J Korean Med Sci 2003; 18: 58-64
ISSN 1011-8934

Tissue Plasminogen Activator and Plasminogen Activator Inhibitor Type 1 Gene Polymorphism in Patients with Gastric Ulcer Complicated with Bleeding

Tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) may be involved in the pathogenesis of peptic ulcers through suppression of fibrinolysis. This study was designed to investigate associations of *t-PA* and *PAI-1* genes with clinical features of the patients with bleeding gastric ulcers. Eighty-four patients with peptic ulcers and 100 controls were studied between January 1998 and April 2000. We used polymerase chain reaction and endonuclease digestion to genotype for 4G/5G polymorphism in the promoter region of the *PAI-1* gene and the Alu-repeat insertion/deletion (I/D) polymorphism in intron h of the *t-PA* gene. Various clinical features, including lesion site, bleeding event, recurrence of ulcer, and rebleeding, were assessed using a multiple logistic regression model. The genotype distributions of both the *t-PA* and *PAI-1* genes did not differ between the patient and control groups. The occurrence of the I/D or D/D genotype of *t-PA* was significantly higher in cases of duodenal ulcer (adjusted OR=4.39, 95% CI=1.12-17.21). When a dominant effect (i.e., 4G/4G or 4G/5G versus 5G/5G) of the 4G allele was assumed, the *PAI-1* 4G/4G genotype was independently associated with rebleeding after hemostasis (adjusted OR=5.07, 95% CI=1.03-24.87). Our data suggest that *t-PA* gene polymorphism is associated with duodenal ulcers, and that the *PAI-1* gene may be a risk factor leading to recurrent bleeding after initial hemostasis.

Key Words : *Tissue Plasminogen Activator; Plasminogen Activator Inhibitor 1; Peptic Ulcer; Fibrinolysis; Hemostasis*

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Received : 4 September 2002
Accepted : 1 November 2002

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INTRODUCTION

Upper gastrointestinal hemorrhage is the most common complication of peptic ulcers. In approximately 20% of patients, ulcer-related hemorrhage occurs without preceding symptoms (1). Bleeding persists or recurs in a subgroup of these patients, which contributes to an overall mortality rate of 6-10%. This rate has remained remarkably stable over the past 20-30 yr (2), despite the tremendous advances in supportive care techniques such as blood-banking, intensive care units, and endoscopy (3-9).

Gastric hemorrhage may occur for many reasons, such as eroded vessels in an active ulcer, portal hypertension, or multiple gastric erosions. It has been assumed that this feature does not occur in all peptic ulcer cases (10). Many independent risk factors for ulcer hemorrhage have been described, such as higher age, use of nonsteroidal anti-inflammatory drugs (NSAIDs), and a history of prior peptic ulcers (11, 12). However, there is no satisfactory explanation of the pathogenesis of ulcer bleeding: *Helicobacter pylori* (HP) has not been shown to be an independent risk factor for ulcer hemorrhage (13), gastric acid hypersecretion is not necessary for hemorrhage in patients with peptic ulcers, and no significant differences in secretory parameters (e.g., baseline, peak, or meal-stimulated acid outputs, and parietal cell sensitivity) have been noted among ulcer patients with and without bleeding (14). Therefore, further studies are needed to determine the exact mechanism of ulcer hemorrhage.

Many reports have indicated that the fibrinolytic system plays a significant role in gastric bleeding (15-18), but most of these have focused on a specific parameter of the fibrinolytic system adjacent to the ulcer. The fibrinolysis, which is mediated mainly by the tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1), is a reparative process that occurs in response to hemostatic plug or thrombus formation (19). The fibrin that is formed during hemostasis must be removed when normal tissue structure and function is restored. Thus, a fibrin clot that forms quickly in a torn blood vessel to stop hemorrhage is remodeled and then removed to restore normal blood flow. The fibrinolytic system is the principal effecter of clot removal and controls the enzymatic degradation of fibrin. Its action is coordinated through the interaction of activators, zymogens, enzymes, and inhibitors

to provide local activation at the site of fibrin deposition.

Various methods have been used to evaluate systemic fibrinolytic activity, such as monitoring the euglobulin fibrinolytic activity, chromogenic bioassay, enzyme-linked immunosorbent assay, and radioimmunoassay for t-PA and PAI-1. However, there are several reasons why these methods do not provide an accurate measure of the patient's physiologic fibrinolytic activity: (a) physiologic fibrinolysis does not occur in the circulating blood but at the fibrin and/or thrombus site, so any method that measures fibrinolytic activity in a sample of circulating blood does not reflect fibrin-associated fibrinolysis; (b) the release of t-PA from endothelial cells can be modified by many factors such as exercise, venous occlusion, mental stress, sympathetic stimulation, and prostaglandin (20); and (c) variations in blood levels of PAI-1 are resulted from changes in the rate of *PAI-1* gene expression in the tissue rather than from the release of stored PAI-1 in the cells (19). With these factors kept in mind, it seems likely that the fibrinolytic activity measured at the gene level may provide a better assessment of fibrinolysis.

There is recent evidence that 4G/5G polymorphism in the promoter region of the *PAI-1* gene is related to circulating PAI-1 levels (21-24). In vitro experiments have shown that both alleles of the 4G/5G polymorphism contain a binding site for a transcription activator, and that the 5G allele also contains a binding site for a transcription repressor that partially overlaps with the activator binding site (21, 25). Therefore, the 4G allele of the *PAI-1* gene is associated with increased basal gene transcription. The 4G/4G genotype of *PAI-1* has been shown to be associated with ischemic heart disease (24, 26) and myocardial infarction (MI) (21, 27).

Similarly, the insertion/insertion (I/I) genotype of *Alu*-repeat insertion/deletion (I/D) polymorphism in the *t-PA* gene has been shown to be associated with the plasma PAI-1 antigen in patients with MI (28). Since t-PA and PAI-1 levels are positively correlated, the Alu-repeat I/D polymorphism could be a marker of a functional mutation in the *t-PA* gene that regulates the interaction between *t-PA* and *PAI-1*. The *Alu*-repeat insertion may also be closely linked to a mutation at or near the *t-PA* gene that produces a functional effect, and an Alu-repeat I/D event can alter mRNA stability and splicing (29). Although *Alu*-repeat I/D polymorphism in the *t-PA* gene is not associated with either t-PA plasma levels (28) or the basal synthesis rate of t-PA in endothelial cells (30), Alu-repeat I/D polymorphism in the *t-PA* gene may be a candidate marker for thrombosis and hemostasis (31).

The major pathogenic mechanisms for peptic ulcer are acid, HP, and NSAIDs (1). Of these factors, only NSAIDs appear to be an important risk factor for the development of bleeding (32-34). In order to avoid any influence of this factor in our study, we excluded any patient who had a history of NSAID within 3 months of the study.

Intramucosal proteolysis has been considered to be an aggressive factor in the development of peptic ulcers, and it has been shown that there is a significantly higher level of protease activity at the ulcer edge and from the antral mucosa of patients with an active duodenal ulcer (15-17). HP can induce peptic ulceration through direct production of several proteases capable of damaging the gastroduodenal mucosa (34, 35). It is well known that plasmin, the end product of the fibrinolytic system, converts latent collagenase to an active form. The maintenance of an adequate mucosal blood flow by adequate fibrinolysis is another essential protective factor (28, 29), and t-PA may be a key enzyme in this process. Nonetheless, the role of the fibrinolytic system on the development of peptic ulcers and/or ulcer bleeding is controversial.

Taking these observations into consideration, we hypothesized that the *t-PA* and *PAI-1* genes are factors leading to peptic ulcer and hemorrhage. In order to determine whether our hypothesis was correct, we analyzed I/D type polymorphism in intron h of the *t-PA* gene and 4G/5G polymorphism in the promoter of the *PAI-1* gene in patients with gastric ulcers with or without ulcer bleeding.

MATERIALS AND METHODS

Study subjects

Between January 1998 and April 2000, diagnostic and/or therapeutic endoscopy was performed in 84 patients with active peptic ulcers. The control group comprised 100 subjects who presented consecutively for multiphasic health check-up at Soonchunhyang University Chunan Hospital. Individuals with an NSAID-induced ulcer, underlying systemic disease, or bleeding tendency were excluded from the study.

The study design was approved by the Ethics Committee of Soonchunhyang University Chunan Hospital. Informed consent was obtained from all subjects.

We used historical information and endoscopic examinations to evaluate the lesion site for the presence of bleeding, recurrency, and HP infection. We performed several tests for HP infection, including a rapid urease test (CLO test; Ballard, Draper, Australia) that was observed for up to 24 hr, histological examination of an endoscopic biopsy, ^{13}C-labeled urea breath test (UBT), and serologic examination for the presence of immunoglobulin G antibody to HP (Radim, Pomezia, Italy). We determined the presence of HP infection in patients when two or more of the above tests produced positive results. Rebleeding was defined as the persistence or redevelopment of overt bleeding (hematemesis, melena, or hematochezia) or a reduction in hemoglobin of more than 1.5 g/dL within 5 days of initial hemostasis.

Blood sampling and DNA isolation

Blood samples were obtained from the antecubital vein between 7 a.m. and 9 a.m. after a fast and a 20 min rest. For DNA

analysis, blood was collected in K-EDTA vacuum tubes, and stored at -70℃. DNA was isolated using a commercial genomic DNA purification kit (QIAamp; QIAGEN, Valencia, CA, U.S.A.). Serum was separated from whole blood and used for biochemical assays.

Determination of genotypes

Allele-specific polymerase chain reaction (PCR) was performed according to Falk's assay (32) with some modifications. The *PAI-1* 4G/5G polymorphism was determined for each subject using two 17-mer allele-specific primers: 5′-GTCTGGACACGTGGGGG-3′ for insertion 5G allele and 5′-GTCTGGACACGTGGGGA-3′ for deletion 4G allele, in combination with the common downstream primer 5′-TGCAGCCAGCCACGACGTGATTGTCTA-3′. The PCR amplified 140-bp DNA fragments with the 5G-specific primer only, whereas in the 4G/4G genotype only 139-bp DNA fragments with the 4G-specific primer, and in heterozygotes both 140- and 139-bp DNA fragments were amplified. As a positive control for the PCR, a 256-bp DNA fragment was generated using a fourth primer, 5′-AAGCTTTTACCATGGTAACCCCTGGT-3′, located upstream of the polymorphic region in combination with the downstream primer. Each DNA amplification was performed in a total volume of 25 μL using 150-200 ng of genomic DNA. The reaction mixture contained 50 mM Tris-HCl buffer (pH 9.0), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate, and 0.6 μM of each primer, except for the upstream control primers which were 0.12 μM and 1.0 U of DNA polymerase. The mixture was subjected to 35 155-sec-long cycles of 94℃ for 35 sec, 65℃ for 45 sec, and 72℃ for 75 sec. The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and viewed under UV light.

An I/D polymorphism resulting from the presence or absence of an *Alu* repeat in intron h of the *t-PA* gene was identified in all subjects. Briefly, 25-50 ng of each genomic DNA sample was amplified by PCR in 25-μL reaction volumes. Upstream (5′-TCCGTAACAGGACACCTCA-3′) and downstream (5′-ACCGTGGCTTCAGTCATGGA-3′) primers were chosen as previously described (31). Briefly, 50 ng of DNA was amplified in a 25 μL reaction containing 45 mM Tris-HCl (pH 8.0), 11 mM ammonium sulfate, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 μM K-EDTA (pH 8.0), 1 mM each of dNTP, 100 μg/mL bovine serum albumin, 0.01% gelatin, 10 pmol of each primer, and 1 unit of *Taq* polymerase. The reaction was initially heated to 94℃ for 5 min, which was followed by 30 3-min-long cycles of 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 1 min. Amplified products (I allele=967 base pairs, D allele=655 base pairs) were resolved on 1.5% agarose gels and visualized by ethidium bromide fluorescence. Samples were classified according to three genotypes: I/I, D/D, and I/D. The classification was determined independently by two individuals who had no knowledge of the case or control status. The PCR direct sequencing was carried out from randomly selected samples to confirm the accuracy of allele-specific oligonucleotide genotyping.

Statistical analysis

Differences in baseline characteristics among three groups were evaluated by analysis of variance (ANOVA) and χ^2 tests for continuous and categorized variables, respectively. The observed numbers of each *PAI-1* genotype and each *t-PA* genotype were compared with those expected for a population in Hardy-Weinberg equilibrium using a χ^2 test. Associations of *PAI-1* and *t-PA* genotypes with the presence of peptic ulcers were estimated using odds ratio (OR) and 95% confidence interval (CI) measures. Both dominant and recessive models were constructed to test the associations. Multiple logistic regression was applied to evaluate the independent association of the *PAI-1* 4G/5G polymorphism and *t-PA* I/D polymorphism with peptic ulcer, whilst controlling for possible confounding factors. Gene-gene interaction between *PAI-1* and *t-PA* on peptic ulcer parameters was also assessed. Statistical analysis was performed using SPSS for Windows V 10.0 (SPSS Inc., Chicago, U.S.A.). *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Profile of patients

The clinical profiles of the patients are given in Table 1. Genotypes for *PAI-1* and *t-PA* were available in 84 patients with peptic ulcer and 100 and 98 of the controls, respectively. The mean (SD) age of the patients was 58.6 yr (15.1 yr), and 67 (79.8%) of them were male. The peptic ulcer was gastric in 56 cases and duodenal in 28 cases; its phase was categorized as initial in 64 cases and recurrent in 20 cases. Clinical bleeding was observed in 56 cases (66.7%). Of 32 patients subjected to the CLO test, 84.4% were positive. Fifty-six (66.7%) of the patients regularly drank alcohol, and 59 (70.2%) were cigarette smokers.

The clinical outcomes are presented in Table 2. Sixty patients required blood transfusion and 4 patients needed surgical operation for hemostasis. Rebleeding was observed in 9 patients. The mean (SD) duration of hospital treatment was 14.1 (8.8) days.

Gene polymorphisms of *t-PA* and *PAI-1*

The distribution of *t-PA* genotypes in 84 patients with a peptic ulcer was: 32 (38.1%) D/D, 30 (35.7%) I/D, and 22 (26.2%) I/I. This distribution was not significantly different from that of 98 control subjects (p=0.180): 25 (25.5%) D/D, 40 (40.8%) I/D, and 33 (33.7%) I/I (Table 3). No significant

Table 1. Clinical profiles of 84 patients with active peptic ulcer

Clinical variables	n (%)
Age (yr), mean±SD	58.6±15.1
Gender	
Male	67 (79.8)
Female	17 (20.2)
Lesion site	
Duodenum	28 (33.3)
Stomach	56 (66.7)
Ulcer bleeding phase	
Initial	64 (76.2)
Recurrent	20 (23.8)
Current bleeding	
No	28 (33.3)
Yes	56 (66.7)
Past ulcer history	
No	38 (45.2)
Yes	46 (54.8)
CLO test*	
Negative	5 (15.6)
Positive	27 (84.4)
Regular alcohol drinker	
No	28 (33.3)
Yes	56 (66.7)
Current smoker	
No	25 (29.8)
Yes	59 (70.2)
Systemic disease	
No	52 (61.9)
Yes	32 (38.1)

*CLO test was performed in 32 patients.

Table 2. Therapeutic and prognostic profiles of 84 patients with active peptic ulcer

Clinical variables	n (%)
Blood transfusion	
No	24 (28.6)
Yes	60 (71.4)
Treatment	
Endoscopy	80 (95.3)
Surgery	4 (4.7)
Rebleeding	
No	75 (89.3)
Yes	9 (10.7)
Hospitalization duration (days), mean (SD)	14.1 (8.8)

difference in frequency of the I allele was observed between control subjects (0.54) and peptic ulcer patients (0.63) (p= 0.193).

The distribution of *PAI-1* genotypes in the patients was: 26 (31.0%) 4G/4G, 42 (50.0%) 4G/5G, and 16 (19.1%) 5G/5G. This distribution was not significantly different from that of the 100 control subjects: 21 (21.0%) 4G/4G, 53 (53.0%) 4G/5G, and 26 (26.0%) 5G/5G (p=0.245, Table 3).

Table 3. Distributions of genetic polymorphisms of *t-PA* and *PAI-1* in patients and controls

Genetic polymorphisms	Patients n (%)	Controls n (%)	*p*-value
t-PA			0.180
II	22 (26.2)	33 (33.7)	
ID	30 (35.7)	40 (40.8)	
DD	32 (38.1)	25 (25.5)	
PAI-1			0.245
4G/4G	26 (31.1)	21 (21.0)	
4G/5G	42 (50.0)	53 (53.0)	
5G/5G	16 (19.1)	26 (26.0)	

Table 4. Associations between gene polymorphisms and site of ulcer

Gene	Model	Adjusted OR*	95% CI	*p* value
t-PA	Dominant			
	stomach vs duodenum	2.05	0.75-5.56	0.161
	Recessive			
	stomach vs duodenum	4.39	1.12-17.21	0.034
PAI-1	Dominant			
	stomach vs duodenum	0.85	0.24-2.97	0.799
	Recessive			
	stomach vs duodenum	1.16	0.41-3.26	0.783

*adjusted for age, gender, alcohol consumption, and smoking.

Table 5. Associations between gene polymorphisms and rebleeding

Gene	Model	Adjusted OR*	95% CI	*p* value
t-PA	Dominant			
	no vs rebleeding	1.83	0.41-8.25	0.429
	Recessive			
	no vs rebleeding	3.95	0.40-38.66	0.237
PAI-1	Dominant			
	no vs rebleeding	5.07	1.03-24.87	0.045
	Recessive			
	no vs rebleeding	0.97	0.20-4.58	0.966

*adjusted for age, gender, bleeding during endoscopy, ulcer site, alcohol consumption, and smoking.

Relations of gene polymorphism with ulcer site and rebleeding

Using a multiple logistic regression model in which a recessive effect (I/I vs. I/D or D/D) of the I allele was assumed, the I/I genotype of *t-PA* was found to be related to the presence of peptic ulcers. The occurrence of the I/D or D/D genotypes of *t-PA* was significantly higher in duodenal ulcers (adjusted OR=4.39, p=0.034). However, *PAI-1* was not related to ulcer site in both dominant and recessive models (Table 4).

When a dominant effect (4G/4G or 4G/5G versus 5G/5G) of the 4G allele was assumed, the *PAI-1* 4G/4G genotype was

significantly and independently related to rebleeding after initial hemostasis (adjusted OR=5.07, p=0.045). However, neither the recessive model of *PAI-1* nor recessive or dominant model of *t-PA* was related to rebleeding (Table 5).

The interactions between *PAI-1* and *t-PA* were calculated in both dominant and recessive models and evaluated for gene-gene interaction on ulcer sites and rebleeding as well as other clinical parameters in a further analysis. However, no significant interactions were observed (data not shown).

DISCUSSION

In the present study, the occurrence of the I/D or D/D genotypes of *t-PA* was higher in duodenal ulcers, and the *PAI-1* 4G/4G genotype was associated with rebleeding of a peptic ulcer. These results suggest that the t-PA and PAI-1 system plays a role in both ulcer formation and ulcer bleeding.

In our study, when age, gender, alcohol consumption, and smoking habits were controlled, there was an association between the I/D or D/D genotype of *t-PA* and the presence of a duodenal ulcer. This result suggests that the *t-PA* gene is an independent factor in the pathogenesis of duodenal ulcers. The so-called peptic ulcer disease encompasses both gastric and duodenal ulcers (1). Duodenal and gastric ulcers share many common features in terms of pathogenesis, diagnosis, and treatment, but several factors distinguish the two. Gastric ulcers tend to occur later in life than duodenal lesions. More than half of gastric ulcers occur in males, and they are less common than duodenal ulcers. As for duodenal ulcers, the majority of gastric ulcers can be attributed to either HP-or NSAID-induced mucosal damage. Gastric ulcers that occur in the prepyloric area or those associated with a duodenal ulcer are similar to duodenal ulcers in terms of pathogenesis (1). The exact mechanism of the formation of a peptic ulcer is beyond the scope of this study. However, our results have shown that the effects of the *t-PA* gene on peptic ulcer formation depends upon whether the ulcer occurs in the stomach or in the duodenum.

The other question relating to our results is the significance of the I/D and D/D genotypes of *t-PA*. *Alu*-repeat I/D polymorphism in the *t-PA* gene was not associated with either t-PA plasma levels (28) or the basal synthesis rate of t-PA in endothelial cells (30). Therefore, it is hard to explain why stimulated fibrinolysis is implicated in the pathogenesis of a duodenal ulcer. However, the I/I genotype of *Alu*-repeat I/D polymorphism in the *t-PA* gene shows an association with plasma *PAI-1* antigen in patients with MI (28). Since t-PA and PAI-1 levels are positively correlated, the *Alu*-repeat I/D polymorphism could be a marker of a functional mutation in the *t-PA* gene that regulates the interaction between t-PA and PAI-1. In this regard, *Alu*-repeat I/D polymorphism in the t-PA gene may be a candidate marker for thrombosis and hemostasis (31). Further studies are needed to clarify the property of the I/D or D/D genotypes of *t-PA* during duodenal ulcer formation.

Contrary to our expectations, the *PAI-1* 4G/4G genotype was an independent risk factor for rebleeding. It is known that an increased PAI-1 concentration induces decreased plasmin formation and leads to an accumulation of fibrin. The 4G allele and the 4G/4G genotype of *PAI-1* have been reported to be associated with increased basal gene transcription, and consequently have been regarded as thrombogenic factors (21, 24, 26, 27). With this kept in mind, our results are suggestive of some other role of PAI-1 in the process of rebleeding, except the effect on fibrinolytic activity.

PAI-1 is synthesized by endothelial cells and hepatocytes, and is present mainly in platelets and plasma. But some PAI-1 is also present in the subendothelial extracellular matrix, where its function may be inhibition of local proteolysis. PAI-1 activity induces changes in remodeling of the vessel wall through activation of metalloproteinases, growth factors, and degradation of the extracellular matrix (26, 27). In addition to its antiprotease activity, PAI-1 participates in the cellular adhesion and migration processes (36). Taken the well-known function of PAI-1 to inhibit local proteolysis into consideration, our results suggest that disturbed vessel wall remodeling may be involved in the rebleeding of peptic ulcers.

In the normal physiological state, the activities of coagulation and fibrinolysis are balanced. However, increased activity of either coagulation or fibrinolysis can overwhelm the other, to the extent of producing a clinical problem. Therefore, increased fibrinolysis and/or decreased coagulation may be involved in the pathogenesis of ulcer hemorrhage. However, we believe that the role of t-PA and PAI-1 in peptic ulcer bleeding differs between current-bleeding and rebleeding situations. Furthermore, it seems likely that peptic ulcer rebleeding is due in large part to the properties of PAI-1, which disturbs remodeling of injured vessel after initial bleeding.

In conclusion, our data suggest that *t-PA* gene polymorphism may be associated with duodenal ulcers, and that the *PAI-1* gene may be a risk factor leading to recurrent bleeding after initial hemostasis.

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