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A novel polymorphism in the PAI-1 gene promoter enhances gene expression. A novel pro-thrombotic risk factor?

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ABSTRACT

Introduction: Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor of tissue-type plasminogen activator in plasma and the most important regulator of the fibrinolytic pathway. The 4G/5G polymorphism (rs1799889) in the PAI-1 promoter is associated with altered PAI-1 transcription. We have identified a new 4G/5G allele, in which a T is inserted near the 4G tract or replaces a G in the 5G tract, forming a T plus 4G (T4G) region.
Materials and Methods: This new variant was first identified in two women, one had experienced juvenile myocardial infarction, the other repeated miscarriage; both had increased PAI-1 plasma activity. In view of the important influence of this promoter region on PAI-1 protein plasma level, we performed *in vitro* evaluation of the effects of the T4G variant on the transcription activity of the PAI-1 gene promoter.
Results and Conclusions: In silico prediction analysis showed that presence of the T4G allele disrupts the E-Box region upstream of the T4G variant, altering the affinity of the target sequence for E-Box binding factors like upstream stimulatory factor-1 (USF-1). Basal T4G promoter activity was 50% higher compared to 4G and 5G variants, but it was less stimulated by USF-1 overexpression. We also analyzed the effects of IL-1β and IL-6 on the PAI-1 promoter activity of our three constructs and showed that the T4G variant was less affected by IL-1β than the other variants. These findings indicate that the T4G variant may be a novel risk factor for thrombotic events.

Introduction

Plasminogen activator inhibitor (PAI)-1 is a monomeric glycoprotein produced by liver and endothelial cells. PAI-1 is a 52 kDa glycoprotein belonging to the serine protease inhibitor superfamily (serpin-1), which includes about 10% of all human plasma proteins. It displays serine protease inhibitor activity, mainly directed at the tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). High PAI-1 activity causes reduced activation of plasminogen and consequently poor fibrinolysis and thus increases the risk of thrombotic events [1,2] such as cardiovascular [3] and cerebrovascular [4] diseases, recurrent loss of pregnancy [5] and pre-eclampsia [6].

The human PAI-1 gene is located on chromosome 7 and contains 9 exons and 8 introns [7]. Several polymorphisms have been described within the gene, including a cytosine-adenine (CA)n dinucleotide repeat, a HindIII restriction fragment-length polymorphism and a common single base-pair polymorphism in the promoter region of the gene 675 bp upstream of the transcriptional start site, which is termed the 4G/5G polymorphism [8].

The common 4G/5G polymorphism was found in the PAI-1 gene promoter, and the 4G allele was associated with greater expression of PAI-1 [9] because it modulates affinity for the upstream stimulatory factor (USF-1) which enhances gene transcription [10]. Half the population is heterozygous (4G/5G) and 25% have a 5G/5G genotype. *In vitro* studies have demonstrated differential binding of transcription-regulating proteins at this site [11].

Because of the association between PAI-1 activity and thrombotic events molecular analysis for the 4G/5G PAI-1 polymorphism is routinely performed to assess individual thrombotic risk [12].

We identified a novel PAI-1 promoter polymorphism, the -675G > T, in two patients. The novel polymorphism significantly enhances gene transcription and may thus increase thrombotic risk.

Abbreviations: PAI-1, plasminogen activator inhibitor-1; SERPIN, serine protease inhibitors; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; USF-1, upstream stimulatory factor-1; ATIII, antithrombin III; HPA-1, human platelet antigen-1; MTHFR, methylenetetrahydrofolate reductase.

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Matherials and methods

Patients

The novel T4G mutation was identified in two young women. The first, a 37 year old woman, was pregnant after several first trimester miscarriages. She had tested negative for all known risk factors for recurrent miscarriage [13,14]. The second patient, a 39 year old woman, had experienced a juvenile myocardial infarction and was negative for known cardiac risk factors including family history [15]. Both subjects were referred to our laboratory for molecular analysis of pro-thrombotic gene mutations.

Light Cycler analysis and sequencing

Gene sequencing was used to analyze antithrombin III (ATIII) mutations [16]. Light Cycler-PCR melting curve analysis (Roche) was used to evaluate pro-thrombotic gene mutations (including PAI-1 polymorphism). The atypical melting peak observed in the PAI-1 profile was studied using sequencing analysis (protocol, primers and conditions available on request).

Plasmid constructs

A PAI-1 promoter region of 1.6 Kb containing 5′ between -1529 bp and +38 bp was amplified directly from human genomic DNA from the patients using the following primers: 5′-GGTACCAACCTCTGGGCC AGG-3′, 3′-GACAGCGCTCTTGGCCCTGCAGCCA-5′. The PCR products were digested with *KpnI* and *XhoI* and ligated into *KpnI* and *XhoI* digested pGL3-Basic vector upstream of the luciferase gene. The 4G, 5G and T4G allele variations were confirmed using sequence analysis. upstream stimulatory factor-1 (USF-1) ORF was amplified using RT (reverse transcription)-PCR and cloned in the pcDNA3 vector.

Statistical analysis

Wild-type and variant PAI-1 promoter activity were compared using the unpaired *t*-test; data were expressed as means ± SD for four different assays.

Cell cultures

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen) as previously described [17]. HuH-7 cells were cultured and maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen) with 10% heat-inactivated fetal bovine serum (HyClone) without the addition of antibiotics. Cells were maintained at 37 °C in a 5% (v/v) CO_2 humidified atmosphere.

Transient transfection and luciferase assay

HepG2 cells were transfected using IBAfect in a 96-well culture dish 24 h before experiments. At 80% confluence cells were cotransfected with 100 ng/3well of plasmid reporter and 50 ng/3well of internal control pRL-CMV containing Renilla Luciferase (Promega) driven by a CMV promoter to normalize transfection efficiency. The cells were incubated at 37 °C for 4 hours. pGL3- has no eukaryotic promoting or enhancing sequence and was therefore used as a negative control. The effects of USF-1 on PAI-1 transcription were investigated by cotransfecting HepG2 cells with 4G-pGL3-Basic-or T4G-pGL3-Basic or 5G- pGL3-Basic promoter constructs at increasing concentrations of human USF-1 expression vector. Experiments were performed independently four times. The effects of IL-1β and IL-6 on PAI-1 promoter activity were tested on transfected HepG2 cells placed on a 48-well culture dish. The medium was replaced by serum-free medium 6 h after transfection. 24 h after the transfection the cells were exposed to serum-free medium containing 1 ng/mL IL-1β, 1 ng/mL IL-6 and harvested 6 h later. Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega).

Table 1
Molecular analysis of pro-thrombotic gene mutations in the two patients.

Gene mutation	Patient #1	Patient #2
Factor V Leiden	wild type	wild type
Factor V HR2	wild type	wild type
FII G20120A	wild type	wild type
MTHFR C677T	heterozygous	mutant homozygous
MTHFR A1298C	heterozygous	wild type
PAI-1 4G/5G	T4G/5G	T4G/5G
HPA 1a/1b	wild type	wild type
Beta-fibrinogen-455G > A	wild type	heterozygous
ATIII	absence of mutations	absence of mutations

Results

We carried out molecular analysis to detect pro-thrombotic gene mutations in two patients (Table 1). In the first patient analysis revealed wild-type factor V Leiden and HR2 mutations; G20120A mutation of FII and 1a/1b polymorphism of human platelet antigen-1 (HPA-1); -455G/A polymorphism of fibrinogen; no ATIII mutations were found and the patient was heterozygous for both the C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene and had normal levels of serum homocysteine (Table 1). In the second patient, molecular analysis revealed wild-type factor V Leiden and HR2 mutations; G20120A mutation of FII; 1a/1b polymorphism of HPA-1 and A1298C polymorphism of MTHFR. No ATIII mutations were found and the patient was heterozygous for the -455G/A polymorphism of fibrinogen and mutant homozygous for the C677T polymorphism of the MTHFR gene and had normal levels of serum homocysteine (Table 1). Both patients had normal Protein C and Protein S activity. PAI-1 plasma activity was 8.3UI/mL and 10.1UI/mL respectively for patients 1 and 2 (reference values: 0.3-3.5UI/mL). Molecular analysis revealed that both patients were 4G/5G heterozygous mutant for the PAI-1 4G/5G polymorphism, with an atypical peak in the light cycler scan (Fig. 1 panel A).

Sequencing analysis of the PAI-1 promoter to determine the sequence of the atypical peak revealed, in both patients, the presence of a heterozygous nucleotide substitution/insertion at -675 bp of the PAI-1 promoter, in which the presence of a T results in a region formed by a T plus 4G (T4G). (Fig. 1 panel B). The T4G variant was not found in 200 unrelated healthy control subjects from southern Italy whose DNA was available in our biological bank.

In silico prediction analysis using the TFSearch software, showed that the presence of the T4G allele should remove the E-Box region upstream to T4G variant (Fig. 2), resulting in an altered affinity of the target sequence for E-Box binding factors e.g., USF-1 [10].

Further investigation of the effects of the T4G variant was carried out using a luciferase assay to compare the activity of 4G, T4G or 5G variants of the PAI-1 promoter. We cloned 1.6 kb of the PAI-1 gene promoter region into the pGL3-Basic vector, obtaining three constructs each bearing a different variant, i.e. 4G, 5G and the T4G. The luciferase activity of pGL3-PAI-1-T4G was about 50% higher than that of both pGL3-PAI-1-4G and pGL3-PAI-1-5G, ($p < 0.05$ and $p < 0.005$ respectively, Fig. 3). Similar results were obtained in the HuH-7 cell line (data not shown).

Because our *in silico* prediction revealed disruption of the E-Box motif upstream of the T4G polymorphism that binds the USF-1 protein [10] we evaluated the activity of our three PAI constructs following overexpression of USF-1 protein. We found that USF-1 overexpression increased pGL3-PAI-1-4G and pGL3-PAI-1-5G promoter activity by 100% and 57%, $p < 0.05$, and $p < 0.001$ respectively (Fig. 4). After overexpression of dominant negative USF-1 the activity of pGL3-PAI-1-4G and pGL3-PAI-1-5G was decreased by 21% and 36% ($p < 0.001$ and $p < 0.05$