A Common 34C>G Variant at the Peroxisome Proliferator-Activated Receptor- γ 2 Gene in Chilean Women with Polycystic Ovary Syndrome and Controls

Polimorfismo 34C>G del Gen del Receptor Activado por Proliferadores Peroxisomales- γ 2 en Mujeres Chilenas con Síndrome de Ovario Poliquístico y Controles

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SUMMARY: The 34C>G (Pro12Ala) polymorphic variant of the peroxisome proliferator-activated receptor- γ 2 (PPAR- γ 2) gene has been associated with polycystic ovary syndrome (PCOS). However, the results between populations are contradictory. Thus, in the present study was investigated the possible association between 34C>G polymorphism at the PPAR- γ 2 gene and PCOS in Chilean women. A total of 50 unrelated women (29.1 ± 8.1 years) with diagnosis of PCOS and 75 healthy controls (29.3 ± 9.3 years) were included in this study. Serum lipids, glucose and uric acid levels were determined by enzymatic-colorimetric methods. The 34C>G variant in the PPAR- γ 2 gene was analyzed by PCR-RFLP. Women with PCOS exhibited a higher levels of glucose, total cholesterol, triglycerides, LDL-C and uric acid, and lower HDL-C levels than controls ($p < 0.05$). The frequency of the 34G allele was 9% in PCOS patients and 12% in control women ($p = 0.589$). The odds ratio for PCOS associated with 34G allele was 0.73 (95% CI = 0.31 - 1.69) confirming the absence of association. We conclude that the 34C>G polymorphism of the PPAR- γ 2 gene is not related to PCOS in Chilean women.

KEY WORDS: Polycystic ovary syndrome; Peroxime proliferator-activated receptor; Pro12Ala polymorphism.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) constitutes an important health public problem in young women, with a prevalence of 5-10% depending on their ethnic background. This disorder involves the combination of chronic anovulation, clinical and endocrinological signs of hyperandrogenism, evident hyperinsulinemia and polycystic ovaries (Norman *et al.*, 2007). In addition, several studies have been associated this disorder with metabolic abnormalities as dyslipidemia, diabetes, hypertension, obesity and cardiovascular diseases (Cussons *et al.*, 2006, 2007).

Although the inheritance mode of PCOS is still uncertain, multiple genetic factors including mutations and polymorphisms to several genes have been associated with PCOS and its phenotypic traits (Diamanti-Kandarakis &

Piperi, 2005; Haap *et al.*, 2005; Luque-Ramirez *et al.*, 2006; Unluturk *et al.*, 2007); specially, the genes involved in the energy homeostasis as the peroxisome proliferator activated receptor- γ (PPAR γ).

The PPAR γ gene is located on 3p25, comprises nine exons and extends over more than 100 kb of genomic DNA (Fajas *et al.*, 1997). Three different PPAR γ mRNAs have been characterized in humans generated by alternative splicing. Among these, the PPAR γ 2 is a protein predominantly expressed in adipose tissue, and has been considerate a regulator of adipocyte differentiation and glucose homeostasis (Beaven & Tontonoz, 2006).

Several genetic variants have been described in the PPAR- γ 2 gene (Unluturk *et al.*). However, there are two

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PPAR- γ 2 gene polymorphisms that have been systematically investigated in various populations. The first is the silent 478C>T substitution which resides in the exon 6 and the second is the missense 34C>G mutation resulting in a change of proline by alanine at codon 12 (Pro12Ala) of the exon 2 (Yen *et al.*, 1997; Meirhaeghe & Amouyel, 2004).

Numerous studies have been conducted with the objective to evaluate the possible relationships between 34C>G mutation (Pro12Ala) of the PPAR- γ 2 gene and dyslipidemia (Zietz *et al.*, 2002; Tai *et al.*, 2004), obesity (Masud & Ye, 2003), type 2 diabetes (Hara *et al.*, 2000) and insulin sensitivity (Ek *et al.*, 2001; Meshkani *et al.*, 2007). Since PCOS and type 2 diabetes share certain phenotypic features such as obesity and insulin resistance, several studies have been also investigated the association between PCOS and 34C>G variant at the PPAR- γ 2 gene (Hara *et al.*, 2002; Korhonen *et al.*, 2003; Orio *et al.*, 2003; San Millán *et al.*, 2004; Hahn *et al.*, 2005; Tok *et al.*, 2005; Wang *et al.*, 2006; Antoine *et al.*, 2007). However, these studies have yielded conflicting results.

Considering a significant interethnic allele frequency variation for 34C>G polymorphism across populations, the aim of the present study was to investigate the frequency and possible association between 34C>G variant at the PPAR- γ 2 gene and the presence of PCOS in Chilean women.

MATERIAL AND METHOD

Subjects. A total of 125 unrelated Chilean women were studied. Fifty were patients with PCOS (16 - 43 years old) and 75 were non-PCOS women (controls, 20 - 50 years old) with normal menstrual cycles (< 32 days) without hirsutism, acne, or male-type alopecia, and not taking hormonal medications. All women were recruited from the Obstetrics and Gynaecology Service of the Hernán Henríquez Hospital of Temuco city, Chile.

The diagnosis of PCOS was assigned using the 1990 National Institute of Health criteria, which define PCOS as ovulatory dysfunction plus hirsutism and/or hyperandrogenemia, with exclusion of other disorders (Zawadzki & Dunaif, 1992). Polycystic ovary syndrome was diagnosed after exclusion of androgen-producing tumors, nonclassic 21-hydroxylase-deficient adrenal hyperplasia, hyperprolactinemia, active thyroid disease, and Cushing's syndrome. Ovulatory dysfunction was defined as menstrual cycles >45 days in length, or a progesterone level <2 ng/mL on days 22-24 of the menstrual cycle, in conjunction with a monophasic basal body temperature chart.

Demographic data and history of hypertension, diabetes mellitus, and hypercholesterolemia were assessed in each subject. Subjects with a history of diabetes or basal glycemia ≥ 126 mg/dl were defined as diabetic. We calculated the BMI [body weight (kg) divided by square of height (m)] to assess obesity.

The study protocol was approved by the Ethics Committee of the University of La Frontera, and all subjects gave written informed consents according basic principle of biomedical investigation enumerated in the Helsinki Declaration.

Laboratory measurements. Biochemical measurements were determined from blood sample collected after overnight (>12h) fast. Triglycerides (TG) and total cholesterol (TC) levels were assayed by enzymatic colorimetric methods (Fossati & Prencipe, 1982; Fossati & Medici, 1987). High-density lipoprotein cholesterol (HDL-C) concentrations were measured by enzymatic assay after phosphotungstic acid and magnesium precipitation (Burstein *et al.*, 1970). Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation when the triglyceride concentrations did not exceed 4.8 mmol/l (Friedewald *et al.*, 1972). Serum glucose and uric acid levels were also determined by enzymatic methods (Barham & Trinder, 1972; Fossati *et al.*, 1980).

DNA analysis. Genomic DNA was extracted from blood leukocytes by salting out procedure optimized by Salazar *et al.* (1998). The PPAR- γ 2 34C>G polymorphism was identified according to conditions described by Tavares *et al.* (2005). A 244-bp fragment was amplified by PCR in a final volume of 50 μ l containing 50 ng of genomic DNA, 100 nM of each primer, 200 mM of each dNTP, 1 unit of Taq DNA polymerase and PCR buffer (KCl 50 mM, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0). After initial denaturation at 98°C for 3 min, the amplification was performed in 30 cycles consisting of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C. A final extension of 10 min at 72°C completed the reaction.

PCR products were submitted to *Fnu*DII cleavage (5U) in a total reaction volume of 20 μ l. Enzymatic digestions were carried out at 37°C over night. The fragments were separated on 3% agarose gel for about 60 min at 100V and stained with 0.5mg/dl of ethidium bromide, and visualized on a UV transilluminator. The correct assessment of genotype for 34C>G (Pro12Ala) polymorphism at the PPAR γ 2 gene was evaluated using a homozygous sample for restriction site as a positive control. In addition, all gels were reread blindly by two persons without any change, and 10% of the analyses were randomly repeated.

Table I. Clinical and metabolic characteristics of the study population.

	PCOS (50)	Controls (75)	P*
Age, years	29.1 $\pm$ 8.1	29.3 $\pm$ 9.3	0.919
BMI, kg/m ²	33.0 $\pm$ 8.2	23.3 $\pm$ 2.6	< 0.001
SBP, mmHg	117.8 $\pm$ 9.9	110.4 $\pm$ 11.0	0.049
DBP, mmHg	76.0 $\pm$ 9.7	69.8 $\pm$ 9.8	0.066
Fasting glucose, mg/dl	103 $\pm$ 18	90 $\pm$ 16	< 0.001
Total cholesterol, mg/dl	201 $\pm$ 36	180 $\pm$ 32	< 0.040
Triglycerides, mg/dl	153 $\pm$ 73	82 $\pm$ 43	< 0.001
HDL-C, mg/dl	43 $\pm$ 7.5	54 $\pm$ 11.8	< 0.001
LDL-C, mg/dl	127 $\pm$ 41	108 $\pm$ 32	0.002
Uric acid, mg/dl	4.7 $\pm$ 1.0	3.7 $\pm$ 0.6	< 0.001

Number of individuals in parenthesis; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; PCOS, polycystic ovary syndrome; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. * Student's t-test.

Table II. Genotype distribution and relative allele frequencies of 34C>G (Pro12Ala) polymorphism at the PPAR- γ 2 gene in Chilean women with PCOS and controls.

	Genotypes			Alleles	
	CC	CG	GG	C	G
PCOS	84%	14%	2%	0.91	0.09
(50)	(42)	(7)	(1)		
Controls	79%	19%	2%	0.88	0.12
(75)	(59)	(14)	(2)		
$\chi^2 = 0.26$; 1 df; $p = 0.610$ *			$\chi^2 = 0.29$; 1 df; $p = 0.589$		

Number of individuals in parenthesis; PCOS: polycystic ovary syndrome; df, degree of freedom; *CC vs. CG + GG

Statistical analysis. Statistical analysis was carried out using the Sigma Stat Software, v. 2.0 (Jandel Sci., San Rafael, CA). Data are presented as mean $\pm$ SD. Differences between the means of the 2 continuous variables were evaluated by Student's t-test. The allelic frequencies and genotype distribution were estimated by gene counting. Differences between noncontinuous variables, genotype distribution and allele frequency were tested by chi-square analysis (χ^2). The Odds Ratio (OR) for PCOS and their 95% confidence interval (CI) associated with the 34G (Ala) variant was also calculated. Statistical significance was at $P < 0.05$.

RESULTS

The clinical characteristics of women enrolled in the study are summarized in Table I. The serum total cholesterol, triglycerides, LDL-C, glucose and uric acid concentrations were higher in the PCOS women ($P < 0.05$). In addition, PCOS patients presented a lower HDL-C levels ($P < 0.05$) and higher systolic blood pressure ($P = 0.049$) and BMI values ($P < 0.001$) when compared to control women.

The genotype distribution and the relative allele frequencies of the Pro12Ala polymorphism at the PPAR- γ 2 gene in PCOS patients and controls

Table III. Clinical and laboratory characteristics (mean $\pm$ SD) of Chilean women with PCOS and controls according to different genotypes of 34C>G (Pro12Ala) polymorphism of the PPAR- γ 2 gene.

	PCOS			Controls		
	CC	CG/GG	P*	CC	CG/GG	P*
	(42)	(8)		(59)	(16)	
Age, years	26 $\pm$ 7	30 $\pm$ 8	0.238	32 $\pm$ 6	27 $\pm$ 3	0.208
BMI, kg/m ²	32 $\pm$ 7	34 $\pm$ 6	0.606	23 $\pm$ 3	24 $\pm$ 2	0.322
SBP, mmHg	124 $\pm$ 8	116 $\pm$ 8	0.192	110 $\pm$ 11	107 $\pm$ 10	0.510
DBP, mmHg	78 $\pm$ 4	77 $\pm$ 6	0.725	70 $\pm$ 9	68 $\pm$ 12	0.566
Glucose, mg/dl	104 $\pm$ 19	96 $\pm$ 11	0.283	88 $\pm$ 19	91 $\pm$ 19	0.723
TC, mg/dl	206 $\pm$ 43	173 $\pm$ 19	0.076	170 $\pm$ 27	196 $\pm$ 33	0.047
HDL-C, mg/dl	43 $\pm$ 8	44 $\pm$ 7	0.695	54 $\pm$ 11	59 $\pm$ 12	0.292
LDL-C, mg/dl	131 $\pm$ 43	104 $\pm$ 13	0.125	94 $\pm$ 26	120 $\pm$ 27	0.027
TG, mg/dl	158 $\pm$ 73	128 $\pm$ 74	0.362	88 $\pm$ 42	81 $\pm$ 34	0.658
Uric acid, mg/dl	4.7 $\pm$ 1.0	4.9 $\pm$ 1.4	0.703	3.7 $\pm$ 0.7	3.8 $\pm$ 0.5	0.792

Number of individuals in parenthesis, BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; PCOS, polycystic ovary syndrome; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

*P values from Student's t test.

are shown in Table II. Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in controls ($\chi^2 = 1.01$; $P = \text{NS}$) and in PCOS women ($\chi^2 = 1.06$; $P = \text{NS}$).

The frequency of G (Ala) allele for 34C>G polymorphism at the PPAR- γ 2 was 9% in PCOS patients and 12% in control women ($P < 0.05$, Table II). The Odds ratio for polycystic ovary syndrome among carriers of G allele was 0.73 (95% CI = 0.31 - 1.69, $P = \text{NS}$) confirming the absence of association. No significant differences were observed between PCOS carriers of the PPAR- γ 2 C alleles (wild) and that of G variant alleles with regard to the anthropometric or metabolic parameters investigated (Table III). On the other hand, was observed that healthy women carrying the 34G allele presented higher serum concentrations of total cholesterol ($p = 0.047$) and LDL-C ($p = 0.027$).

DISCUSSION

Polycystic ovary syndrome is a metabolic disorder resulting from the interaction of genetic predisposition and environmental risk factors (Crosignani & Nicolosi, 2001; Carmina, 2003). In the current study, we examined the possible association between 34C>G (Pro12Ala) polymorphism at the PPAR- γ 2 gene and the presence of polycystic ovary syndrome in Chilean women.

When we evaluated the clinical and laboratory characteristics in PCOS patients and controls, we observed that the BMI values, total cholesterol, triglycerides, LDL-C, fasting glucose and uric acid concentrations were significantly greater in PCOS patients. On the other hand, the serum levels of HDL cholesterol were lower in this group when compared to controls. These data confirm the well-know associations between PCOS and traditional cardiovascular risk factors (Quiñonez *et al.*, 2000; Cussons *et al.*, 2006; Shroff *et al.*, 2007).

Our data also shown that the 34C>G polymorphism at the PPAR- γ 2 gene was not associated with PCOS in Chilean

women. The frequency of G (Ala) allele for 34C>G polymorphism at the PPAR- γ 2 was 9% in PCOS patients and 12% in control women. This result is in accordance with other previous studies (Urbanek *et al.*, 1999; Orio *et al.*; San Millán *et al.*; Tok *et al.*; Wang *et al.*; Antoine *et al.*). On the other hand, Korhonen *et al.* (2003) in Finnish population found a significant association between 34C>G polymorphism and PCOS. In another study, Hara *et al.* (2002) showed that Caucasian PCOS patients with 34G allele are more insulin sensitive than those with 34C allele. Recently, Hahn *et al.* also reported that the 34C>G polymorphism is associated with increased insulin sensitivity as well as lower hirsutism scores in PCOS women. Similarly, Yilmaz *et al.* (2005) suggest that 34C>G polymorphism may be protective against insulin resistance and might prevent the development of diabetes mellitus, in the first-degree relatives of subjects with PCOS.

These contradictory results may be explained, at least in part, by undetected ethnic admixture in cases or controls that falsely distort allele frequencies in some situations (Colhoun *et al.*, 2003). Several studies have been described a significant interethnic allelic variation for 34C>G polymorphism of the PPAR- γ 2 gene across populations, with a higher allele frequency in Caucasians and minor frequency in Asian subjects (Ruiz-Narvaez, 2005).

In addition, was reported that there are a widely spectrum of allele frequencies within and between populations, irrespective of disease status (Cardon & Palmer, 2003). PCOS is a common and complex disorder. Similar to other multifactorial traits such as type 2 diabetes and obesity, the complexity of the underlying genetic model as well as potential gene-gene and gene-environment interactions pose a difficulty for genetic analysis (Unluturk *et al.*). Other major limitations of genetic studies in PCOS are lack of universally accepted diagnostic criteria and definition, relatively small sample size of the study populations, and variable penetrance and expressivity.

In summary, our data suggest that the 34C>G polymorphism of the PPAR- γ 2 gene is not associated with increased susceptibility to PCOS in Southern Chilean women.

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RESUMEN: El polimorfismo 34C>G del gen del receptor activado por proliferadores peroxisomales- γ 2 (PPAR- γ 2) ha sido relacionado con el síndrome de ovario poliquístico (SOP). Sin embargo, los resultados obtenidos entre poblaciones son contradictorios. En el presente estudio se investigó la posible asociación entre el polimorfismo 34C>G del gen PPAR- γ 2 y SOP, en mujeres chilenas. Fueron analizadas 50 mujeres no relacionadas con diagnóstico de SOP (29.1 ± 8.1 años) y 75 mujeres controles (29.3 ± 9.3 años). Se evaluaron las concentraciones séricas de lípidos, glucosa y ácido úrico mediante métodos enzimáticos-colorimétricos. La genotipificación de la variante 34C>G del gen PPAR- γ 2 fue realizada mediante la técnica de PCR-RFLP. Los datos muestran que las mujeres con SOP presentan elevados niveles de glucosa, colesterol total, triglicéridos, LDL-C y ácido úrico; y bajos niveles de HDL-C al ser comparadas con las mujeres controles ($p < 0.05$). La frecuencia del alelo mutado 34G fue 9% en las mujeres con SOP y 12% en las mujeres controles ($p = 0.589$). La odds ratio para PCOS asociada al alelo 34G fue 0.73 (I.C. 95% = 0.31 - 1.69) confirmando la ausencia de asociación. En conclusión, nuestros datos sugieren que el polimorfismo 34C>G del gen PPAR- γ 2 no está relacionado a SOP, en mujeres chilenas.

PALABRAS CLAVE: Síndrome de ovario poliquístico; Receptor activado por proliferadores peroxisomales; Polimorfismo Pro12Ala.

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