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ORIGINAL PAPER

4G/5G Polymorphism of *PAI-1* gene and Alu-repeat I/D polymorphism of *TPA* gene in Turkish patients with polycystic ovary syndrome

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Abstract
Purpose Polycystic ovary syndrome (PCOS) is one of the most encountered endocrine malfunctions. PCOS patients have enhanced activation of the blood coagulation system.
Methods Eighty-six young women with PCOS and 70 healthy control women were included in our study. PCOS patients and controls were matched for age, body mass index, and allele frequency. Genetic analysis of TPAI and PAI-1 were performed in all subjects.
Results and conclusions No statistically significant differences have been detected about the ratios of genotypes resulting from PAI-1 promotor 4G/5G gene polymorphism. PAI-1 765 4G/5G gene polymorphism and TPA gene's Alu-repeat insertion/deletion (I/D) polymorphism ratios were not different from the controls. In this study it is shown by the analysis of TPA gene's Alu-repeat insertion/deletion (I/D) polymorphism the PCOS patients with genotype II had lowers total cholesterol and LDL-cholesterol levels.

Keywords *PAI-1* gene · TPA gene · Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is one of the most encountered endocrine malfunctions, which typically occur with chronic anovulation and hyperandrogenism [1]. Almost 15% of the women at reproductive period are diagnosed as PCOS [2].

Cardiovascular disease, dyslipidemia and hypertension are more frequently seen in patients with PCOS than in normal patients [3–5]. The disorder has also been reported to be associated with an increase in sub-clinical atherosclerotic disease [5]. These findings suggest that women with PCOS are at a higher risk of early-onset cardiovascular disease (CVD). The majority of ischemic CVD occur because of thrombotic or thromboembolic occlusions.

PCOS patients have enhanced activation of the blood coagulation system. Thrombus formation results from the imbalance between prethrombotic and anti-thrombotic factors that controls clotting homeostasis: this imbalance may occur due to an ongoing stimulus to thrombogenesis, a defect of the natural anti-coagulant or fibrinolytic system [6].

PAI-1, a member of the serine protease inhibitor (SERPIN) family, is a main regulator of the endogenous fibrinolytic system. It inhibits fibrinolysis activity of the tissue-type plasminogen activator, tPA, which produces active plasmin from plasminogen, that then cleaves the fibrin [7].

Thus, PAI-1 determines in part fibrinolysis activity and modulates the progression of thrombosis [8]. PAI-1 is expressed and secreted in a variety of tissues, including liver, spleen [9]. PAI-1 synthesis is regulated by various agents, including insulin [9, 10], very-low-density lipoprotein, VLDL [10], low-density lipoprotein and glucose [11].

Women with PCOS also show elevated levels of plasminogen activator inhibitor-1 (PAI-1), which is a potent inhibitor of fibrinolysis in obese [12] and non-obese women with PCOS [13].

The present study was undertaken to evaluate the 4G/5G polymorphism in the promoter region of *PAI-1* gene and Alu-repeat I/D polymorphism in intron 8 of *TPA* gene in a

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Turkish population with PCOS and controls, and also to investigate the association of the *TPA* and *PAI-1* gene polymorphisms with clinical, metabolic parameters in Turkish PCOS patients.

Material and methods

Patients

Eighty-six young women (mean age, 24.27±5.44SD years) with PCOS and 70 healthy control women (mean age, 26.41±5.65SD years) were included in our study. Patient and control subjects were accepted from same ethnic populations. PCOS was defined by the Rotterdam PCOS consensus criteria [14].

Patients who had DM, hyperprolactinemia, congenital adrenal hyperplasia (diagnosed with the ACTH stimulation test), thyroid disorders, Cushing's syndrome, hypertension, hepatic or renal dysfunction were excluded from the study. Confounding medications, including oral contraceptive agents, hypertensive medications and insulin-sensitizing drugs and those that may affect the metabolic criteria were questioned and the patients were excluded if they used anyone of them.

Another 70 healthy young volunteer females matched for age, body mass index (BMI), and allele frequency, were included as the control group. Their health statuses were determined by medical history, physical and pelvic examination, and complete blood chemistry. The patients with PCOS and the control group were genetically unrelated.

Study protocol

At study onset, all subjects underwent venous blood drawing for complete hormonal assays, lipid profile, glucose, insulin, cortisol, ACTH levels, and genetic study. All blood samples were obtained in the morning between 08.00 and 09.00 hours after an overnight fasting, and resting in bed during early follicular phase of the spontaneous or progesterone-induced menstrual cycle.

During the same visit, all subjects underwent anthropometric measurements including BMI and detailed history, systolic and diastolic blood pressure.

Biochemical assay

Serum concentrations of hs-CRP were determined by an immunonephelometric assay (N-high-sensitivity CRP; Dade Behring); intra and interassay CV were 1.72% and 2.80%, respectively.

Serum total cholesterol, LDL and HDL-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) were measured by Olympus AU 2700 automated analyzer. Immunolite 2000 using two-site chemiluminescent immunometric assay determined plasma insulin concentrations. Insulin resistance was calculated using the homeostasis model assessment insulin resistance index (HOMA-IR) [15], according to the following formula:

$$\mathrm{HOMA-IR} = \frac{\text{fasting serum insulin (mU/ml)} \times \text{fasting plasma glucose (mmol/l)}}{22.5}$$

Genomic DNA preparation

Two millilitres of whole-blood samples was collected into EDTA-anticoagulated tubes by the standard venipuncture method. Genomic DNA was extracted from whole-blood samples using a commercially available genomic DNA purification kit (Nucleospin Blood, Macherey-Nagel, and Düren, Germany) following the manufacturer's instructions. DNA concentration was determined by the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR, USA) according to the manufacturer's instructions and diluted as 100-ng/μl.

Genotyping of the TPA gene

Alu-repeat (I/D polymorphism in intron 8 of the *TPA* gene were genotyped by a polymerase chain reaction (PCR) [16]. Synthetic oligonucleotides obtained from TIB MOLBIOL Syntheselabor (Berlin, Germany) were used. Primer sequences were for upstream (5′-TCCGT AACAGGA CAGCTCA-3′) and downstream (5′-ACCGTGGCTTCAG TCAT GGA-3′), respectively.

Amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA) in a 25 μl reaction mixture in 0.2 ml thin-walled PCR strip tubes (Axygen Scientific Inc., Union City, CA, USA) containing 1 μl genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris–HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 2.5 mmol $MgCl_2$, 50 μmol/l each of the dGTP, dATp, dTTP and dCTP (Promega, Madison, WI, USA), 5 pmol each of forward and reverse primers and 1.0 U AmpliTaq Gold polymerase (PE Applied Biosystems).

The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 45 s, 58°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 7 min. Amplified products (I allele=967 bp, D allele=655 bp) were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. Samples were classified according to three genotypes: I/I, I/D, and D/D.

Table 1 Clinical and biochemical data in women with PCOS and healthy controls

	Patient (n=91)	Control (n=100)	P value
Age (years)	24.06±1.31	25.02±2.04	>0.05
BMI (k/m^2)	24.28±4.23	23.47±4.111	>0.05
Fasting blood glucose (mg/dl)	90.85±6.23	88.78±7.13	>0.05
Fasting insulin (mIU/ml)	16.4±31.3	4.74±2.15	<0.05
HOMA-IR	3.57±7.8	1.32±0.43	<0.05
LH (mIU/ml)	7.71±4.53	3.26±1.65	<0.05
FSH (mIU/ml)	6.42±2.12	4.16±2.26	<0.05
Estradiol (pg/ml)	34.86±31.2	35.8±37.22	>0.05
DHEA-S (μg/dl)	228±104.3	212.4±99.18	>0.05
TSH (μIU/dl)	2.93±4.07	2.58±3.64	>0.05
17-OHP (ng/ml)	4.47±20.8	0.74±0.12	<0.05
f-testosterone (pg/ml)	3.34±2.26	1.23±0.65	<0.05
T-testosterone (ng/ml)	0.93±1.63	0.82±0.76	>0.05
Prolactin (ng/ml)	16.6±8.15	17.05±7.13	>0.05
Cholesterol (mg/dl)	195.26±44.3	190.13±28.34	>0.05
Triglyceride (mg/dl)	114.18±53.29	113.47±30.23	>0.05
LDL-cholesterol (mg/dl)	115.16±22.52	109.93±20.34	>0.05
HDL-cholesterol (mg/dl)	54.23±34.4	55.2±6.57	>0.05
Fibrinogen (mg/dl)	368.44±88.7	262.53±60.25	<0.05
hs-CRP (mg/dl)	0.397±0.64	0.25±0.37	<0.05

Genotyping of the PAI-1 gene

4G/5G polymorphism, which is located in the promoter region 675 bp upstream from the transcriptional start site of the *PAI-1* gene, was genotyped by PCR and endonuclease digestion [17].

Amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems) in a 25 μl reaction mixture in 0.2 ml thin-walled PCR strip tubes (Axygen Scientific) containing 100 ng genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris–HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 1.5 mmol $MgCl_2$, 50 μmol/l each of the dGTP, dATP, dTTP, and dCTP (Promega), 5 pmol each of forward and reverse primers, and 0.5 U AmpliTaq Gold polymerase (PE Applied Biosystems). The sequences of the forward and reverse primers were used; 5′-CCAACAGAGG ACTCTTGGTCT-3′ and 5′-CACAGAG AGAGTCTGGCCACGT-3′, respectively. The cycling conditions comprised a denaturation step at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. The 100-bp PCR products for *PAI-1* gene were analysed on a 2.0% agarose gel prestained with ethidium bromide.

Genotyping was performed by using the *Bsl*I (MBI Fermentas, Vilnius, Lithuania) restriction enzyme as manufactured. Briefly, 10 μl of PCR product was mixed with 5 U *Bsl*I and appropriately buffered and incubated at 55°C during a 4-h period. The fragments, a single one of 99 bp for the 4G allele and two fragments of 77 and 22 bp, respectively, for the 5G allele, were separated on a 3% metaphor agarose gel (FMC BioProducts, BioConcept, Allschwill, Switzerland) stained with ethidium bromide, and visualized under ultraviolet light.

Results

The clinical and metabolic parameters of PCOS patients and controls are shown in Table 1. HOMA-IR, fasting glucose, LH, FSH, 17-OPH, free-testosterone, total testosterone, fibrinogen and hs-CRP levels of PCOS patients were detected in statistically high levels according to controls.

TPAI II/ID gene polymorphism

The genetic part of the study started with 105 controls and 95 PCOS patient DNAs. But 100 participants in control group (genotype success ratio=95%, genotype failure ratio=5%) and 91 participants in patient group (genotype success ratio=96%, genotype failure ratio=4%) could be genetically analysed.

TPAI gene polymorphism of genetically analysed 100 control and 91 PCOS patients are shown in Table 2. No statistically significant difference has been detected between the patient and control groups according to TPAI II/ID and DD genotypes. D and I alleles were similar in the patient and control groups. The distribution of clinical and metabolic parameters of II/ID and DD genotypes of PCOS

Table 2 Genotype distribution and allele frequency of TPAI II/ID polymorphism for the PCOS and Controls group

Genotypes	Hasta		Control		*P*
	n	%	*n*	%	
II	23	25.6	24	24	ns
DI	39	43.3	45	45	ns
DD	28	31.1	31	31	ns
Alleles					
D	85		93		ns
I	95		107		ns

No statistically significant difference was determined between two groups ($P>0.05$).

patients are shown in Table 3. Between these parameters II genotypes had lower LDL and total cholesterol according to DD and ID genotypes. HOMA-IR, fasting insulin, LH, FSH, 17-OPH, free-testosterone, total-testosterone, fibrinogen and hs-CRP, HDL -cholesterol, triglyceride, fibrinogen, fasting glucose levels and BMI showed no significant difference between the groups.

PAI-1 promoter 4G/5G gene polymorphism

The genetic assay of our study started with 105 controls and 95 PCOS patient DNAs. But 100 participants from the control group (genotype success ratio=95%, genotype failure ratio=5%) and 91 participants from the patient group (genotype success ratio=96%, genotype failure ratio=4%) were able to be genetically analysed.

The results of the genetical analysis of PAI-1 promoter 4G/5G gene polymorphism in 100 control and 91 PCOS patients are shown in Table 4. No statistically significant difference has been detected about the ratios of 4G4G/4G5G and 5G5G genotypes resulting from PAI-1 promoter 4G/5G gene polymorphism. The 4G and 5G allels were in similar ratios in both the patient and control groups.

The distributions of clinical and metabolic parameters between 4G4G/4G5G and 5G5G genotypes of PCOS patient group are shown in Table 5. No significant differences including HOMA-IR, fasting insulin, LH, FSH, 17-OHP, free-testosterone, total-testosterone, fibrinogen and hs-CRP, LDL-cholesterol, total-cholesterol, HDL-cholesterol, triglyceride, fibrinogen, fasting glucose and BMI were detected between the groups.

Discussion

The 4G allele of the PAI-1 gene was more common in women with PCOS than in normal women in agreement with Diamanti-Kandarakis et al. [18] and contributed to hypofibrinolytic miscarriage-promoting PAI-1 activity levels in concert with obesity, hyperinsulinemia, and hypertriglyceridemia. In other studies of women with PCOS, we have previously shown that the 4G4G PAI-1 mutation [19] was an independent determinant of serious pregnancy complications and that PAI-1 activity was an independent determinant of miscarriage [20–23] and recurrent pregnancy loss [24].

Table 3 Clinical and laboratory data for the t-PAI II/ID polymorphism genotypes

	DD *n*=23	ID *n*=38	II *n*=27	*P* value
Age (years)	24.43±5.54	24.89±5.9	23.04±4.9	ns
BMI (kg/m^2)	23.54±5.21	25.87±6.49	23.712±4.43	ns
Glucose (mg/dl)	88.0±7.37	92.53±8.1	93.85±10.5	ns
Insulin (pmol/l)	12.97±11.12	19.45±45.14	10.07±3.908	ns
HOMA-IR	2.4±1.49	5.1±13.4	2.49±1.46	ns
Cholesterol (mg/dl)	204.6±46.4	208.78±37.98	178.8±36.8	0.027
LDL (mg/dl)	114.74±38.648	127.61±30.683	105.24±23.569	0.042
HDL (mg/dl)	58.74±19.975	57.55±15.32	55.76±10.07	ns
Triglyceride (mg/dl)	133.9±71.8	132.84±60.8	102.0±76.59	ns
Fibrinogen (mg/dl)	392.62±89.597	379.42±127.73	347.3±104.9	ns
DHEAS (mmol/l)	252.7±116.06	204.97±114.43	207.0±113.1	ns
FSH (mIU/ml)	5.12±2.18	5.78±2.08	5.2±2.1	ns
LH (mIU/ml)	6.5±4.4	6.2±4.08	6.42±4.9	ns
Free-testosterone (pg/ml)	3.74±1.81	2.64±1.14	3.7±2.87	ns
Total-testosterone (ng/ml)	1.622±3.3	0.7±0.86	0.67±0.4	ns
17-OHP (ng/ml)	1.76±0.94	1.83±0.91	1.83±0.91	ns
Prolactin (ng/ml)	19.02±9.1	16.64±6.95	15.6±8.6	ns
Estradiol (pg/ml)	35.22±26.89	29.54±13.93	44.69±43.7	ns

No statistically significant difference was determined between two groups ($p>0.05$).

Table 4 Genotype distribution and allele frequency of PAI-1 promoter 4G /5G polymorphism for the PCOS and Controls group

Genotypes	Hasta		Control		*P*
	n	%	*n*	%	
4G4G	22	24.2	38	38	ns
4G5G	48	52.7	41	41	ns
5G5G	21	23.1	21	21	ns
Alleles					
4G	92		117		ns
5G	90		83		ns

No statistically significant difference was determined between two groups ($p>0.05$).

Elevated levels of PAI-1 have been associated with increased cardiovascular risk and increased thrombogenic tendency [25–27]. 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 [11], venous thromboembolism [27] or type 2 diabetes mellitus [28, 29].

There may be differences between the PAI-1 gene polymorphism of the populations. In this study no significant differences were detected between 4G4G/4G5G and 5G5G genotypes of PAI-1 gene of the patient and control groups. At the same time no significant relation have been detected about cardiovascular indicators such as LDL-cholesterol, HDL-cholesterol, hs-CRP and fibrinogen levels in 4G4G/4G5G and 5G5G genotypes of PCOS patient group.

Nevertheless, some investigators have provided evidence that PAI-1 activity is not raised in women with PCOS independent of obesity [30]. 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 [33], environmental parameters, which may be involved in the regulation of plasma PAI-1 level in PCOS, should also be considered. In this study no relation between PAI-1 genotype and BMI was detected. And also no significant relation has been detected between 4G4G/4G5G and 5G5G genotypes and HOMA-IR, plasma glucose and insulin levels.

It was recommended that Alu-repeat I/D polymorphism could be a marker of a functional mutation in the *TPA* gene that regulates the communication between tissue plasminogen activator (TPA) and PAI-1 owing to the positive correlation between TPA and PAI-1 levels [28]. The I/I genotype of Alu-repeat I/D polymorphism in the *TPA* gene was shown to be associated with plasma PAI-1 antigen in patients with myocardial infarction [32].

In our study, patient and control subjects were accepted from same ethnic populations. Ethnicity may be a contributing factor, as described previously in Afro-Caribbeans, Asians, Pima Indians, Japanese, and, more recently, blacks [33–36]. Both retrospective and prospective studies have found associations with high TPA antigen levels and MI [37, 38]. Other studies have found similar links with coronary artery disease (CAD) as well as with stroke [39]. The Alu-repeat insertion/deletion (I/D) polymorphism of the

Table 5 Clinical and laboratory data for the PAI-1 promoter 4G/5G polymorphism genotypes

	4G/4G *n*=22	4G/5G *n*=47	5G/5G *n*=20	*P* value
Age (years)	23.95±5.98	24.13±5.05	24.6±6.29	ns
BMI (kg/m^2)	25.02±6.1	24.4±5.8	24.3±4.7	ns
Glucose (mg/dl)	94.8±6.8	89.77±10.2	92.55±6.7	ns
Insulin (pmol/l)	12.97±11.12	19.45±45.14	10.07±3.908	ns
HOMA-IR	3.03±2.58	4.2±11.7	2.2±0.8	ns
Cholesterol (mg/dl)	202.8±34.4	193.88±44.3	204.06±44.7	ns
LDL (mg/dl)	116.12±22.4	116.4±36.02	118.76±34.4	ns
HDL (mg/dl)	61.18±21.4	54.53±10.67	59.94±16.01	ns
Triglyceride (mg/dl)	138.72±80.6	121.95±66.2	109.7±65.82	ns
Fibrinogen (mg/dl)	393.89±116.6	346.13±84.1	408.7±134.02	ns
DHEAS (mmol/l)	221.15±122.85	222.58±116.24	214.53±114.57	ns
FSH (mIU/ml)	6.26±1.6	5.2±2.4	5.1±1.7	ns
LH (mIU/ml)	6.7±4.34	6.36±4.49	5.96±4.48	ns
Free-testosterone (pg/dl)	3.42±2.54	3.38±2.17	2.84±1.46	ns
Total-testosterone (ng/dl)	0.736±0.46	0.8±0.76	1.4±1.2	ns
17-OHP (ng/ml)	1.97±1.4	1.86±0.93	1.80±0.8	ns
Prolactin (ng/ml)	16.19±8.49	17.51±7.9	17.1±8.9	ns
Estradiol (pg/ml)	35.22±26.89	29.54±13.93	44.69±43.7	ns

For all parameters $p>0.05$.

TPA gene was recognized within the intron 8, between exons 8 and 9 of this gene, which was sequenced and mapped on chromosome 8p12-p11.2 [40].

The TPA gene polymorphism was found to be related with increased MI risk among Dutch participants in a Dutch study [41]. These researchers found both the II genotype and increased TPA antigen levels are risk factors for nonfatal MI.

In contrast to the Dutch findings, other studies conducted in Italy and the United Kingdom as well as the United States Physicians Health Study found no relation between the TPA I/D polymorphism and MI [40]. In the study we carried out, it is shown by the analysis of TPA genes Alu-repeat insertion/deletion (I/D) polymorphism that genotype II had lower total cholesterol and LDL-cholesterol levels.

Fibrinogen has been established by many studies to be an independent cardiovascular risk factor [42–44]. It is still a matter of debate if its elevated concentrations are the cause or result in the progress of atherosclerosis. No statistically significant relation has been found by the genetically analysis of both TPA and PAI-1.

Conclusions

Plasminogen activator inhibitor-1 765 4G/5G gene polymorphism and TPA gene's Alu-repeat insertion/deletion (I/D) polymorphism ratios were not different from the controls. This study shows that the PCOS patients with genotype II determined by of TPA gene's Alu-repeat insertion/deletion (I/D) polymorphism had lowers total cholesterol and LDL-cholesterol levels.

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