percentage, abdominal wall fat thickness, and fasting serum glucose, triglyceride and total cholesterol levels (Table 3).

DISCUSSION

NAFLD is a clinicopathological syndrome with a complex pathogenesis. NAFLD encompasses several clinical signs, including insulin resistance, oxidative stress, free fatty acid-induced lipotoxicity, ER stress,

Table 3. Clinical characteristics of 97 NAFLD and 51 healthy subjects classified according to their genotype of Pro12Ala polymorphism of the *PPARγ* gene ($\bar{x} \pm s$)

Clinical parameters	Pro/Pro (n=134)	Pro/Ala (n=14)	T	P
Age (years)	49.02 ± 13.61	50.93 ± 14.13	-0.497	0.620
Height (cm)	164.72 ± 7.33	162.43 ± 6.56	1.123	0.263
Weight (kg)	67.27 ± 11.37	66.38 ± 9.64	0.282	0.779
Body mass index (kg/m^2)	24.7 ± 3.23	25.09 ± 2.64	-0.445	0.657
Hip circumference (cm)	96.11 ± 5.94	95.93 ± 5.25	0.111	0.912
Waist circumference (cm)	84.35 ± 9.51	86.5 ± 6	-1.195	0.246
Body fat percentage (%)	28.96 ± 6.76	29.84 ± 7.74	-0.456	0.649
Abdominal wall fat thickness (cm)	2.78 ± 0.75	2.98 ± 0.7	-0.961	0.338
Systolic blood pressure (mmHg)	128.03 ± 17.84	133.21 ± 18.02	-1.030	0.305
Diastolic blood pressure (mmHg)	79.73 ± 8.58	81.07 ± 9.63	-0.548	0.584
Total protein (g/L)	74.4 ± 3.56	74 ± 2.74	0.405	0.686
Albumin (g/L)	47.4 ± 4.86	46.48 ± 1.75	0.706	0.481
ALT (U/L)	38.78 ± 28.43	27.43 ± 13.94	1.472	0.143
Triglyceride (mmol/L)	1.86 ± 1.32	2.28 ± 1.63	-1.101	0.273
Total cholesterol (mmol/L)	4.74 ± 0.9	4.88 ± 0.51	-0.549	0.584
High-density lipoprotein (mmol/L)	1.3 ± 0.32	1.15 ± 0.36	1.592	0.113
Glucose (mmol/L)	5.02 ± 0.79	5.02 ± 0.32	0.013	0.990

cytokine secretion and gut flora alterations. These abnormalities contribute to steatosis, liver injury and disease progression. PPARγ protein is a nuclear receptor that regulates adipocyte differentiation and possibly lipid metabolism and insulin sensitivity. Thus, *PPARγ* is a promising candidate gene for several human disorders including obesity, T2DM, and NAFLD; it is also considered to participate in the pathogenesis of fatty liver disease. Screening for mutations at codon 12 of *PPARγ2* in NAFLD and healthy subjects is important.

Yen et al. (1997) found that the allele frequency of the Pro12Ala *PPARγ2* variant was 12% in Caucasian Americans, 10% in Mexican Americans, 8% in Samoans, 3% in African Americans, 2% in Nauruans and 1% in Chinese individuals. In the present study, the total allele heterozygote frequency was 9.46% for all subjects, 11.34% in NAFLD patients, and 5.88% in the healthy population. The *PPARγ2* Pro12Ala genotype frequency and the allelic gene frequency were not significantly different between NAFLD and healthy subjects, and there was no significant difference between *PPARγ2* Pro12Ala polymorphism distribution and blood pressure, weight, body mass index, hip and waist circumference, waist-hip ratio, body fat percentage, abdominal wall fat thickness, and fasting serum glucose, triglyceride, and total cholesterol levels when compared between the genotypes.

Dongiovanni et al. (2010) also found that *PPARγ2* Pro12Ala did not differ between patients and healthy controls. In the study of Rey et al. (2010), no association was found between *PPARγ2* Pro12Ala and NAFLD; however, there was a significantly higher risk (odds ratio [OR] = 2.50, 95% confidence interval [CI]: 1.05–5.90, P = 0.028) of development of inflammatory alterations in patients with alcoholic fatty liver disease who possessed the mutated Ala12 allele. In another study in China, it was found that the C/C genotype of *PPARγ2* was significantly associated with NAFLD (OR = 1.87, 95%CI: 1.13–2.85, P = 0.009), which differed from our results (Yang et

al., 2012). Gawrieh et al. (2012) found that *PPARγ2* Pro12Ala was associated with histologically advanced NAFLD.

Although no association between the Pro12Ala polymorphism and NAFLD was established in the present study, future studies should include a larger sample size to better evaluate the relationship between the *PPARγ* Pro12Ala polymorphism and the development of NAFLD.

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