Gene Polymorphisms of Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 in Patients with Antiphospholipid Antibodies

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ABSTRACT. ***Objective.*** Impaired fibrinolytical outcomes may be one of the pathogenic factors for thrombotic events in patients with antiphospholipid antibodies (aPL). We investigated the consequences of the gene polymorphisms of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) in patients positive for aPL.

Methods. Seventy-seven Japanese and 82 British patients with aPL were examined for Alu-repeat insertion (I)/deletion (D) polymorphism of the tPA gene by polymerase chain reaction (PCR), and 4G/5G polymorphism in the PAI-1 promoter gene by site-directed mutagenesis-PCR and restriction fragment length polymorphism analysis. Correlations between these polymorphisms and clinical symptoms of antiphospholipid syndrome (APS) (arterial thrombosis, venous thrombosis, miscarriage) were analyzed.

Results. Significant differences in the allele frequencies of these genes did not exist between patients and controls. There was no significant correlation between these gene polymorphisms and clinical symptoms of APS in patients with aPL.

Conclusion. Polymorphisms of the tPA or PAI-1 genes probably do not significantly influence the risk of arterial thrombosis, venous thrombosis, or pregnancy morbidity in patients with aPL. (J Rheumatol 2002;29:1192–7)

Key Indexing Terms:
FIBRINOLYSIS GENOME PATHOGENESIS
ANTIPHOSPHOLIPID ANTIBODY SYNDROME THROMBOSIS

Antiphospholipid antibody syndrome (APS) is one of the most frequent causes of acquired thrombophilia. Detection of antiphospholipid antibodies (aPL) is carried out mostly by $ß_2$-glycoprotein I dependent anticardiolipin assays and lupus anticoagulant (LAC) assays. The clinical value of these assays is widely acknowledged, and the tests are included in the classification criteria of APS[1]. The major clinical features of this syndrome are recurrences of arterial thrombosis, venous thrombosis, and pregnancy loss. Other clinical manifestations include thrombocytopenia, neurological disorders, hemolytic anemia, migraine, and pulmonary hypertension. The thrombotic events tend to recur: arterial thrombosis is often followed by another arterial thrombotic event and likewise for venous thrombosis[2]. An explanation for this heterogeneity and the tendency toward recurrence has remained elusive. A well-accepted mechanism of pregnancy loss is placental insufficiency due to vascular occlusion[3], although several other mechanisms have been proposed.

Antiphospholipid antibodies are a significant factor in the pathophysiology of thrombotic events: aPL impair anticoagulants such as protein C or antithrombin III[4,5], activate platelet function[6], induce a procoagulant state by activating endothelial cells[7], or induce early atherosclerotic lesions at least in animal models[8]. However, no correlation between methods of detection of aPL and other clinical symptoms has been found, suggesting that APS is a multifactorial disorder in which some factors that may not directly relate to aPL may nevertheless influence the occurrence and nature of thrombotic events.

Dysregulation of fibrinolysis may also play a significant role in determining the clinical course of patients with aPL. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) are 2 major regulators in thrombolysis. Plasmin is converted from plasma plasminogen by tPA, a serine protease that promotes the initial step in the fibrinolytic cascade on the surface of fibrin. On the other hand, PAI-1 is one of the most potent serine protease inhibitors that inactivate tPA. Polymorphisms of Alu-repeat insertion (I)/deletion (D) in intron h of the tPA gene, and 4G/5G in the 5'-untranslated region at -675 of the PAI-1 gene are known, as are the

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differences in productivity of these proteins between genotypes[9,10]. Whether these polymorphisms affect the occurrence of thrombosis and their relationships with arterial or venous thrombosis have been given attention[11-17]. We speculated that impairment of fibrinolysis induced by the tPA and PAI-1 gene polymorphisms may modulate the initiation or the severity of thrombotic events in APS and analyzed these genetic factors in Japanese and Caucasian patients positive for aPL.

MATERIALS AND METHODS

Patients and controls. This retrospective study comprised 77 Japanese (8 men, 69 women) and 82 Caucasian patients (10 men, 72 women), all positive for aPL. Their clinical records were carefully reviewed and the clinical symptoms such as arterial thrombosis, venous thrombosis, and pregnancy loss were characterized. Of the 77 Japanese patients, 41 were diagnosed as APS [14 primary APS, 21 secondary to systemic lupus erythematosus (SLE), and 6 APS with autoimmune disorders other than SLE]. Thirty-six subjects, albeit positive for aPL, had no clinical manifestations of APS, and had other autoimmune disorders (26 SLE, 4 rheumatoid arthritis, 4 Sjögren's syndrome, one mixed connective tissue disease, 1 polymyalgia rheumatica). Of these patients, 17 were taking warfarin, 36 were being treated with antiplatelet agents, and 48 with corticosteroids. The followup period varied from 2 to 27 years with an average of 11.2 years. Of the 82 Caucasian patients, 76 were diagnosed as APS (51 primary APS, 25 secondary to SLE). Three had SLE and 3 thrombocytopenia, all positive for aPL. Of these patients, 40 were taking warfarin, 43 were treated with antiplatelet agents, and 23 with corticosteroids. The followup period varied from 1 to 38 years with an average of 10.1 years. Diagnoses of APS and SLE were based on proposed criteria for APS[1] and American College of Rheumatology criteria for classification of SLE[18]. Clinical features of these patients are given in Table 1. Arterial thromboses were diagnosed by magnetic resonance imaging, computed tomography, or angiography. Venous thromboses were diagnosed by venography or radioisotope-labelled venography. Pulmonary embolisms were diagnosed using perfusion scintigraphy. DNA samples were obtained from all these patients. DNA from 144 healthy Japanese and 38 healthy Caucasians served as controls. Written permission was obtained from each patient to use DNA for this purpose. As the patients had antithrombotic therapy after the first thrombotic event, the difference of the treatment did not confound the results. Among each subgroup of patients, no difference was found in the length of followup.

Analysis of Alu-repeat polymorphism in the tPA gene. I/D polymorphism resulting from the presence/absence of an Alu repeat in the exon h of the tPA gene was identified by polymerase chain reaction (PCR) and electrophoresis. We utilized primers described by Yang-Feng, *et al*[19]; 5' primer; 5'-TCCGTAACAGGACAGCTCA-3' (PR-TPAOL-1; nt 25,216-25,234), 3' primer; 5'-ACCGTGGCTTCAGTCATGGA-3' (PR-TPAOL-2; nt 26,181-26,162). PCR conditions were as follows: 10 μl of reaction liquid containing 5 pmol of each primer, 1 μl of 10 × PCR buffer II, 0.8 μl of dNTP mixture (2 mM of each dNTP), 0.6 μl of $MgCl_2$ (25 mM), and 0.08 μl of ampli Taq Gold (Perkin-Elmer, Norfolk, CT, USA), was subjected to the initial denature step for 9 min at 94°C, followed by 35 cycles of denaturing at 94°C for 1 min, annealing step at 56°C for 1 min, extension step at 72°C for 2 min, and an additional extension step at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gels. Nine hundred and sixty-seven bp and 655 bp bands correspond to I and D alleles, respectively.

Table 1. Clinical features of the patients. Values are the number of positive/number of patients tested (%).

	Japanese	Caucasian
Arterial thrombosis	21/77 (27.3)	41/82 (50.0)
Venous thrombosis	7/77 (9.1)	41/82 (50.0)
Pregnancy loss	10/51 (19.6)	30/72 (41.7)
Anticardiolipin	39/77 (50.6)	60/82 (73.2)
Lupus anticoagulant	56/73 (76.7)	49/82 (59.8)

Analysis of 4G/5G polymorphism in PAI-1 promoter gene. Site-directed mutagenesis PCR (SDM-PCR) and subsequent restriction fragment length polymorphism (RFLP) analysis, using DraIII (New England Biolabs, Beverly, MA, USA) were done to analyze the 4G/5G polymorphism of the PAI-1 promoter gene. DraIII restriction enzyme site was introduced into amplification products of the 4G allele by SDM-PCR. The upstream primer, described by Kimura, *et al*[20], 5'-TCCAACCTCAGCCAGACAAG-3' (PAI-1 pr1) and the newly designed downstream primer 5'-TGATACACGGCTGACTCACC-3' (underlining indicates the mutation introduced to form the restriction site for DraIII) were utilized. PCR was done in the same fashion except for the annealing temperature (65°C) and each reaction time of denaturing, annealing, and extension steps (30 s). Five microliter of SDM-PCR products were digested overnight with 10 units of Dra-III at 37°C and resolved on a 4% agarose gel. DraIII digests the amplified products from the 4G alleles into 2 fragments of 71 and 19 bp. Products amplified from genes with the 5G allele remain undigested (Figure 1). The accuracy of SDM-PCR and RFLP system for detection of the PAI-1 4G/5G gene polymorphism was confirmed by sequence analysis in several randomly selected samples.

Determination of aPL. Anticardiolipin enzyme immunoassays and LAC assays were done in these patients. IgG/M aCL were measured by standard aCL ELISA[21]. Existence of LAC was determined, according to guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-dependent Antibodies[22], by prolonged activated partial thromboplastin time, dilute Russell's viper venom time or kaolin clotting time, and their correction by phospholipid or platelets.

Statistical analysis. Correlations between the polymorphisms of tPA and PAI-1 genes and clinical symptoms (arterial thrombosis, venous thrombosis, pregnancy loss) were analyzed. Contingency table analyses were done using Fisher's exact test. Both univariate and multivariate analysis were done. P values ≤ 0.05 were considered to have statistical significance.

RESULTS

Genotypes and allele frequencies of I/D polymorphism of tPA and 4G/5G polymorphism of PAI-1 genes of both Japanese and Caucasian healthy volunteers are similar to those documented[14,20,23]. As shown in Table 2, these data are consistent with the distribution predicted by the Hardy-Weinberg equilibrium. Significant differences in both genotypes and allele frequencies of both polymorphisms of tPA and PAI-1 genes did not exist between patients and controls, or between Caucasian and Japanese.

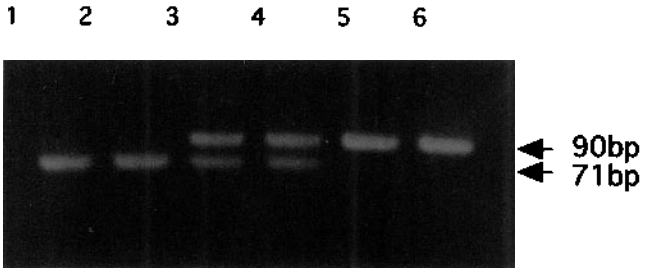

Figure 1. Analysis of the plasminogen activator inhibitor-1 (PAI-1) gene. Site-directed mutagenesis PCR and subsequent RFLP analysis were used to analyze 4G/5G polymorphism in the promoter region of PAI-1 gene. The Dra III restriction site was introduced into 4G alleles (71+19bp), while the 5G alleles remained undigested (90 bp). Lane 1 and 2: homozygous for 4G allele; lane 3 and 4: 4G/5G; lane 5 and 6: homozygous for the 5G allele.

Table 2. Prevalence of I/D polymorphism of tPA gene and 4G/5G polymorphism of PAI-1 gene. Values are numbers of genotypes or alleles (%).

Genotype	tPA II	tPA DI	tPA DD	PAI-1 4G/4G	PAI-1 4G/5G	PAI-1 5G/5G
Japanese						
aPL+ patients	22 (28.6)	35 (45.5)	20 (26.0)	23 (29.9)	45 (59.7)	8 (10.4)
Healthy controls	43 (30.0)	66 (45.8)	35 (24.3)	38 (26.4)	73 (50.7)	33 (22.9)
Caucasian						
aPL+ patients	30 (35.7)	34 (40.5)	18 (21.4)	25 (30.5)	38 (46.3)	19 (23.2)
Healthy controls	9 (23.7)	21 (55.3)	8 (21.1)	10 (26.3)	22 (57.9)	6 (15.8)

Allele	I Allele	D Allele	4G Allele	5G Allele
Japanese				
aPL+ patients	79 (51.3)	75 (48.7)	92 (59.7)	62 (40.3)
Healthy controls	152 (52.8)	136 (47.2)	149 (51.7)	139 (48.3)
Caucasian				
aPL+ patients	90 (57.3)	70 (42.7)	88 (53.7)	76 (46.3)
Healthy controls	39 (51.3)	37 (21.1)	42 (55.3)	34 (44.7)

aPL: antiphospholipid antibodies; tPA: tissue plasminogen activator, PAI-1: plasminogen activator inhibitor-1.

In monovariate analysis, I allele of tPA, when compared with D allele, had a weak correlation with a history of arterial thrombosis (p = 0.029) in Japanese patients, while in Caucasians, D allele of tPA, compared with I allele, had a weak correlation with a history of venous thrombosis (p = 0.018). Statistical significance was lost when multivariate analyses were done (Table 3A). There was no significant correlation between the gene polymorphism of PAI-1 and clinical symptoms (Table 3B). No allele investigated in this study was commoner in patients with multiple symptoms than in the others (Table 3). We also investigated the association between APS symptoms and both of I/I genotype of tPA (compared with non-I/I genotypes) and 4G/4G genotype (compared with non-4G/4G genotypes), finding no significant correlations (Table 4).

DISCUSSION

The consequences of tPA or PAI-I polymorphisms have been discussed. In 1992, Ludwig, *et al*[23] found an Alu-repeat I/D polymorphism in intron h of the tPA gene. Basal tPA release rates were unaffected by this polymorphism when human umbilical vein endothelial cells were used[24], but *in vivo*,

Table 3A. Correlation between clinical features in patients with antiphospholipid antibodies and alleles of tissue plasminogen activator. Odds ratio as the relative risk of I allele for having each manifestation.

		Allele Count I Allele	Allele Count D Allele	Odds Ratio	95% CI	p Value Univariate	p Value Multivariate
Japanese							
Arterial thrombosis	(+)	28	14				
	(–)	51	61	2.39	1.14–5.02	0.029	0.876
Venous thrombosis	(+)	5	9				
	(–)	74	66	0.50	0.16–1.55	0.269	
Pregnancy loss	(+)	13	7				
	(–)	42	40	1.77	0.64–4.88	0.323	
Multiple symptoms	(+)	6	2				
	(–)	88	68	2.32	0.45–11.85	0.468	
Caucasian							
Arterial thrombosis	(+)	47	35				
	(–)	47	35	1.00	0.54–1.86	> 0.9999	
Venous thrombosis	(+)	39	43				
	(–)	55	27	0.45	0.24–0.84	0.018	0.053
Pregnancy loss	(+)	33	27				
	(–)	50	34	0.83	0.43–1.62	0.616	
Multiple symptoms	(+)	21	25				
	(–)	72	45	0.53	0.26–1.05	0.793	

Table 3B Correlation between clinical features in patients with antiphospholipid antibodies and alleles of plasminogen activator inhibitor-1. Odds ratio as the relative risk of I allele for having each manifestation.

		Allele Count 4G Allele	5G Allele	Odds Ratio	95% CI	p Value Univariate
Japanese						
Arterial thrombosis	(+)	20	22			
	(–)	72	40	0.51	0.25–1.03	0.067
Venous thrombosis	(+)	9	5			
	(–)	83	57	1.24	0.39–3.88	0.783
Pregnancy loss	(+)	13	7			
	(–)	44	38	1.60	0.58–4.43	0.454
Multiple symptoms	(+)	6	2			
	(–)	86	60	2.09	0.41–10.73	0.476
Caucasians						
Arterial thrombosis	(+)	42	40			
	(–)	46	36	0.82	0.44–1.52	0.639
Venous thrombosis	(+)	42	40			
	(–)	46	36	0.82	0.44–1.52	0.639
Pregnancy loss	(+)	35	25			
	(–)	43	41	1.34	0.68–2.60	0.742
Multiple symptoms	(+)	26	20			
	(–)	62	56	1.17	0.59–2.33	0.728

Table 4. Associations of I/I genotype of tPA or 4G/4G genotype of PAI-1 gene and APS symptoms. Values are number of patients positive/number of patients tested. p Values of I/I genotype vs non-I/I genotypes and 4G/4G genotype vs non-4G/4G genotypes were calculated.

	Genotypes of tPA I/I	Non-I/I	p	Genotypes of PAI-1 4G/4G	Non-4G/4G	p
Japanese						
Arterial thrombosis	10/22	11/54	0.122	3/23	18/53	0.174
Venous thrombosis	1/22	6/54	0.667	2/23	5/53	> 0.999
Pregnancy loss	4/14	6/37	0.462	5/14	5/37	0.261
Multiple symptoms	2/22	2/54	0.579	2/23	2/53	0.585
Caucasians						
Arterial thrombosis	15/30	9/52	> 0.999	12/25	29/57	> 0.999
Venous thrombosis	9/30	31/52	0.150	13/25	28/57	> 0.999
Pregnancy loss	10/26	20/46	0.824	10/22	20/50	0.817
Multiple symptoms	4/30	19/52	0.129	8/25	15/57	0.800

release rates were markedly higher in subjects homozygous for the I allele[9]. In a study done in The Netherlands, the I allele was significantly more frequent than the D allele in patients with myocardial infarction[11]. However, subsequent studies in other populations found no such association[12,13].

In 1993, 4G/5G polymorphism located in the 5'-untranslated region at position -675 of the PAI-1 gene was described by Dawson, *et al*[10], and an *in vitro* study using HepG2 cells indicated that the 4G allele is associated with enhanced gene expression under stimulation. In addition, there are reports showing that the 4G allele corresponds to significantly higher PAI-1 levels than does the 5G allele[15,25-27]. In studies done in Sweden, the 4G allele was reported to be a risk for myocardial infarction[25] and deep vein thrombosis[16]. In contrast, other studies found that this polymorphism was not a risk factor for thrombosis[26,28].

A possible relationship between impaired fibrinolysis and APS has to be considered. These polymorphisms may be used as a discriminator value to stratify patient populations with respect to incremental function of these proteins. In patients with aPL, some studies noted impairments in the fibrinolytic system, which is vital for preventing clot formation and occlusion in vessels. Francis, *et al*[29] reported that, in some patients with LAC and thrombosis, increments in tPA activity after venous occlusion were not evident. Jurado, *et al*[30] reported higher PAI-1 levels before and after venous occlusion in patients with autoimmune diseases. Decreased tPA release and higher mean PAI-1 levels were investigated[31,32], although in

the former report there was no correlation with high level of aPL. Reduced fibrinolytic response to venous occlusion was also noted in patients with aPL who did not have SLE[33].

We speculated that the reported perturbation in fibrinolysis by tPA and PAI-1 gene polymorphisms may alter the risks of thrombosis in APS patients more significantly than in patients with other thrombotic disorders. However, our current study, revealed neither the I allele of tPA nor the 4G allele of PAI-1 to be significant risk factors for thrombosis or pregnancy loss in patients positive for aPL. While preparing our study, the positive correlation between PAI-1 polymorphism and thrombosis in APS was reported in a Catalan population[34]. In Japanese and in British Caucasians, however, we found no correlation between these gene polymorphisms and symptoms of APS. Polymorphism of the tPA and the PAI-1 gene does not seem to significantly influence the risk of thrombosis or other symptoms in patients with APS.

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