Impact of the 4G/5G polymorphism in the plasminogen activator inhibitor-1 gene on primary nephrotic syndrome

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Abstract. The aim of the present study was to investigate whether the four guanosines (4G)/five guanosines (5G) polymorphism in the gene coding for plasminogen activator inhibitor-1 (PAI-1) affects the clinical features of primary nephrotic syndrome (PNS). A cohort of 200 biopsy-diagnosed PNS patients was studied, with 40 healthy subjects as controls. The *PAI-1* gene polymorphism was detected by polymerase chain reaction and DNA sequencing. Associations between the *PAI-1* 4G/5G polymorphism and clinical features and pathological types of PNS were analyzed. The results indicated that the *PAI-1* genotype distribution is significantly different between patients with PNS and healthy controls, with significantly higher numbers of the 4G/4G genotype and lower numbers of the 5G5G genotype detected in PNS patients compared to controls (both $P<0.05$). The frequency of the 4G allele was also significantly higher in PNS patients compared to healthy controls ($P<0.01$). Among the different pathological types of PNS, IgA nephropathy (IgAN) and membranous nephropathy (MN) were associated with significantly increased frequencies of the 4G/4G and 4G/5G genotypes, as well as of the 4G allele. The increased 4G frequency was also detected in patients with minimal change disease (MCD). Significantly increased international normalized ratio (INR) and prolonged activated partial thromboplastin time (APTT) were observed in 4G/4G compared to 5G/5G PNS subjects. The response to steroids was not significantly different among the three genotypes. In conclusion, the 4G allele of the *PAI-1* gene appears to be associated with PNS, especially in MN and IgAN patients. These findings suggest that specific targeting may be required for the treatment of PNS patients with the 4G/4G genotype.

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Introduction

Plasminogen activator inhibitor (PAI)-1 is a key inhibitor of urokinase plasminogen activator (u-PA), and tissue plasminogen activator (t-PA), both of which mediate the conversion of plasminogen to plasmin. Plasmin degrades fibrin and other protein substrates, promoting fibrinolytic processing and degradation of the extracellular matrix (ECM). PAI-1 overexpression may compromise fibrin clearance mechanisms and promote pathological fibrin deposition and thrombotic events (1), a complication commonly seen in patients in hypercoagulable state, including primary nephrotic syndrome (PNS).

Thromboembolism occurs in ~25% of adults with PNS (2), and its incidence varies with the pathological types of PNS. In membranous nephropathy (MN), the incidence of renal vein thrombosis (RVT) can be as high as 37%, whereas the cumulative incidence is only ~24% in the remaining common types of nephrotic syndrome, including membranoproliferative glomerulonephritis (MPGN), minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) (3,4). It has been shown that the serum PAI-1 level in patients with membranous glomerulopathy is increased by 6-fold compared to controls, accompanied by diminished glomerular fibrinolysis (4).

The human *PAI-1* gene is located on chromosome 7q22 (5). A genetic polymorphism of this gene has been identified in the promoter region, where one allele has a sequence of four guanosines (4G) and the other has five guanosines (5G), upstream (-675 bp) of the mRNA initiation site (6). Both 4G and 5G alleles have a binding site for an activator of transcription. However, the 5G allele has an additional binding site for a repressor, leading to lower transcription rates and thereby, lower PAI-1 activity (7). The plasma level of PAI-1 in patients with the 4G/4G genotype is 25% higher compared to that of patients with the 5G/5G genotype, while 4G/5G genotypes show intermediate PAI-1 levels (8,9). The 4G/5G polymorphism has been linked to a number of pathological conditions and diseases. The 4G allele appears to increase the risk of venous thrombosis (10), and is associated with poor outcome in meningococcal disease (11).

Evidence is accumulating that PAI-1 is crucial in the acute-phase response (12). PAI-1 is an acute-phase protein, as PAI-1 levels markedly increase in response to inflammation or injury. The nature of the polymorphism can thus be described as response polymorphism, suggesting that the difference in PAI-1 levels between 4G and 5G becomes clearer

in the presence of environmental and/or disease factors, which stimulate PAI-1 expression. Therefore, for the other determinants of plasma PAI-1, we will also describe the evidence for interactions with the 4G/5G-polymorphism. In this study, we evaluated whether the *PAI-1* gene 4G/5G polymorphism is associated with PNS in a Chinese population.

Materials and methods

Patients and control subjects. A cohort of 200 biopsy-diagnosed PNS patients were recruited for the study. Exclusion criteria were: i) secondary nephrotic syndrome; ii) pregnancy and nursing females; iii) severe trauma, burn or major surgeries; iv) renal insufficiency with serum creatinine level ≥177 μmol/l. Medical records of all the patients were reviewed, and clinical information was retrieved. Recorded patient characteristics included: demographic variables, clinical and laboratory data of the disease progression, and treatment regimens as well as response to these regimens. Another 40 healthy subjects were included and served as controls. Informed consent was obtained from all participating individuals. The study protocol complied with the ethical guidelines of our hospital.

DNA isolation and genotyping. Genomic DNA was extracted from peripheral blood leukocytes of all 200 PNS patients and 40 healthy controls using a genomic DNA extraction kit (Qiagen, Hilden, Germany). A pair of PCR primers (forward, 5'-CAC GTT GGT CTC CTG TTT CCT T-3' and reverse, 5'-TGC TTT TCC TTT GGC GAA C-3') targeting a 443-bp region bordering the *PAI-1* gene promoter polymorphism at -675 bp was designed and used to amplify the region. The PCR products were purified, sequenced (ABI3730 analyzer; Applied Biosystems, Foster City, CA, USA), preprocessed with Chromas (Technelysium Pty Ltd., South Brisbane, Australia) and aligned with ClustalX version 1.83 software (European Bioinformatics Institute, Hinxton, UK) to identify the *PAI-1* 4G/5G gene polymorphism.

The DNA sequence of the PCR products was identical among the patients, control subjects and the published GenBank sequence (Gene ID: 5054) except for the -675 site of the *PAI-1* promoter. The genotypes associated with the 4G/5G polymorphism were 4G/4G, 4G/5G and 5G/5G (Fig. 1).

Statistical analysis. Continuous variables were expressed as means ± standard error. One-way ANOVA was performed to determine differences among groups when the data were normally distributed, whereas when data were not normally distributed, the Kruskal-Wallis test was used. For comparisons between two groups, t-tests or Mann-Whitney U-tests were used when appropriate. The χ^2-test or Fisher exact test was used to assess significance of differences when variables were dichotomous. Two-sided test P-values <0.05 were considered to indicate significant differences. Analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Distribution of the PAI-1 gene 4G/5G polymorphism and allele frequency in PNS. To determine whether the 4G/5G polymorphism is associated with PNS, a total of 240 participants (40 healthy controls, 200 PNS patients) were genotyped. The distribution of the 4G/4G (50.5%), 4G/5G (38.0%) and 5G/5G (11.5%) genotypes in PNS patients was significantly different compared to corresponding distributions observed in healthy controls (30.0, 45.0 and 25.0%, respectively; P<0.05). The allele frequency of 4G (69.5%) and 5G (30.5%) in PNS subjects also differed from that observed in healthy controls (52.5% for 4G and 47.5% for 5G, P<0.05) (Table I).

Distribution of the 4G/5G polymorphism and allele frequency in different glomerulonephropathies. Considering the diverse pathological types of PNS, we compared the distribution of the *PAI-1* 4G/5G genotype and the 4G and 5G allele frequencies across different glomerulonephropathies. Among the 200 PNS patients, there were 25 diagnosed with IgA nephropathy (IgAN) and 38 with MN, and these showed a higher frequency of the 4G/4G genotype (72.0 and 52.6%, respectively) and a lower frequency of the 5G/5G one (8.0 and 2.6%) compared to healthy controls (4G/4G, 30.0% and 5G/5G, 25.0%, P<0.05) (Fig. 2). The frequency of the 4G allele was significantly higher in the patients diagnosed with MCD, IgAN and MN compared to healthy controls. There were no significant differences in the distribution of 4G/5G polymorphism and allele frequency in patients with FSGS, MsPGN and MPGN (Table II).

Relationship between PAI-1 genotypes and clinical features of PNS. The clinical features of PNS patients with the three different *PAI-1* genotypes are shown in Table III. There was no difference in gender distribution, age of onset, serum creatinine, blood urea nitrogen (BUN), albumin, total cholesterol and triglyceride levels, proteinuria and platelet count. The therapeutic response to steroids did not differ among the genotypes. Among the different clinical features examined, only the international normalized ratio (INR) and the activated partial thromboplastin time (APTT) showed significant differences between the three genotypes, with an increase observed in PNS patients with the 4G/4G genotype compared to 5G/5G patients (Table III).

Discussion

A significant body of evidence indicates that activation of clotting factors and thrombin formation occur in nephrotic syndrome (13). First, deep venous thrombosis in the nephrotic syndrome occurs preferentially in the renal veins. Second, elevated fibrinogen levels in renal vein blood correlate with intraglomerular fibrin deposition, and may contribute to the development of glomerular injury (14). Glomerular fibrin depositions are observed in various glomerulopathies (15), as a result of intra-rena1 thrombin formation (13). Third, glomeruli contain haemostatic elements that promote *in situ* coagulation (16). Expression of PAI-1 is also increased in glomeruli and may promote fibrin deposition and lead to a higher propensity for the development of RVT. Our data are not sufficient to establish that intraglomerular coagulation is the primary abnormality accounting for increased thromboembolic complications, but do suggest that the 4G/5G *PAI-1* polymorphism may play a role in nephrotic syndrome.

Glomerular fibrinolysis is reduced in nephrotic syndrome patients. Plasminogen levels are also decreased in nephrotic

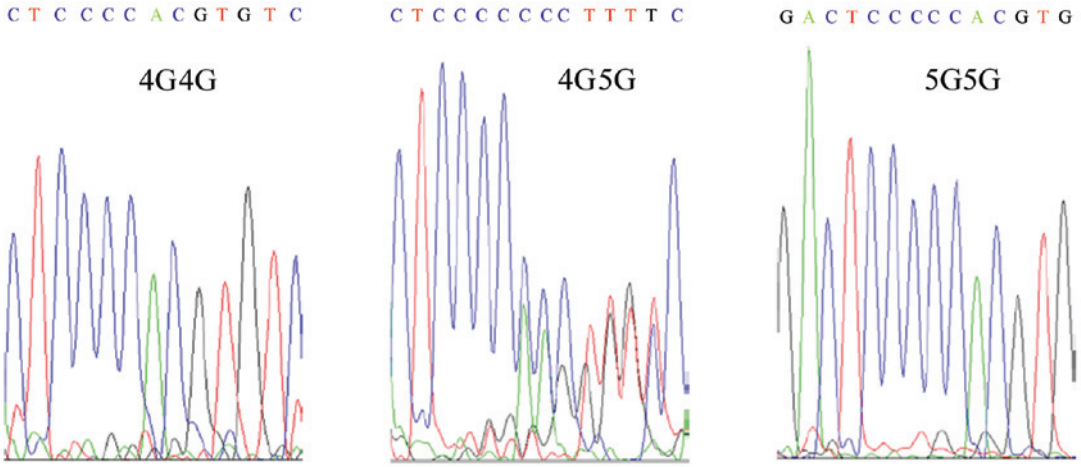

Figure 1. Chromatograms showing different genotypes based on the presence of the four guanosines (4G)/five guanosines (5G) polymorphism at -675 bp of the plasminogen activator inhibitor-1 gene.

Table I. Characteristics of primary nephrotic syndrome patients and healthy controls.

Features	PNS	Control	P-value
No.	200	40	
Gender (M/F)	129/71	13/27	<0.05
Age (years)	31.7±13.5	28.3±8.8	NS
PAI-1 genotype			<0.05
4G/4G	101 (50.5)	12 (30.0)	
4G/5G	76 (38.0)	18 (45.0)	
5G/5G	23 (11.5)	10 (25.0)	
Allele frequency			<0.05
4G	278 (69.5)	42 (52.5)	
5G	122 (30.5)	38 (47.5)	

M, male; F, female; NS, not significant; 4G, four guanosines; 5G, five guanosines.

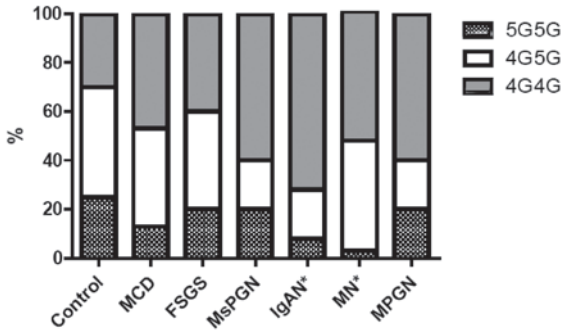

Figure 2. Distribution of different genotypes based on the presence of the four guanosines (4G)/five guanosines (5G) polymorphism in the plasminogen activator inhibitor-1 gene in different glomerulonephropathies associated with primary nephrotic syndrome. *P<0.05 vs. control.

syndrome, correlating with the degree of proteinuria (17). Furthermore, hypoalbuminemia itself has been postulated to contribute to fibrinolysis. Albumin is a cofactor for the binding of plasminogen to fibrin and their interaction with t-PA (14). PAI-1 is a key regulator of the fibrinolytic system that converts plasminogen to plasmin. A previous study showed that the urinary PAI-1 level, and not the plasma PAI-1 level, is higher in a nephrotic group of patients compared to a non-nephrotic group, suggesting overproduction of PAI-1 in the kidneys or increased urinary losses of PAI-1 in patients with primary nephrotic syndrome (18).

The exact mechanism by which the *PAI-1* 4G allele exerts its deleterious effect is not fully understood. Bioinformatic analysis (19) showed that there is an E-box motif (CACGTG) from -682 to -677 bp, overlapping with the 4G/5G site by a guanosine nucleotide (E-4G/5G). In the same study, the activity of the 4G-*PAI-1* promoter was found to be higher than that of the 5G-*PAI-1* promoter in stimulated mast cells (MCs); however, following substitution of the central dinucleotide CG with AT in E-4G/5G by mutagenesis, the activities of 4G- and 5G-*PAI-1* promoters were decreased, and notably, these activities became comparable. These results suggest that E-4G/5G is a positive regulatory element for *PAI-1* expression in stimulated MCs, and more importantly, it is critical for 4G/5G polymorphism-dependent *PAI-1* expression. Using the supershift assay with antibodies targeting a number of potential E-box-binding factors, it was found that the upstream stimulatory factor (USF)-1 binds to the E-4G/5G in the form of a homodimer (19). Similar results were observed in renal epithelial cells, where the E-box motif locating on the promoter (-165 to -160 bp) of the *PAI-1* rat ortholog was bound only by USF-1 but not USF-2 (20). However, in adipocytes and epidermal keratinocytes, the E-4G/5G was occupied by both USF-1 and USF-2 (21). These results suggest that the binding pattern of USFs to the E-box is cell type-dependent. Further analysis (19) indicated that USF-1 binds to the E-4G site with a higher affinity than to the E-5G site. When the 3' flanking sequences of E-5G were changed, the DNA-binding affinity of USF-1 was increased. This suggests that the 3' sequence of E-4G and E-5G determines the binding affinity of USF-1 to these sites, which is consistent with previous findings showing

Table II. Distribution of the 4G/5G polymorphism in glomerulonephropathies associated with primary nephrotic syndrome.

PAI-1 polymorphism	Control n=40	MCD n=94	FSGS n=15	MsPGN n=23	IgAN n=25	MN n=38	MPGN n=5
Genotype							
4G/4G (no.)	12	44	6	10	18*	20*	3
(%)	(30.0)	(46.8)	(40.0)	(43.5)	(72.0)	(52.6)	(60.0)
4G/5G (no.)	18	38	6	9	5	17	1
(%)	(45.0)	(40.4)	(40.0)	(39.1)	(20.0)	(44.7)	(20.0)
5G/5G (no.)	10	12	3	4	2	1	1
(%)	(25.0)	(12.8)	(20.0)	(17.4)	(8.0)	(2.6)	(20.0)
Allele							
4G (no.)	42	126*	18	29	41*	57*	7
(%)	(52.5)	(67.0)	(60.0)	(63.0)	(82.0)	(75.0)	(70.0)
5G (no.)	38	62	12	17	9	7	3
(%)	(47.5)	(33.0)	(40.0)	(37.0)	(18.0)	(25.0)	(30.0)

4G, four guanosines; 5G, five guanosines; MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; MsPGN, mesangial proliferative glomerulonephritis; IgAN, IgA nephropathy; MN, membranous nephropathy; MPGN, membranoproliferative glomerulonephritis. *P<0.05 vs. control.

Table III. Relationship between plasminogen activator inhibitor-1 *(PAI-1)* genotypes and clinical features of primary nephrotic syndrome.

Clinical features	4G/4G	4G/5G	5G/5G	P-value
No.	101	76	23	
Gender (M/F)	65/36	50/26	14/9	NS
Age at biopsy (years)	34.2±1.5	30.9±1.4	31.4±2.5	NS
Serum Cr (μmol/l)	93.5±3.5	90.0±3.3	85.6±4.9	NS
BUN (mmol/l)	6.5±0.4	6.1±0.3	6.7±1.4	NS
Proteinuria (0-4)	3.1±0.1	3.1±0.1	3.0±0.2	NS
Serum albumin (g/l)	26.6±2.7	23.4±0.9	24.1±1.7	NS
Cholesterol (mol/l)	9.1±0.3	10.0±0.4	10.0±0.9	NS
Triglyceride (mmol/l)	3.0±0.2	3.1±0.2	3.0±0.4	NS
Platelet count (x109/l)	250±9	260±10	251±16	NS
PT (s)	11.7±0.2	11.5±0.4	10.8±0.3	NS
INR	0.96±0.01	0.94±0.01	0.89±0.02	<0.05
APTT (s)	32.0±1.0	30.8±0.9	26.3±1.5	<0.05
Response to steroids (sensitive vs. insensitive cases)	49 vs. 52	35 vs. 41	10 vs. 13	NS

4G, four guanosines; 5G, five guanosines; M, male; F, female; Cr, creatinine; PT, prothrombin time; INR, international normalized ratio; APTT, activated partial thromboplastin time BUN, blood urea nitrogen; NS, not significant.

that the flanking sequence of the E-box core motif contributes to the binding affinity of USFs (22).

The PAI-1 protein is strongly expressed in various forms of renal disease, and it may play an important role in disease severity and progression. In addition, PAI-1 has been associated with accumulation of ECM, glomerulosclerosis and tubulointerstitial fibrosis. Krag *et al* (23) reported that a deficiency in the *PAI-1* gene attenuates TGF-β1-induced kidney disease in an animal model, by causing a decrease in glomerular and interstitial ECM deposition. Those authors concluded that PAI-1 mediates some of the biological effects of TGF-β1 *in vivo*, although they did not demonstrate that the effect is mediated by increased protease activity. However, Wang *et al* (24) found that Chinese patients with systemic lupus erythematosus having the 4G/4G genotype show significantly increased proteinuria and a higher lupus nephritis activity index than those having the 4G/5G or 5G/5G genotypes. The authors were not able to find a correlation between *PAI-1* gene polymorphisms and chronicity of lupus nephritis. A few studies have indicated that *PAI-1* polymorphisms may play a role

in the progression of IgA and diabetic nephropathy (25,26). Our study did not find a significant association between the *PAI-1* gene polymorphism and renal function or proteinuria, suggesting that PAI-1 plays a systemic, rather than a specific role in the kidney of PNS patients. No significantly different responses to steroid therapy were observed between the 4G/4G and 5G/5G genotype groups, which suggests that treatment specifically targeting PAI-1 may be useful in therapy of PNS patients, especially those diagnosed with IgAN and MN.

Analysis of prothrombin time (PT) and activated partial thromboplastin time (APTT) showed that these are prolonged in the 4G/4G genotype compared to the 5G/5G one. In general, PAI-1 has no effect on coagulation. There is no spontaneous bleeding or other major adverse effects in *PAI-1*-deficient mice or patients. Unlike other types of antithrombotic agents such as anticoagulation or antiplatelet agents, the PAI-1 antagonist does not prolong bleeding time and has almost no effect on APTT/PT time and platelet activity (27). However, most abnormal bleeding in *PAI-1*-deficient patients has been observed following trauma or surgery (28-30). In our study, hyperaggregability, hyperlipidemia and low serum albumin, combined with the presence of the *PAI-1* 4G allele in PNS patients may be responsible for the observed prolongation in PT.

In conclusion, the 4G/5G polymorphism of the *PAI-1* gene is associated with PNS. Findings from this and other studies may prompt for specific considerations for the treatment of PNS patients having the 4G/4G genotype.

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