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PRO12ALA GENE POLYMORPHISM IN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA AS A RISK FACTOR FOR THE ONSET OF TYPE 2 DIABETES MELLITUS IN THE SERBIAN POPULATION

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Abstract - The peroxisome proliferator-activated receptor gamma (PPARγ) is a gene candidate for the onset of type 2 diabetes mellitus (T2DM). We investigated the association of the PPARγ Pro12Ala gene with the onset of T2DM for the first time in the Serbian population. The study population consisted of 197 controls and 163 T2DM patients. The 12Ala allele tended to be more frequent in the group of T2DM patients (0.11) compared to the control subjects (0.09). The results from this study indicate that the PPARγ$_2$ 12Ala allele presents a non-significant risk factor for T2DM development in the Serbian population.

Key words: PPARγ$_2$, Pro12Ala polymorphism, type 2 diabetes mellitus, insulin

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INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the PPAR subfamily of nuclear receptors and appears to be an important regulator of adipogenesis (Kubota et al., 1999). This transcription factor is activated by prostaglandin derivatives and antidiabetic synthetic thiazolidindiones, resulting in a powerful adipogenic response and enhanced insulin (INS) sensitivity (Brun et al., 1996). PPARγ is encoded by a single gene that gives rise through alternative splicing to four isoforms (γ$_{1,}$ γ$_2$, γ$_3$ and γ$_{4),}$ which are transcribed from four different promoters and differ in their first exons. All of the transcripts yield the same protein, except for the *γ*2 transcript, which codes for 30 additional amino acids on the N terminus (Ackert-Bicknell and Rosen, 2006). The PPARγ$_2$ isoform is almost exclusively expressed in adipose tissue, while PPARγ$_1$ is widely expressed (Fajas et al., 1997).

Homozygous PPARγ-deficient mice had lethal embryos due to placental dysfunction (Kubota et al., 1999). Unexpectedly, the heterozygous PPARγ-deficient mice were partially protected from high-fat (HF) diet-induced obesity and INS resistance. Thus, it appears that the amount of PPARγ plays a critical role in adypocyte hypertrophy and the development of INS resistance under a HF diet (Kubota et al., 1999). A Pro12Ala substitution has been detected in the PPARγ$_2$ gene (Yen et al., 1997) and this amino acid is located in the PPARγ$_2$ domain that enhances ligand-independent activation (Werman et al., 1997). This amino acid is highly conserved. Codon 12 in the mouse PPARγ$_2$ gene is proline (Yen et al., 1997). The Pro12Ala substitution is associated with decreased receptor activity, lower body mass index (BMI) and improved INS sensitivity (Deeb et al., 1998). It is likely that two alleles of PPARγ interact with environmental factors such as a HF diet leading to an increase in the

incidence of type 2 diabetes mellitus (T2DM) (Heikkinen et al., 2009).

Many studies were undertaken in order to point out the association of PPARγ$_2$ Pro12Ala polymorphism with a susceptibility to T2DM. The majority of them showed the association of this gene polymorphism with T2DM development and also observed that the Pro12 allele is the risk allele for its onset (Mori et al., 2001; Douglas et al., 2001; Memisoglu et al., 2003; Ghoussaini et al., 2005). Studies from Germany (Ringel et al., 1999) and Italy (Mancini et al., 1999) have shown no association between Pro12Ala polymorphism and T2DM onset. The opposite was observed in four other studies (Sramkova et al., 2002; Malecki et al., 2003; Lindi et al., 2002; Evans et al., 2001) where results suggested a trend of increased 12Ala allele frequency in the T2DM patients compared to the control subjects, but with no statistical significance. Taking into consideration these controversial results about the association of PPARγ$_2$ Pro12Ala polymorphism with T2DM onset across the populations and the absence of data for our population, in this study we have analyzed, for the first time, the PPARγ$_2$ Pro12Ala polymorphism in both non-diabetic and diabetic patients in Serbia. Thus, the aim of this study was to investigate which of these two alleles, if either, represents a possible risk factor for development of T2DM.

MATERIALS AND METHODS

Study population

A local Ethical Review Committee approved the case-control study and each participant gave written informed consent to participate in it. The study population consisted of 360 Caucasian subjects from Belgrade, Serbia. The control group (n=197) consisted of healthy volunteers who were undergoing an annual medical check-up at the Occupational Medicine Center, INN Vinča. The T2DM patients (n=163) were recruited from the Endocrinology Clinic of the Military Medical Academy (MMA), Belgrade, and from the Institute of Endocrinology, Diabetes and Metabolism Diseases of the Clinical Center of Serbia (CCS), Belgrade. T2DM was diagnosed according to World Health Organization criteria (WHO, 1985).

Anthropometric and biochemical measurements

Anthropometric measurements included BMI (kg/m^2) and waist-to-hip ratio (WHR). The waist circumference was measured at the midpoint between the iliac crest and the lower rib margin, and the hip circumference was measured around the maximum circumference of the buttocks (posteriorly) and the symphysis pubis (anteriorly). Systolic and diastolic blood pressures were measured twice in the right arms of the subjects who had been resting for at least 10 min in a comfortable position. Fasting blood samples for the analyses of plasma glucose (GLU) and insulin (INS) concentration were obtained in the morning after 12-h fasting, and 120 min after breakfast. Plasma GLU concentration was analyzed using the GLU oxidase method by Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA) and the hexokinase method by Olympus AU2700 (Olympus Diagnostics GmbH, Hamburg, Germany). Plasma INS concentration was determined by RIA kit (INEP, Zemun, Belgrade, Serbia). INS resistance was estimated according to the homeostasis model assessment (HOMA-IR) method from fasting GLU and INS concentrations using the formula: INS (μU/ml) x GLU (mmol/L)/22.5 (Matthews et al., 1985). Glycosylated hemoglobin (HbA_{1c}) was measured following an overnight fast using Olympus AU2700 (Olympus Diagnostics GmbH, Hamburg, Germany).

Lipids Measurement

Lipid concentrations were determined in serum collected after overnight fasting. The total plasma cholesterol (TC) and triglyceride (TG) levels were determined on Monarch Plus apparatus (Instrumentation Laboratory, Lexington, KY) using enzymatic colorimetric methods. High-density lipoprotein cholesterol (HDLC) was determined after dextran sulfate-Mg21 precipitation of VLDL and low-density lipoprotein cholesterol (LDLC), using the CHOD-PAP method. LDL cholesterol was calcula-

ted according to the Friedewald formula for participants with TG levels < 4.5mmol/L (WHO, 1985).

Genetic analysis

Genomic DNA was isolated from whole blood samples collected with EDTA and purified by the proteinase K/phenol extraction method (Kunkel et al., 1977). The Pro12Ala polymorphism was detected by the PCR (polymerase chain reaction) method. The sequences of the primers were 5'-TCTGGGAGATTCTCCTATTGGC – 3' (forward primer) and 5' – CTGGAAGACAAACTACAAGAG – 3' (reverse primer) (Hara et al., 2000). The forward primer contained one nucleotide mismatch (underlined), which made it possible to use the restriction enzyme *Hin*6I for the detection of Pro12Ala polymorphism. The conditions for PCR were in a 25 μl reaction mixture containing 200 ng of genomic DNA, 0.15μM of the primers, 2 mM $MgCl_2$, 200 μM dNTP each and 1U of *Taq* polymerase. The reaction mixtures were incubated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s. The PCR products were digested with *Hin*6I at 37°C overnight. The Pro12 allele gave one 154 bp fragment whereas the 12Ala allele gave 133 bp and 21 bp fragments. The digestion products were loaded onto a 10% polyacrylamide gel for genotyping and run for 2 h in an electric field at 12 V/cm. The gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products, Inc., Upland).

Statistical analysis

All statistical analyses were carried out using Statistica Version 5 (1997) software package (StatSoft, Inc.). Differences with two-tailed alpha-probability (P) ≤ 0.05 were considered significant. The allelic frequencies and genotype distribution were estimated using the gene counting method. Differences in both allele and genotype frequency distribution between the studied groups were estimated by the chi-square (χ^2) test. Deviation from Hardy-Weinberg equilibrium was also assessed using the χ^2 test. For cross comparison (2x2) of parameters, which have one of two possible outcomes (the presence of T2DM according to sex, smoking and hypertension), the Fisher exact two-tailed test was used. Differences in continuous variables between the groups were tested with Student's *t*-test and one way ANOVA with adequate *post hoc* tests when the distribution of the variable or the logarithmically transformed variable approached a normal distribution. Otherwise, the Mann-Whitney U test and Kruskal-Wallis ANOVA tests were used. To estimate an association of the PPARγ$_2$ Pro12Ala polymorphism genotypes with the onset of T2DM, both univariate and multivariate logistic regression analyses were performed and the results were presented as both unadjusted and adjusted odds ratios (OR) and their confidence intervals (95% CI). The crude effect of the Pro12Ala polymorphism was adjusted for the variables that were statistically significant for the onset of T2DM in the univariate logistic regression analysis.

RESULTS

Description of the population

The clinical parameters for both control and of T2DM patient groups are shown in Table 1. Differences between the groups were significant for gender, age, BMI, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and hypertensive status. In general, the patients with T2DM were significantly older and had significantly higher BMI values, SBP and DBP levels, TC, LDLC and TG plasma levels and lower HDL levels compared to the control subjects.

Effect of PPARγ Pro12Ala gene polymorphism on susceptibility to T2DM

The genotype frequency distribution for PPARγ$_2$ Pro12Ala gene polymorphism was in Hardy-Wein-

Table 1. The clinical and histopathological characteristics of patients

Characteristics	Control subjects	Type 2 diabetic subjects	p
			t-test
M/F n (%)	95(48.22)/ 102(51.78)	127(77.91)/ 36(22.09)	<0.01*
Age (years)	34.9±12.2	55.1±9.3	<0.01
BMI (kg/m^2)	24.3±3.7	28.3±4.5	<0.01
SBP (mmHg)	117.7±10.2	139.9±18.9	<0.01#
DBP (mmHg)	76.2±6.4	86.2±11.9	<0.01#
TC (mmol/L)	5.6±1.2	6.3±1.3	<0.01
HDLC (mmol/L)	1.40±0.36	1.05±0.28	<0.01
LDLC (mmol/L)	3.56±1.04	4.11±.25	<0.01
TG (mmol/L)	1.29±0.76	2.84±2.71	<0.01
Hypertensive - n (%)	2 (1.02)	66 (44.30)	<0.01*
Smokers - n (%)	100 (50.76)	65 (49.24)	NS*

BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC – total cholesterol; HDLC high-density lipoprotein cholesterol; LDLC – low-density lipoprotein cholesterol; TG – triglycerides. Values are mean ± SD for age, BMI, SBP, DBP, TC, HDLC, LDLC and TG; * -– Fisher exact two-tailed test (χ2 - test); # –-Mann-Whitney U test ; NS – non-significant.

berg equilibrium for both control and T2DM patients. The 12Ala allele frequency was slightly higher in the T2DM patients (0.11) compared to the control (0.09), without any statistical significance (Table 2). The distribution of the genotype frequencies was not significantly different between the studied groups (Table 2).

The 12Ala allele T2DM female carriers had significantly ($p<0.05$) lower fasting INS concentration than T2DM women with Pro12Pro genotype (Table 3.).

The carriers of 12Ala allele had non-significant unadjusted OR (1.10) for the onset of T2DM. After an adjustment of the factors that have been significantly associated with T2DM in univariate analysis (age, BMI, TC, LDLC, HDLC, TG and hypertensive status), Ala12 allele carriers show increased OR (1.81), CI (0.83-3.95) for DMT2 onset, however it was not statistically significant (Table 4 and Fig. 1).

DISCUSSION

This study examined PPARγ_2 Pro12Ala gene polymorphism as a risk factor for the onset of T2DM in the Serbian population. No association between Pro12Ala polymorphism and T2DM was found in the population sample. A non-significant increase in the risk for T2DM onset was found for Ala12 allele carriers. A significantly lower fasting INS concentration associated with 12Ala allele carriers was observed only in the group of T2DM women.

Table 2. Genotype distribution and allele frequencies for PPARγ2 Pro12Ala gene polymorphism in the groups of control and T2DM patients

	Control subjects		Type 2 diabetic patients		p
Genotype	n	%	n	%	χ^2
ProPro	160	81.22	130	79.75	
ProAla + AlaAla	37	18.78	33	20.25	NS[a]
Pro allele/Ala allele	0.91/0.09		0.89/0.11		NS

[a] - Fisher exact two-tailed test

Table 3. Anthropometric and biochemical measurements of T2DM patients stratified by gender according to the Pro12Ala polymorphism of the PPARγ2 gene.

Characteristic	T2DM women (n=36)			T2DM men (n=127)		
	Pro/Pro	Ala-carriers	p	Pro/Pro	Ala-carriers	p
Age (years)	59.3±9.4	56.67±7.02	NS	53.4±8.5	56.7±8.0	NS
BMI (kg/m^2)	26.8±4.0	29.9±8.3	NS	28.4±4.1	28.2±5.4	NS
TC (mmol/L)	6.5±1.9	6.0±0.93	NS	6.2±1.3	6.2±1.3	NS
HDLC (mmol/L)	1.24±0.32	1.25±0.03	NS	1.01±0.26	1.05±0.25	NS
LDLC (mmol/L)	4.19±1.65	3.99±0.72	NS	4.07±1.29	4.11±0.95	NS
TG (mmol/L)	2.30±1.43	1.72±0.61	NS	2.61±2.13	2.42±1.40	NS
Fasting glucose(mmol/L)	7.8±2.0	10.6±2.9	NS	8.4±2.8	8.5±2.2	NS
2h glucose(mmol/L)	11.3±4.7	15.1±1.3	NS	11.4±4.0	10.7±3.3	NS
Fasting insulin(mU/L)	20.2±10.8	9.5±4.5	<0.05	24.5±14.3	31.1±15.9	NS
2h insulin(mU/L)	86.4±88.0	23.8±12.4	NS	68.8±51.9	72.7±50.1	NS
WHR	0.87±0.07	0.86±0.05	NS	1.03±0.07	1.04±0.05	NS
HOMA-IR	7.40±6.16	4.24±1.99	NS	8.89±4.92	11.78±7.65	NS
HbA_{1C} (%)	6.70±1.20	8.47±0.51	NS	7.48±1.53	7.12±1.26	NS
SBP (mmHg)	138.5±14.5	136.2±14.1	NS	137.7±17.1	136.6±14.7	NS
DBP (mmHg)	81.0±7.4	77.7±2.5	NS	86.1±10.6	88.9±4.5	NS

BMI – body mass index; TC – total cholesterol; HDLC – high-density lipoprotein cholesterol; LDLC – low-density lipoprotein cholesterol; TG – triglycerides; WHR – waist-to-hip ratio; HOMA-IR – Homeostasis Model assessment – insulin resistance; HbA1C – glycosylated haemoglobin; SBP – systolic blood pressure; DBP – diastolic blood pressure. Values are mean ± SD for all measured parameters. NS – non-significant.

Table 4. PPARγ$_2$ Pro12Ala gene polymorphism relative odds ratios (ORs) for onset T2DM unadjusted and adjusted for differences in significant clinical risk factors #

Genotype classes	Unadjusted OR (95% CI)	p	Adjusted OR(95% CI)	p
ProPro vs. ProAla + AlaAla	1.10 (0.65-1.86)	0.73	1.81 (0.83-3.95)	0.14

see results

Results involving the role of PPARγ$_2$ Pro12Ala gene polymorphism in T2DM onset vary across populations as well as the frequency of 12Ala allele in the T2DM patients compared to controls, suggest genetic heterogeneity (Ludovico et al., 2007). Our finding of 12Ala allele frequency in the controls is in agreement with the previous study that found a north-south frequency decrease gradient in Europe (Ludovico et al., 2007). Considering the risk of T2DM onset and 12Ala allele frequency, our finding is in accordance with previous results (Ringel et al., 1999; Mancini et al., 1999; Ludovico et al., 2007). A reduced risk of T2DM in the Ala12 carriers is greater in Asia than in Europe. Among Europeans, it is higher in northern Europe, barely significant in central Europe, and nonexistent in southern Europe (Ludovico et al., 2007). The same nonexistence of reduced risk for T2DM in Ala12 carriers is present in our population, as one of the southern European populations. The trend for increased risk for T2DM in PPARγ$_2$ 12Ala allele carriers compared with non-carriers was observed in our population. This is similar to results from other (Czech, Polish, Finnish and German) diabetes studies (Sramkova et al., 2002, Malecki et al., 2003; Lindi et al., 2002; Evans et al., 2001).

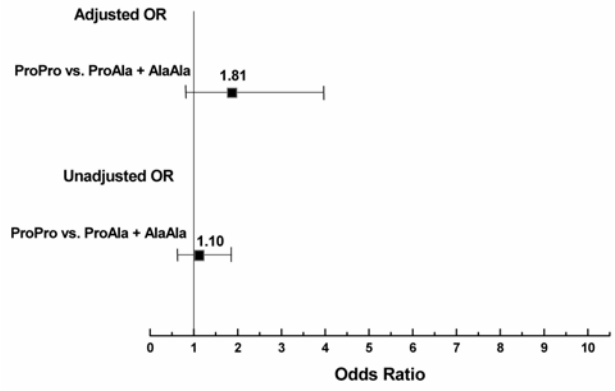

Figure 1.

However, the present data show that T2DM female carriers of 12Ala allele had significantly lower fasting INS than those with a Pro12Pro genotype. This is in agreement with the results of impaired INS secretion in 12Ala T2DM individuals in two other populations (Mori et al., 2001; Sramkova et al., 2002). In addition, the Pro12Ala polymorphism in the PPARγ$_2$ gene might be involved in a differential regulation of INS secretion in response to increased free fatty acids (FFAs) in healthy humans (Stefan et al., 2001). The 12Ala allele carriers have a reduced capacity for INS secretion (Stefan et al., 2001).

The PPAR-gamma is expressed in human beta-cells (Dubois et al., 2000). Therefore, Pro12Ala gene polymorhism could interfere with pancreatic function and have an impact on INS secretion and sensitivity and GLU homeostasis. 12Ala allele might be a risk factor for INS deficiency, and therefore contribute to the progression of T2DM. Heikkinen et al., (2009) reported differences in gene expression changes associated to the Pro12Ala variant between the two diets. This supports the hypothesis that the Pro12Ala phenotype depends on nutritional status and suggests PPARγ$_2$ as an environmental sensor (Heikkinen et al., 2009). Hence, PPARγ$_2$ may provide a better, probably Pro12Ala genotype-dependent treatment strategy for INS resistance in T2DM.

In conclusion, the non-existence of reduced risk of T2DM in the Ala12 carriers in the Serbian population is similar to that of populations with the same geographic origin. The results of our case-control study indicate a trend towards 12Ala allele as a

risk factor for T2DM development. Considering the number of the participants in this study, as well as the existence of the European north-south gradient, there is a need to confirm the present findings in larger replication studies in different populations, particularly those from surrounding south-eastern European countries. Further results are needed for establishing PPARγ_2 Pro12Ala gene polymorphism as a risk factor for the onset of complex, chronic and polygenic disease such as T2DM.

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ПОЛИМОРФИЗАМ PRO12ALA У ГЕНУ ЗА РЕЦЕПТОР КОЈИ СЕ АКТИВИРА ПРОЛИФЕРАТОРОМ ПЕРОКСИЗОМА ГАМА КАО ФАКТОР РИЗИКА ЗА НАСТАНАК ДИЈАБЕТЕСА ТИПА 2 У СРПСКОЈ ПОПУЛАЦИЈИ

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Рецептор активиран пролифератором пероксизома гама (PPARγ) је ген кандидат за настанак дијабетеса типа 2 (DMT2). Испитивали смо по први пут у српској популацији асоцијацију полиморфизма Pro12Ala у гену за PPARγ са настанком DMT2. Студију је чинила популација од 197 контрола и 163 пацијента са DMT2. Алел 12Ala је био чешћи у групи пацијената са DMT2 (0.11) у односу на контролну групу (0.09). Резултати ове студије указују да алел 12Ala $PPAR\gamma_2$ не представља значајан фактор ризика за настанак DMT2 у српској популацији.