CLINICAL STUDY

The prevalence of 4G5G polymorphism of plasminogen activator inhibitor-1 (PAI-1) gene in polycystic ovarian syndrome and its association with plasma PAI-1 levels

Evanthia Diamanti-Kandarakis, Gaetano Palioniko, Krystallenia Alexandraki, Angeliki Bergiele, Thenia Koutsouba and Michael Bartzis

Endocrine Section of First Department of Internal Medicine, Athens University School of Medicine, Laiko General Hospital, 17 Aghiou Thoma str, Athens 115 27, Greece

(Correspondence should be addressed to E Diamanti-Kandarakis, Athens University School of Medicine, Laiko General Hospital, 1A Zefyrou str, Athens 145 78, Greece; Email: akandara@otenet.gr)

Abstract

Objective: To investigate the prevalence of 4G5G polymorphism of plasminogen activator inhibitor-1 (PAI-1) gene in polycystic ovary syndrome (PCOS) and its functional significance.

Design: Case–control study.

Methods: We studied 98 patients and 64 controls. Body mass index (BMI) and waist-to-hip (WHR) ratio were determined. Blood samples were obtained for DNA analysis. PAI-1 plasma levels, serum total testosterone, fasting insulin and fasting glucose were measured and the glucose-to-insulin ratio was estimated in all subjects.

Results: There was a statistically significant difference in the distribution of PAI-1 gene variations among the groups. The PCOS group had significantly higher 4G/4G and 4G/5G combinations than the control group, whereas there were significantly less 5G/5G. Among the PCOS women, 39.8% had the genotype 4G/4G, 39.8% 4G/5G and 20.4% 5G/5G. From the control group, 20.3% had genotype 4G/4G, 28.1% 4G/5G and 51.6% 5G/5G. In the 4G/4G genotype subgroup 75% were PCOS and 25% were controls, in the 4G/5G were 68.42% and 31.58% and in the 5G/5G were 31.58% and 62.26% respectively. The population of PCOS women had significantly higher PAI-1 levels, WHR, total testosterone, and fasting glucose than the population of controls.

Conclusions: 1)The genotypic subtypes 4G/4G and 4G/5G, in PCOS, were present with a statistically higher frequency compared with controls. 2)PCOS women have higher levels of PAI-1 compared with the control group. 3)The presence of the 4G allele in PAI-1 promoter region of the gene further increases the PAI-1 levels.

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Introduction

Polycystic ovary syndrome (PCOS) is considered to be the most common endocrinopathy among women of reproductive age (4–6%) (1, 2) and, according to the 1990 National Institute of Child Health and Human Development conference, is defined by hyperandrogenism and chronic anovulation (3).

In addition, important metabolic aberrations like insulin resistance, glucose intolerance, dyslipidemia, coagulopathy and elevated cardiovascular risk factors have been associated with PCOS (4–6).

Among the cardiovascular risk factors, the presence of increased activity of plasminogen activator inhibitor-1 (PAI-1) has been well documented (7–9). Recently, the polymorphism 4G5G in the promoter region of PAI-1 gene has been associated with increased plasma PAI-1 levels in patients with diabetes type II and

myocardial infarction (7). The levels of PAI-1 in homozygous 4G/4G persons are approximately 25% higher than they are in homozygous 5G/5G subjects (7, 10–13).

The present study investigates the 4G5G polymorphism of the PAI-1 gene in Greek women with PCOS and its association with the circulating levels of PAI-1, compared with weight- and age-matched controls.

Materials and methods

Subjects and protocol

A total of 162 Greek Caucasian women were studied: 98 PCOS patients and 64 women with normal menstrual cycles (<35 days) and normal androgen levels.

Each patient with PCOS met the diagnostic criteria for PCOS presented at the 1990 National Institute of Health Conference on PCOS and at the 1995 Sero Symposium on PCOS. These criteria include hyperandrogenism and chronic anovulation, with the exclusion of secondary causes such as non-classical adrenal 21-hydroxylase deficiency, hyperprolactinemia and androgen-secreting neoplasms. The hyperandrogenism was assessed as total testosterone levels above the 95th percentile of the levels detected in a group of normal cycling women with normal cycles, and clinically, as hirsutism and acne. Chronic anovulation was assessed as oligomenorrhea, i.e. less than 8 cycles per year.

The enrolled population was clinically healthy and not suffering from chronic or acute disease. Oral contraceptives or other drugs that could interfere with the hormonal and metabolic studies were discontinued for at least three months before the study. To gain entry to the study, body weight had to remain stable for at least two months before and subjects participating in dietary or exercise program or weight reduction, were excluded.

We calculated body mass index (BMI, kg/m²) to assess obesity and the waist-to-hip ratio (WHR) to assess body fat distribution.

All women submitted were during the follicular phase, either in automatic menstrual cycle or post-progesterone withdrawal bleeding. They were subjected to a single venipuncture and peripheral blood was immediately processed for DNA extraction and PAI-1 plasma levels (ng/ml). Serum total testosterone (T, ng/dl, nmol/l), serum fasting insulin (I₀, µIU/ml, pmol/l) and serum fasting glucose (G₀, mg/dl, mmol/l) were measured and fasting glucose to fasting insulin ratio (I₀/G₀) was estimated in all subjects.

All participants were recruited during the past four years in the Endocrine Section of the First Department of Medicine, Athens University School of Medicine, Laiko Athens General Hospital.

The protocol was approved by the Institutional Review Committee of Laiko Athens General Hospital and written informed consent was obtained from each subject before entry into the study.

Assays

PAI-1 levels (ng/ml) in plasma were determined by a chromogenic method. The COALIZA PAI-1 kit (CHROMOGENIX, Instrumentation Laboratory SpA, Milano, Italy) was used. Total testosterone levels in the sera of all patients and controls were determined by RIA. The TESTO: CTZ Ria kit (CIS International, Gif-sur-Yvette, France) was used. Plasma glucose (mg/dl, mmol/l) was determined by the glucose oxidase method (Beckman Glucose Analyser, Palo Alto, CA, USA). Serum insulin levels (µIU/ml, pmol/l) were measured using radioimmunoassay INSULIN-CT Kits by CIS Bio

International. Blood samples were centrifuged immediately and serum was stored at -20 °C until assayed.

The intra- and interassay CVs for PAI-1 were 4.1% and 6.3% for 5 ng/ml, 5.5% and 7.3% for 50 ng/ml and 3.7% and 5.8% for 100 ng/ml respectively. The intra- and interassay CVs for insulin were 8.2% and 8.8% and 5.4% and 6.4%, for low and high levels respectively and for T were 4.5% and 5.1% and 5.5% and 4.8%, for low and high levels respectively.

4G/5G polymorphism genotype analysis

Genomic DNA was isolated from peripheral blood of all individuals by the standard phenol-chloroform method.

A 890 bp sequence spanning the 4G/5G polymorphic site of the PAI-1 gene promoter region was amplified by the polymerase chain reaction (PCR). The primers used in PCR were: P1 primer (sense) 5'-AAGCTTTTAC-CATGGTAACCCCTGGT-3' and P2 primer (antisense) 5'-GGAGCTGCAGGAATTCAGCTGCTGGA-3'. PCR was performed in a Perkin-Elmer Cetus thermocycler (Norwalk, CT, USA). The reaction mixture had a total volume of 50 µl and contained: 500 ng of genomic DNA, 50 pmol/µl of each primer, 1 × reaction buffer, 100 µM each of dATP/ dGTP/ dCTP/ dTTP and 1 unit of Taq DNA polymerase (Amersham Life Science, Athens, Greece). PCR was completed after 30 cycles of: 1 minute denaturation at 94 °C, 1 minute annealing at 65 °C and 1 minute extension at 72 °C. An initial denaturation step of 5 min at 95 °C and a final extension of 10 min at 72 °C were employed.

PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide to permit visualization of DNA bands upon ultraviolet irradiation, in order to check the quality of the PCR product. For the preparation of the agarose gel, 2 g of agarose were dissolved in 100 ml TBE buffer. When the gel was ready, 5 µl of each PCR product were loaded into the gel slots mixed with 12 µl of bromophenol blue. Phage ΦX174 *Hae*III Digest was used as a marker to verify the amplification of the 890 bp DNA fragment which contains the 4G/5G polymorphism (Fig. 1).

PCR products (25 µl) were then denatured using a NaOH/EDTA solution. Denatured DNA was then transferred to two Hybond-N + membranes using the

Figure 1 Agarose gel electrophoresis (2%) of PCR-amplified fragments from the PAI-1 promoter region, showing the 890 bp product that includes the 4G/5G polymorphic site from the 1st to the 12th lane (from the left). The 13th lane (from the left) shows the negative control. The size of the fragments was estimated using the Phage ΦX174 *Hae* III digest (14th lane). The gel was stained with ethidium bromide to permit visualization of DNA bands upon ultraviolet irradiation.

vacuum apparatus. DNA was stabilized on the membrane using the UV-Stratalinker.

The determination of the 4G/5G polymorphism was performed by hybridization of the membranes with two allele-specific oligonucleotide (ASO) probes. The two 14-mer oligonucleotides were designed by covering the polymorphic site, one representing each allele sequence. The probes were: (4G-specific): 5'-CACGTGG-GGAGTCA-3' and (5G-specific): 5'-ACGTGGGGGAGTC-A-3'. They were phosphorylated at their 5'ends with [γ - 32 P]dATP using T4 polynucleotide kinase. Membranes were prehybridized individually in a solution containing 5 $\times$ SSPE, 5 $\times$ Denhards and 0.5 $\times$ SDS at 45 °C for 10 min. The labeled probes were then added separately to each solution at a final concentration of 10⁶ c.p.m./ml and hybridized at 45 °C for 1 h. Membranes were then washed three times with a SSPE/SDS solution for 15 min at a time. Subsequently, they were washed in another SSPE/SDS solution for 15 min at 49 °C. Autoradiograms were prepared by exposing films overnight to membranes at -70 °C in order to produce a readable result of the PAI-1 4G/5G polymorphism genotype analysis (Fig. 2).

Statistical analysis

Results are reported as mean value $\pm$ S.E. BMI and age are reported as mean value $\pm$ S.D. Statistical analysis of differences in genotype frequencies between groups was performed using the chi-squared test. Multivariate analysis of variance was performed using Group (controls, PCOS) and genotype (4G/4G, 4G/5G, 5G/5G) as the independent variables and PAI-1 plasma levels, WHR, total testosterone, insulin, glucose, and glucose-to-insulin ratio as independent variables. Statistical analysis was accepted at a *P* value < 0.05. Analysis was performed using Statistica (version 6.1, software package for Windows XP (Microsoft Corp.)).

Results

The population of 98 PCOS women had mean ($\pm$ S.D.) age of 23.3 $\pm$ 4.2 years and BMI 24.9 $\pm$ 5.7 kg/m².

The population of 64 normal women had mean age 23.5 $\pm$ 4.3 years and BMI 23.85 $\pm$ 2.0 kg/m². The following parameters in PCOS women were statistically different compared with control group of women: WHR 0.77 $\pm$ 0.01 vs 0.73 $\pm$ 0.01 (*P* < 0.01), total testosterone 85.19 $\pm$ 5.12 ng/dl (2.95 $\pm$ 0.17 nmol/l) vs 50.44 $\pm$ 4.80 ng/dl (1.75 $\pm$ 0.16 nmol/l) (*P* < 0.0001), fasting glucose levels 86.43 $\pm$ 1.84 mg/dl (4.79 $\pm$ 0.10 mmol/l) vs 79.83 $\pm$ 1.82 mg/dl (4.43 $\pm$ 0.10 mmol/l) (*P* < 0.01), and the ratio G₀/I₀ was 8.13 $\pm$ 0.77 vs 11.53 $\pm$ 1.45 (*P* < 0.02) respectively. Fasting insulin levels 15.34 $\pm$ 1.32 μ IU/ml (106.53 $\pm$ 9.16 pmol/l) vs 11.56 $\pm$ 1.52 μ IU/ml (80.28 $\pm$ 10.55 pmol/l) approached, but did not reach statistical significance (Table 1).

There was a statistically significant difference in the distribution of gene variations among the groups (chi-squared = 17.56, *P* < 0.0001). The PCOS group had significantly higher 4G/4G and 4G/5G combinations than the control group, whereas there were significantly less 5G/5G. The opposite applied for the control group (Table 2). The differences between the 4G/4G and 4G/5G combinations were not statistically significant. The differences between the 4G/4G and 4G/5G, taken together, were statistically different from those of 5G/5G (chi-square = 17.07, *P* < 0.01).

Among the population of PCOS women, 39 (39.8%) had the homozygous genotype 4G/4G, 39 (39.8%) the heterozygous 4G/5G and 20 (20.4%) the homozygous genotype, 5G/5G. From the control group, 13 (20.3%) had genotype 4G/4G, 18 (28.1%) were heterozygous 4G/5G and 33 (51.6%) were 5G/5G (Table 3). In the 4G/4G genotype subgroup 75% were PCOS and 25% were controls, in the 4G/5G were 68.42% and 31.58% and in the 5G/5G were 37.74% and 62.26% respectively (Table 4) (16).

Multivariate analysis of variance was performed using group (controls, PCOS) and genotype (4G/4G, 4G/5G, 5G/5G) as the independent variables and PAI-1 plasma levels, WHR, total testosterone, insulin, glucose and G₀/I₀ as the dependent variables. The multivariate analysis revealed a significant group effect (*F* = 5.162, *P* < 0.0001) and a significant genotype

Figure 2 Autoradiograms of the two hybridized membranes loaded with the denatured PCR products. Membrane 1 is on the left and membrane 2 on the right. Each membrane was hybridized with a 32 P-labelled allele-specific probe, in order to identify the presence of the 4G (on the left) or the 5G (on the right) allele. Each membrane was loaded with the same PCR products in the relative spots.

Table 1 Anthropometric characteristics, metabolic and hormonal profile in PCOS patients and normal women.

Study group	PCOS	Controls	P
Age (years)*	23.3±4.2	23.5±4.3	ns
BMI (kg/m ²)*	24.9±5.7	23.85±2.0	ns
Waist-hip ratio**	0.77±0.01	0.73±0.01	0.01
Total T (ng/dl, nmol/l)**	85.19±5.12 (2.95±0.17)	50.44±4.80 (1.75±0.16)	0.0001
Insulin (μIU/ml, pmol/l)**	15.34±1.32 (106.53±9.16)	11.56±1.52 (80.28±10.55)	0.06
Glucose (mg/dl, mmol/l)**	86.43±1.84 (4.79±0.10)	79.83±1.82 (4.43±0.10)	0.01
G ₀ /I ₀	8.13±0.77	11.53±1.45	0.02

* Values are means±S.D. ** Values are means±S.E. *P* < 0.05.

Table 2 Observed minus expected frequency of genotype subgroup in PCOS and control group.

Observed minus expected frequencies	Genotype		
	4G/4G	4G/5G	5G/5G
Controls	-7.54	-4.52	12.06
PCOS	7.54	4.52	-12.06

Table 3 The genotype frequencies of 4G/4G, 4G/5G and 5G/5G in PCOS patients and normal women.

	Genotype subgroups			Totals
	4G/4G	4G/5G	5G/5G	
Controls (<i>n</i>)	13	18	33	64
Controls (%)	20.31	28.13	51.56	100
PCOS (<i>n</i>)	39	39	20	98
PCOS (%)	39.80	39.80	20.41	100
Total population (<i>n</i>)	52	57	53	162
Total population (%)	32.10	35.19	32.72	100

effect ($F = 2.831$, $P < 0.001$). There was no statistically significant interaction. The group effect was significant for PAI-1 plasma levels ($P < 0.001$), WHR ($P < 0.01$), total testosterone ($P < 0.0001$) and fasting glucose ($P < 0.01$). The group effect for fasting insulin approached, but did not reach statistical significance. The genotype effect was significant for the PAI-1 plasma levels only ($P < 0.001$) (17).

The population of PCOS women had significantly higher PAI-1 levels than the population of controls (total: 72.35 ± 5.14 vs 48.89 ± 6.31 ng/ml, respectively; $P < 0.001$) (Table 5). Specifically, in PCOS women, PAI-1 plasma levels were 98.78 ± 9.67 for 4G/4G

genotype, 63.23 ± 5.80 for 4G/5G and 45.78 ± 2.66 for 5G/5G. In the control group the 5G/5G had 26.95 ± 2.72 PAI-1 plasma levels, the 4G/5G 64.74 ± 13.07 and the 4G/4G 83.00 ± 14.97 (Table 6).

There was no statistical significance in the inter-action group genotype.

The intragroup comparison of the PAI-1 levels of different genotypes in the two studied groups revealed statistical significance when the 4G/4G genotype was compared with 5G/5G ($P < 0.001$) or 4G/5G ($P < 0.001$) in the PCOS group, and when the 5G/5G genotype was compared with 4G/4G ($P < 0.001$) or 4G/5G ($P < 0.001$) in the control group (Table 7).

The intergroup comparison of the PAI-1 levels of different genotypes in the two studied groups revealed statistical significance when the 4G/4G genotype of the PCOS group was compared with 5G/5G ($P < 0.001$) or 4G/5G ($P < 0.01$) of the control group, when the 4G/5G genotype of the PCOS group was compared with 5G/5G ($P < 0.001$) of the control group and when the 5G/5G genotype of the PCOS group was compared with 4G/4G ($P < 0.05$) of the control group (Table 8).

Discussion

In the present study, it has been demonstrated for the first time that the homozygous 4G/4G and the heterozygous 4G/5G genotypes were present with statistically higher frequency in women with PCOS as compared with normally menstruating and normo-androgenic women of the same ethnicity (Table 3). Subsequently, plasma PAI-1 levels of PCOS phenotypic group were statistically higher than those of controls

Table 4 The percentages of PCOS patients and controls in genotypes 4G/4G, 4G/5G and 5G/5G.

Genotype subgroups	Controls (<i>n</i>)	Controls (%)	PCOS (<i>n</i>)	PCOS (%)	Total population (<i>n</i>)	Total population (%)
4G/4G	13	25	39	75	52	32.10
4G/5G	18	31.58	39	68.42	57	35.19
5G/5G	33	62.26	20	37.74	53	32.72
Totals	64	100	98	100	162	100

Table 5 Total PAI-1 plasma levels of PCOS patients, normal women and of each genotype.

		PAI-1 (ng/ml)
Groups	Controls	48.89±6.31
	PCOS	72.35±5.14
Genotypes	4G/4G	95.00±8.15
	4G/5G	63.75±5.77
	5G/5G	34.09±2.59

Values are means±S.E.

Table 6 PAI-1 plasma levels of PCOS patients and normal women in each genotype.

Genotypes		PAI-1 (ng/ml)
Controls	4G/4G	83.00±14.97
	4G/5G	64.74±13.07
	5G/5G	26.95±2.72
PCOS	4G/4G	98.78±9.67
	4G/5G	63.23±5.80
	5G/5G	45.78±2.66

Values are means±S.E.

(Table 6), which is in accordance with previous studies (8, 9).

In this study, women with PCOS with 4G/4G genotype had higher PAI-1 levels than any other genotype in the studied groups except for 4G/4G of the control group (Table 6). This finding suggests that the presence of the 4G/4G increases the PAI-1 levels further. Elevated levels of PAI-1 have been associated with increased cardiovascular risk and increased thrombotic tendency (4, 7, 10). The 5G/5G genotype in controls is associated with lower levels of PAI-1 in either group except 5G/5G genotype group of PCOS. This finding suggests that the presence of 5G has a decreased effect on PAI-1 levels as opposed to the 4G allele.

The intragroup comparisons of the genotypic subgroups in the PCOS group suggest that the presence of the homozygosity 4G/4G is associated with higher levels of PAI-1. Corresponding findings, on the low side, were observed with the 5G/5G genotype of PCOS having a non-statistical difference with the low PAI-1

Table 8 Comparison intergroup between the plasma PAI-1 levels (ng/ml) in genotype sub-groups.

PCOS			Control		
Genotype	PAI-1 (ng/ml)	vs	Genotype	PAI-1 (ng/ml)	P
4G/4G	98.78±9.67	vs	5G/5G	26.95±2.72	0.001
4G/4G	98.78±9.67	vs	4G/5G	64.74±13.07	0.01
4G/5G	63.23±5.80	vs	5G/5G	26.95±2.72	0.001
5G/5G	45.78±2.66	vs	4G/4G	83.00±14.97	0.05

Values are means±S.E.

levels from the 5G/5G group of controls. These findings imply that the presence of 4G/4G is deleterious and the opposite could be suggested for the 5G/5G genotype, with a beneficial effect on PAI-1 levels.

The higher prevalence of homozygosity 4G/4G and heterozygosity 4G/5G population and its association with higher PAI-1 levels may further increase the cardiovascular risks in this population of young women, as it has been reported in patients who suffer from coronary heart disease (7, 11, 12), venous thromboembolism (10) or type 2 diabetes mellitus (18, 19).

The observed difference in plasma PAI-1 levels between PCOS patients and controls cannot be attributed to the influence of BMI since this anthropometric parameter did not differ between the two phenotypic groups (Table 1). Nevertheless, other investigators have provided evidence that PAI-1 activity is not raised in women with PCOS independent of obesity (20).

Since the expression of PAI-1 is regulated by a number of factors and the regulation has been found to take place at the level of transcription (10, 14, 15), environmental parameters which may be involved in the regulation of plasma PAI-1 levels in PCOS, should also be considered. It has been demonstrated that insulin resistance correlates positively with plasma PAI-1 levels (21). Hyperinsulinemia, when present in PCOS, may further increase PAI-1 levels, especially when it acts on the genetic background of homozygosity like 4G/4G.

Since there are data to suggest that PAI-1 is involved in luteolysis, modifying the reproductive potential, it could be intriguing to speculate on a dual role of elevated levels of PAI-1 in PCOS (22, 23).

Table 7 Comparison intragroup between the plasma PAI-1 levels in genotype subgroups.

Genotype		PAI-1 (ng/mL)	P
PCOS	4G/4G vs 5G/5G	98.78±9.67 vs 45.78±2.66	P < 0.001
	4G/4G vs 4G/5G	98.78±9.67 vs 63.23±5.80	P < 0.001
	4G/5G vs 5G/5G	63.23±5.80 vs 45.78±2.66	n.s.
Controls	5G/5G vs 4G/4G	26.95±2.72 vs 83.00±14.97	P < 0.001
	5G/5G vs 4G/5G	26.95±2.72 vs 64.74±13.07	P < 0.001
	4G/5G vs 4G/4G	64.74±13.07 vs 83.00±14.97	n.s.

Values are means±S.E.

In conclusion, the PCOS women had higher PAI-1 levels compared with the control group. The 4G/4G and 4G/5G polymorphisms are present with higher frequency in PCOS women compared with the control group. These genotypes appear to contribute to the elevated PAI-1 levels. Finally, the combination of genetic and metabolic abnormalities may indeed be responsible for elevated PAI-1 levels which, along with several other cardiovascular risk factors, pose an increased health risk to women with PCOS.

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