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Association of Peroxisome Proliferator-Activated Receptor-Gamma2 Pro12Ala Gene Variants with Insulinoresistance in Coronary Heart Disease Patients

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Abstract

Peroxisome proliferator-activated receptor-gamma2 (PPAR-γ2), a member of nuclear receptor super family, has a key role in the regulation of adipocyte differentiation, lipid and carbohydrates metabolism.

The aim of the present study was to determine whether the Pro12Ala polymorphism of PPARγ2 gene affects the acute forms of coronary heart disease (CHD) in Russian population. The second aim of this study was to identify the association of Pro12Ala polymorphism of PPARγ2 gene with insulin resistance.

In the current study 278 patients aged 36 to 78 years (232 male and 46 female) with verified diagnosis of coronary heart disease were enrolled. The comparison group consisted of 220 subjects without CHD of comparable age (mean age - 60, 09 ± 0, 72 years). The identification of polymorphic variants of Pro12Ala polymorphism of PPAR-γ2 gene based on the method of polymerase chain reaction (PCR) followed by restriction analysis. Serum immunoreactive insulin level was revealed by ELISA (DRG Diagnostics, Germany), HOMA index of insulin resistance was calculated.

The frequency of 12Ala allele in CHD patients was higher than in the control group (0.15 and 0.08 respectively, p=0.0003). 12Ala allele carriage was associated with increased risk of CHD (OR=2, 00, p=0, 0006). No significant differences in Pro12Pro and Pro12Ala + Ala12Ala genotype distribution in CHD patients with or without obesity, smoking factor, family history were revealed. Pro12Ala and Ala12Ala genotypes detected significantly more often in patients with myocardial infarction at a young age (45 years and younger). Carriage of Ala12 allele was associated with an increased risk of myocardial infarction at a young age to 2.49 times (p<0, 05).

In CHD patients with diabetes Ala allele detected significantly more rare, than in CHD patients with diabetes – non Ala allele carriers (in 17% and 30% of cases, p=0, 01). 12Ala allele carriage was associated with diminished risk of diabetes type 2. Serum immunoreactive insulin level was lower in the group of CHD patients – 12Ala allele carriers, than in patients with Pro12Pro genotype (11, 06±1, 12 μU/ml and 21, 02±1, 97 μU/ml respectively, p=0,008). The same tendency was revealed in HOMA index assessment.

Conclusion: The study has demonstrated that Ala12 PPAR γ allelic variant influence the predisposition for CHD in human subjects and was associated with the risk of myocardial infarction at the age 45 years and younger in the population of North-West region of Russian Federation. 12Ala allele carriage is associated with reduced risk of type 2 diabetes in CHD patients and with lower content of immunoreactive insulin and HOMA index.

Introduction

Peroxisome proliferator-activated receptor-gamma2 (PPAR-γ2), a member of nuclear receptor superfamily, play a key role in the regulation of adipocyte differentiation, lipid metabolism and glucose homeostasis as transcriptional regulators of a number of genes included in these pathways [1]. Activation of PPAR-γ2 is necessary to maintain the body's sensitivity to insulin [2].

PPAR-γ2 control on transcriptional level different genes, associated with insulin resistance and lipid metabolism: GLUT4, glucokinase, acyl-CoA synthase, lipoprotein lipase, fatty acid-binding protein, genes, involved in the transport of free fatty acids and some other genes [3].

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PPAR-γ2 performs a crucial role in adipogenesis, adipocyte differentiation from preadipocytes. High levels of circulating free fatty acids in blood plasma activate of PPAR-γ2, resulting in a growing mass of adipose tissue and insulin resistance. Besides the adipogenesis, PPAR-2 regulate lipid metabolism and secretion of various adipokines by adipocytes (adiponectin, resistin, leptin, adipsin) [4,5]. PPAR-γ2, activated by binding with the appropriate ligands, increase synthesis of glucose transporters 1st and 4th types, so tissues become more sensitive to insulin and overcome insulin resistance [2,6].

PPAR-γ2 contributes to the suppression of immune inflammation in the vascular wall [3]. Activated PPAR-γ2 potentiate expression of C-terminal fragment of α_1-antitrypsin on the surface of monocytes and macrophages. The next step is inhibition of monocyte production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-18 (IL-18) and matrix metalloproteinase-9 (MMP-9, especially in areas of atherosclerotic lesions [3,5].

PPAR-γ2 gene is located on chromosome 3r25. Its most common Pro12Ala polymorphism is a substitution of cytosine to guanine in exon B (codon 12), which in turn leads to the substitution of proline by alanine in the encoded protein [7].

Polymorphism Pro12Ala was first identified in 1997 [8] and is associated with decreased gene transcriptional activity [7], which reduces insulin release during eating. This process helps to overcome insulin resistance and reduce the risk of developing Type 2 diabetes by 21% [5].

The aim of the present study was to determine whether the Pro12Ala PPAR-γ2 gene polymorphism affects the acute forms of coronary heart disease in Russia. The second aim of this study was to identify the association of Pro12Ala polymorphism of PPAR-γ2 gene with insulin resistance.

Materials and Methods

Patients were prospectively enrolled into a CHD registry of Coronary Care Unit of BFSEI HE "Academician I. P. Pavlov First St. Petersburg State Medical University". All patients underwent diagnostic coronary angiography between October 2012 and April 2014. In the current study 278 patients aged 36 to 78 years (232 male and 46 female) with verified diagnosis of coronary heart disease were enrolled. All the women were postmenopausal for over 10 years. Inclusion criteria: patients, enrolled into a CHD registry with written inform consent. Exclusion criteria: anamnesis of cancer, active and clinically significant systemic or inflammatory disease, liver and kidney disease, infective endocarditis, uncontrolled hypertension, diabetes mellitus with clinically significant complications, thyroid disorders.

The control group consisted of 220 people without coronary heart disease of comparable age (mean age - 61, 09 ± 0, 72 years). Consent for inclusion in the study was obtained from the subjects. The research protocol was approved by the Ethics Committee of FSBEI HE "Academician I.P. Pavlov First St. Petersburg State Medical University".

CHD group characterized by a mean age of 62,3 ± 0,51 years, the average age of developing coronary heart disease - 56,1 ± 0,49 years. The history of myocardial infarction was in 192 (69%) of all patients. Myocardial infarction at the age of 45 years or less was diagnosed in 34 patients.

The diagnosis of myocardial infarction was verified according to the Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction guideline.

Arterial hypertension was present in 262 (94%) of CHD patients. Obesity and overweight were observed in 86 (31%) of patients. A history of smoking at the time of the development of coronary heart disease was observed in 167 patients (60%). Family history of coronary heart disease was revealed in 97 examinees (35%). Type 2 diabetes was observed in 71 (25.5%) patients, while fasting hyperglycemia was detected in 109 (39.2%) examinees. All patients with type 2 diabetes were on a diet or sulfonylurea or biguanides.

Diabetes mellitus type 2 was diagnosed in accordance with the recommendations of the International Diabetes Federation (IDF 2012, 2013, 2014), the American Diabetes Association (ADA, 2012, 2015), the American Association of Clinical Endocrinologists (AACE, 2015) of the Russian Association of Endocrinologists (RAE 2015) with an increase in the level of glucose in the morning on an empty stomach, before and more than 7.0 mmol/ L and glycosylated hemoglobin up to or more than 6.5%.

Anthropometric evaluation included measurement of height, weight, waist and hip circumferences. Calculated body mass index (BMI) = weight / height^2 (kg / m^2) (A. Quetelet formula). Normal body weight consistent with BMI values from 18.5 to 24.9 kg / m^2, overweight - BMI of 25 to 29.9 kg / m^2, and obesity - a BMI over 30 kg /m^2.

Molecular-genetic examination

Molecular-genetic examination of patients with CHD and a control group of comparable age without CHD was performed. Fasting peripheral venous blood was obtained and centrifuged at 4°C and 3360 g for 15 min. All of the samples were then stored at –70°C until analysis. Deoxyribonucleic acid (DNA) extraction from venous blood leukocytes was carried out on the column "K-SORB-100" ("Syntol", Russian Federation).

The identification of polymorphic variants of Pro12Ala PPAR-2 gene polymorphism based on the method of polymerase chain reaction (PCR) followed by restriction analysis, as described previously in the literature [9]. PPAR-γGene ID: 5468.The Pro12Ala PPAR- γ 2 variant (rs1801282) was detected by polymerase chain reaction, as described by Mori and co-workers [9]. The 154-bp PCR products of the human PPAR-γ gene were digested with HhaI, subjected to electrophoresis on a 10% polyacrylamide gel, and visualized by staining with ethidium bromide. Amplification of the fragment of the gene was performed on an automated thermocycler Tertsik (DNA Technology, Moscow) using the following oligonucleotide sequences (Beagle, Russian Federation):

- forward primer: 5'TCTGGGAGATTCTCCTATTGGC–3;
- reverse primer: 5'CTGGAAGACAAACTACAAGAG–3.

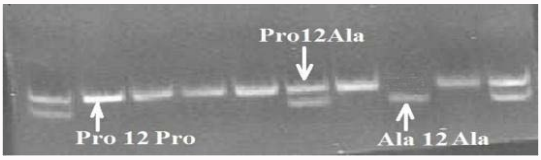

Figure 1: The image of PCR product: in the case of12Ala allele the PCR product was cleaved to fragments of 133 bp and 24 bp. In the case of 12Pro allele the PCR product remained undigested of 154 bp in length.

Table 1: Pro12Pro, Pro12Ala, Ala12Ala genotype distribution, and frequency of 12Pro alleles and 12Ala alleles of PPAR-γ2 genssse in patients with coronary heart disease and in control group of individuals without coronary heart disease.

Groups	PPAR-γ2 genotype					Allele frequency	
	Pro12 Pro	Pro12 Ala	Ala12 Ala	Pro12Pro	Pro12Ala + Ala12Ala	12Pro	12Ala
CHD patients (n=278)	205 (74%)	64 (23%)	9 (3%)	205 (74%)	73 (26%)	0,85	0,15
Control group without CHD (n=220)	187 (85%)	31 (14%)	2 (1%)	187 (85%)	33 (15%)	0,92	0,08
p	0,004			0,0008		0,0003	
OR	—			2,02, CI:1,28÷3,19, p=0,001		2,00, CI:1,32÷3,04, p=0,0006	

Note – P−confidence probability when checking the homogeneity of the distribution of genotypes and alleles when compared of group of CHD patients and control group without CHD

Table 2: Traditional risk factors in coronary heart disease patients - Pro12Pro and Pro12Ala + Ala12Ala PPAR-γ2 genotype carriers.

Traditional CHD risk factor	PPAR-γ2 genotypes		P
	Pro12Pro	Pro12Ala + Ala12Ala	
Arterial hypertension	197 (75%)	65 (25%)	0,06
Without arterial hypertension	9 (56%)	7 (54%)	
Family history of coronary heart disease	71 (73%)	26 (27%)	0,15
No family history of coronary heart disease	132 (73%)	49 (27%)	
Smokers	121 (72%)	46 (28%)	0,12
Non smokers	79 (71%)	32 (29%)	
Male	172 (74%)	60 (26%)	0,07
Female	31 (67%)	15 (33%)	
Obesity	64 (74%)	22 (26%)	0,13
Without obesity	143 (74%)	49 (26%)	
Diabetes type 2	59 (83%)	12 (17%)	0,01
Without diabetes type 2	158 (70%)	68 (30%)	

Note – P - confidence probability when checking the homogeneity of the distribution of Pro12Pro and Pro12Ala + Ala12Ala genotypes of PPAR-γ2 gene when compared CHD patients with or without traditional risk factors.

The next step was carried out restriction analysis. The reaction mix for restriction analysis included 10 mkl of the PCR product, 1mkl of restriction enzyme Hha I (Thermo Scientific). Incubation of mix produces at 37°C for 8 hours.

Restriction analysis was performed using a vertical electrophoresis in 8% polyacrylamide gel followed by staining with ethidium bromide and visualization in the ultraviolet. In the case of12Ala allele the PCR product was cleaved to fragments of 133 bp and 24 bp. In the case of 12Pro allele the PCR product remained undigested of 154 bp in length (Figure 1).

Serum immunoreactive insulin level was revealed by ELISA. We used "DRG INSULIN ELISA KIT" (DRG Diagnostics, Germany). Incubation was performed in serum samples from micro well plate coated with specific antiserum to insulin. After fixation, the wells were detected by the AI binding a conjugate of anti-insulin and addition of the enzyme complex comprising horseradish peroxidase. Adding stop solution, which is a 0.5 M sulfuric acid increased the sensitivity. AI levels were determined by measuring the absorbance of the solution at 450 nm using a microplate reader.

HOMA index of insulin resistance was calculated by the formula:

HOMA-IR = (fasting glucose (mmol/L) x immunoreactive insulin level (μU / ml) / 22.5.

Statistical analysis was performed using the statistical software package Statistica 10 (Stat Soft Inc., version 10.0.228.8, Oklahoma, USA). Analysis of the conformity of the type distribution characteristic normal distribution was carried out using the Shapiro-Wilk test. To evaluate the quantitative parameters of the normal distribution calculated following parameters: arithmetic mean (M), an error of the arithmetic mean (m), standard deviation (SD). In the absence of signs of a normal distribution statistical analysis was performed using non-parametric mathematical criteria. Analysis of qualitative binary signs held by means of Fisher's exact test. An analysis of the relationship of the two signs was performed using Spearman correlation analysis. Significant differences were considered when the probability of the null hypothesis (P) does not exceed a value of 0.05. The sample size calculation was based on formula: $n = f(\alpha/2, \beta) \times [p1 \times (100 - p1) + p2 \times (100 - p2)] / (p2 - p1)^2$.

Results

There was significant difference in the distribution of genotypes and alleles of Pro12Ala PPAR-2 polymorphism when compared of group of CHD patients and control group without CHD (Table 1). Patients were divided into two groups: patients bearing at least one 12Ala allele (Pro12Ala + Ala12Ala genotypes), and patients with no 12Ala allele (Pro12Pro genotype).

The frequency of Pro12Ala + Ala12Ala genotypes of PPAR-γ2 gene in coronary heart disease patients was higher than in control group without CHD (26% and 15% respectively, p=0.0008, Table 1). The frequency of 12Ala allele in patients with coronary heart disease was higher than in the control group (0.15 and 0.08 respectively, p=0.0003). 12Ala allele carriage was associated with risk of CHD (OR=2, 00, CI: 1, 32÷3, 04, p=0, 0006).

Table 3: Pro12Pro, Pro12Ala, Ala12Ala genotype distribution, and frequency of 12Pro alleles and 12Ala alleles of PPAR-γ2 gene in patients with myocardial infarction incidence at the age 45 years and younger and in individuals without coronary heart disease of the same age.

Groups	PPAR-γ2 genotype					Allele frequency	
	Pro12 Pro	Pro12 Ala	Ala12 Ala	Pro12 Pro	Pro12Ala + Ala12Ala	12Pro	12Ala
CHD patients with MI incidence at the age 45 years and younger (n=34)	20 (59%)	12 (35%)	2 (6%)	20 (59%)	14 (41%)	0,76	0,24
Comparison group without CHD (n=91)	73 (80%)	16 (18%)	2 (2%)	73 (80%)	18 (20%)	0,89	0,11
p	0,038			0,011		0,008	
OR				2,84; CI:1,21÷6,68		2,49; CI:1,2÷5,16	
Note – P - confidence probability when checking the homogeneity of the distribution of genotypes and alleles when compared of group of CHD patients and control group without CHD							

Table 4: Serum immunoreactive insulin level and HOMA-IR in coronary heart disease patients - carriers of Pro12Pro and Pro12Ala+Ala12Ala genotypes of the PPAR-γ2 gene.

Groups		PPAR-γ2 genotype		P
		Pro12Pro	Pro12Ala +Ala12Ala	
CHD patients (n=110)	Immunoreactive insulin, μU/ml	21,02±1,97	11,06±1,12	0,008
Subjects without CHD (n=60)	Immunoreactive insulin, μU/ml	18,12±1,41	14,64±2,98	0,391
CHD patients (n=110)	Fasting glucose, mmol/l	6.32	6,04	0,256
Subjects without CHD (n=60)	Fasting glucose, mmol/l	6,24	6,05	0,324
CHD patients (n=110)	HOMA-IR	5,94±0,55	2,82±0,36	0,007
Subjects without CHD (n=60)	HOMA-IR	5,03±0,62	3,94±0,41	0,214
Note – P – confidence probabilitywhen checking the homogeneity of the distribution of serum immunoreactive insulin level and HOMA index in patients with coronary aheart disease and subjects without CHD - carriers of different genotypes of the PPAR-γ2 gene: dispersionanalysis (ANOVApost-hoc, Sheffetest).				

No significant differences in Pro12Pro and Pro12Ala + Ala12Ala genotype distribution in CHD patients with or without obesity, smoking factor, family history were revealed (Table 2). There was no significant differences in total cholesterol level in CHD patients – Pro12Pro and Pro12Ala + Ala12Ala genotype carriers: 4, 92±0, 07 mmol/l 4, 95±0, 23, p=0, 12 respectively).

Pro12Ala and Ala12Ala genotypes detected significantly more often in patients with myocardial infarction at a young age (45 years and younger) compared to patients of control group without CHD aged 45 years and younger (41% and 20% respectively, p = 0,011, Table 3). Carriage of 12Ala allele was associated with an increased risk of myocardial infarction at a young age to 2.49 times (OR = 2, 49; CI: 1, 2 ÷ 5, 16, Table 3).

Pro12Pro genotype was detected in 59 of 71 (83%) patients with coronary heart disease combined with type 2 diabetes and in 158 of 226 (70%) patients with coronary heart disease without combination with diabetes, the difference of this genotype distribution was significant (p=0,01). In CHD patients with diabetes 12Ala allele detected significantly more rare, than in CHD patients with diabetes – non 12Ala allele carriers (in 17% and 30% of cases, p=0, 01). 12Ala allele carriage was associated with diminished risk of diabetes type 2 (OR=0, 47; CI: 0, 23 ÷ 0, 93).

Insulin resistance, hyperinsulinemia and elevated values of HOMA index are risk factors for coronary heart disease and its complications in patients with type 2 diabetes and those without diabetes [10].

Serum immunoreactive insulin level was investigated in 110 patients with coronary heart disease and 60 people from the comparison group without clinical and angiographic evidence of CHD.

Average serum immunoreactive insulin level in the group of CHD patients was 19.38 ± 1.68 m U / L, while in the control group of subjects without CHD - 18.38 ± 1.38 m U / L (p = 0.68). Thus, the level of serum immunoreactive insulin was not significantly different in patients with coronary artery disease and in the control group. There was no difference in serum immunoreactive insulin level in the groups CHD patients with and without concomitant diabetes mellitus type 2 (24,02±2,98 μU/ml and 18,07±1,98 μU/ml, p=0,142).

Serum immunoreactive insulin level was assessed in the groups of CHD patients – Pro12Pro and Pro12Ala + Ala12Ala genotype carriers. The results are presented in (Table 4). Serum immunoreactive insulin level was significantly lower in the group of CHD patients – Ala12 allele carriers, than in patients with Pro12Pro genotype (11, 06±1, 12 μU/ml and 21, 02±1, 97 μU/ml respectively, p=0,008). The same tendency was revealed in HOMA index assessment – (Table 4).

There was no difference in serum immunoreactive insulin level and HOMA-IR in the groups of subjects without CHD - Pro12Pro and Pro12Ala + Ala12Ala genotype carriers.

Discussion

The present work revealed that 12Ala allele carriage is associated with risk of coronary heart disease. Meta-analysis of Z. Wu and co-workers (2012) established the increased CHD risk in patients-Ala12Ala genotype carriers [7]. The results of this meta-analysis demonstrated that the *PPAR-γ2* Pro12Ala polymorphism might be risk-conferring locus for the progression of CHD among Caucasians, but not among Asians. H. Aydoğan and co-workers noted the importance of Pro12Ala PPAR-γ2 gene polymorphism as one of the risk factors for CHD [11].

12Ala allele encodes a gene with reduced transcriptional activity [12]. The 12Ala allele leads to a diminished stimulation of PPAR-γ target genes, subsequent lowered level of C-terminal fragment of α1-antitrypsin on the surface of monocytes and macrophages. The

next step is diminished signal pathway of proinflammatory cytokines supression, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-18 (IL-18), C-reactive protein and matrix metalloproteinase-9 [3,13]. The activity of immune inflammation is one of the leading mechanisms of coronary arteriosclerosis progression.

This study established that the carriage of 12Ala allele is associated with an increased risk of myocardial infarction at a young age. There is evidence that carriers of the 12Ala allele have increased leptin levels in blood plasma. This, in turn, helps to increase platelet aggregation, oxidation of free fatty acids, cholesterol accumulation in macrophages involved in arteriosclerosis [13]. The above mentioned mechanisms contribute in the pathogenesis of acute coronary syndrome.

Carriage of Pro12Pro genotype was associated with an increased risk of type 2 diabetes in patients with coronary heart disease. This corresponds to the data described D. Sanghera and co-work esl in 2010, and S. Akoum in 2014 [13,14].

Missence12Alamutation leads to a diminished stimulation of PPAR-γ target genes, subsequent lowered levels of adipose tissue accumulation, which, in turn, may improve insulin sensitivity. Reduced risk of diabetes in CHD patients - 12Ala allele carriers is due to the fact that it encodes a gene with reduced transcriptional activity [7,12]. Correspondingly, the secretion of insulin during meals is low. This helps to overcome insulin resistance and reduce the risk of type 2 diabetes [13]. The present study revealed that CHD patients - carriers of 12Alaallele had lower content of immunoreactive insulin and HOMA index. It has been shown that type 2 diabetes in carriers of 12Ala PPAR-γ2 allele observed much less frequently, but was associated with more severe co-morbidities with the cardiovascular system [13-15].

Conclusion

The study has demonstrated that 12Ala PPAR-γ allelic variant influence the predisposition for CHD in human subjects and was associated with the risk of myocardial infarction at the age 45 years and younger in the population of North-West region of Russian Federation.

12Ala allele carriage is associated with reduced risk of type 2 diabetes in CHD patients and with lower content of immunoreactive insulin and HOMA index.

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