Diabetic retinopathy, PAI-1 4G/5G and −844G/A polymorphisms, and changes in circulating PAI-1 levels in Tunisian type 2 diabetes patients

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Received 16 April 2008; received in revised form 29 November 2008; accepted 1st December 2008
Available online 5 May 2009

Abstract

Aim. – The association of altered plasminogen activator inhibitor (PAI)-1 levels and PAI-1 polymorphisms (4G/5G and −844G/A) with diabetic retinopathy (DR) was investigated in 856 type 2 diabetes (T2D) patients, of whom 383 presented with (DR group), and 473 presented without (DWR group), retinopathy.

Methods. – PAI-1 4G/5G and −844G/A genotyping were done by PCR-RFLP, and PAI-1 levels were measured by ELISA testing.

Results. – The genotype distribution of 4G/5G and −844G/A polymorphisms did not deviate from the Hardy-Weinberg equilibrium model among healthy subjects. Higher frequencies of the 4G/4G genotype, and lower frequencies of the −844A allele, −844G/A and −844A/A genotypes, were seen in DR patients, conferring disease susceptibility and protection, respectively. While PAI-1 levels were significantly elevated in the 4G/4G compared with other PAI-1 genotypes, significant differences in PAI-1 levels between DR and DWR patients were seen in the 4G/−844A, 4G/−844G and 5G/−844A haplotype carriers among DR patients. However, comparable distributions of 4G/5G and −844G/A alleles, genotypes and haplotypes, and similar PAI-1 levels, were seen in the proliferative retinopathy (PR) and non-proliferative retinopathy (NPR) patients, indicating that neither PAI-1 variants nor changes in PAI-1 levels were linked to DR severity. Multivariate analyses identified 4G/−844A and 4G/−844G haplotypes as negatively and positively associated, respectively, with DR, but not with DR severity (PR vs NPR) after adjusting for a number of covariates.

Conclusion. – The present study identifies changes in PAI-1 levels and genetic variations at the PAI-1 locus as risk factors for DR, but not DR severity, that may serve as useful markers of increased DR susceptibility.

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Keywords: Diabetes; Retinopathy; Plasminogen activator inhibitor-1; Polymorphism; PCR

Résumé

Rétinopathie diabétique, polymorphismes du gène PAI-1 (4G/5G et −844G/A) et variations des taux du PAI-1 chez des diabétiques de type 2 tunisiens.

Objectif. – L’association des variations du taux de l’inhibiteur de l’activateur du plasminogène (PAI)-1 et des polymorphismes (4G/5G et −844G/A) du gène PAI-1 avec la rétinopathie diabétique (RD) a été étudiée chez 856 patients diabétiques de type 2 (DT2), dont 383 présentaient une rétinopathie diabétique (groupe RD) et 473 qui en étaient indemnes (groupe SRD).

Méthodes. – Le génotype des génotypes 4G/5G et −844G/A du PAI-1 a été réalisé par PCR-RFLP et les taux de PAI-1 ont été dosés par Elisa.

Résultats. – La distribution des génotypes de polymorphismes 4G/5G et −844G/A obéit à l’équilibre de Hardy-Weinberg chez les sujets témoins. Une fréquence plus élevée du génotype 4G/4G, ainsi que des fréquences plus faibles de l’allèle −844A et des génotypes −844G/A et −844A/A ont été observées chez les patients avec RD, conférant ainsi respectivement une protection et une susceptibility à la maladie. Les taux de PAI-1 étaient significativement élevés en présence du génotype 4G/4G comparés aux autres génotypes de PAI-1. Une différence significative des taux de PAI-1 entre les patients RD et SRD a été observée uniquement chez les porteurs de génotype −844G/G et chez les patients porteurs des haplotypes contenant 4G mais non pas chez ceux contenant 5G. Une distribution comparable des allèles, des génotypes, des haplotypes des 4G/5G

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et -844G/A, ainsi une similarité des taux plasmatiques du PAI-1 ont été observées aussi bien chez les patients avec rétinopathie diabétique proliférative (RDP) que chez ceux avec rétinopathie diabétique non-proliférative (RDNP), indiquant que ni les variants du PAI-1 ni les variations de son taux plasmatique ne sont associés à une sévérité de la RD. L’analyse multivariée, après ajustement sur de multiples covariables, a montré que les haplotypes 4G/844A et 4G/844G étaient respectivement associés négativement et positivement à la RD, mais non à la sévérité de RD (RDP versus RDNP).

Conclusion. – Cette étude identifie les modifications du taux de PAI-1 et la variation génétique au niveau du locus PAI-1 comme facteurs de risque de la RD, mais non de sa sévérité, ce qui pourrait servir de marqueur utile de l’augmentation de la susceptibilité à la RD.

Mots clés : Diabète ; Rétinopathie ; L’inhibiteur des activateurs du plasminogène-1 ; Polymorphisme ; PCR

1. Introduction

Type 2 diabetes (T2D) is a metabolic disease associated with serious micro- and macrovascular complications, including diabetic retinopathy (DR), a major cause of blindness among diabetic adults, that are aggravated by poor glycaemic control, hypertension and longer disease duration [1]. DR is associated with a strong genetic predisposition, highlighted by the familial clustering of DR [2,3] and the association of several gene polymorphisms with DR [4–11]. These include the aldose reductase [10,11], advanced glycation end-products receptor [6,7], adhesion molecules [8], and coagulation – and fibrinolytic – system gene polymorphisms, including the plasminogen activator system (PAS) [4,5,9].

PAS comprises distinct serine proteases (tissue-type plasminogen activator) and their inhibitors (plasminogen activator inhibitor [PAI]) [12], which control plasminogen activation [12,13]. High PAI-1 activity is associated with atherosclerosis and thromboembolism [14], and several polymorphisms within the PAI-1 gene polymorphisms, including the plasminogen activator inhibitor system (PAI) [12], which control plasminogen activation [12,13]. High PAI-1 activity is associated with atherosclerosis and thromboembolism [14], and several polymorphisms within the PAI-1 gene influence PAI-1 levels [15]. These include the 844G/A variations and PAI-1 activity, independent of 4G/4G genotype, may be implicated in DR pathogenesis [18,22]. The aim of the present study was to investigate the role of the 4G/5G and 844G/A polymorphisms and changes in PAI-1 levels as risk factors of DR among adult T2D patients in Tunisia.

2. Subjects and methods

2.1. Subjects

This was a retrospective case-control study involving 856 unrelated adult T2D patients, recruited from the outpatients’ endocrinology services at Farhat Hached University Hospital (Sousse) and Fattouma Bourguiba University Hospital (Monastir) in Tunisia. Written informed consent was obtained from all participants, and the study was carried out in accordance with the guidelines of the Helsinki declaration of 1975, and approved by the University of Monastir ethics committee. T2D diagnosis was based on clinical features; none of the patients had ever had ketoacidosis, and their initial T2D treatment included diet and/or oral antidiabetic drugs. Patients who required insulin had been treated with oral drugs for at least two years (Table 1).

For all study patients, demographic details were obtained and the patients’ histories verified from clinic records where available. Venous blood samples were collected after an overnight fast for glucose, HbA1c and serum lipid measurements. Total haemoglobin (Hb) and HbA1c levels were measured by colorimetric and immunoturbidimetric methods, respectively, using a COBAS Integra 800 analyzer; the Hb-to-HbA1c ratio yielded the percent HbA1c levels. Blood pressure (BP) was measured twice using a mercury sphygmomanometer with participants in the sitting position following a 5-min rest; the mean of two readings was used. Hypertension was defined as BP readings greater than 140/90 mmHg and/or the use of antihypertensive medications.
1.0 ng/mL, with a working range of 2.5–115 ng/mL. Stago, Asnières sur Seine, France). The detection limit was measured twice by quantitative ELISA, using the Asserachrom kit according to the manufacturer’s specifications (Diagnostica Stago, Asnières sur Seine, France). The detection limit was 1.0 ng/mL, with a working range of 2.5–115 ng/mL.

<table>
<thead>
<tr>
<th>Allele/genotype</th>
<th>DR (n = 383)</th>
<th>DWR (n = 473)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G/5G 4G</td>
<td>321 (0.42)</td>
<td>350 (0.37)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.072</td>
<td>1.20 (0.99–1.46)</td>
</tr>
<tr>
<td>5G/5G</td>
<td>139 (0.36)</td>
<td>177 (0.37)</td>
<td>0.788</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>5G/4G</td>
<td>167 (0.44)</td>
<td>242 (0.51)</td>
<td>0.217</td>
<td>0.83 (0.62–1.11)</td>
</tr>
<tr>
<td>4G/4G</td>
<td>77 (0.20)</td>
<td>54 (0.11)</td>
<td>0.015</td>
<td>1.64 (1.10–2.43)</td>
</tr>
<tr>
<td>−844G/A −844A</td>
<td>277 (0.36)</td>
<td>417 (0.44)</td>
<td>0.001</td>
<td>0.71 (0.59–0.87)</td>
</tr>
<tr>
<td>−844G/G −844G</td>
<td>164 (0.43)</td>
<td>143 (0.30)</td>
<td>&lt; 0.001</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>−844G/A −844A/A</td>
<td>161 (0.42)</td>
<td>243 (0.51)</td>
<td>0.001</td>
<td>0.61 (0.46–0.82)</td>
</tr>
<tr>
<td>−844A</td>
<td>58 (0.15)</td>
<td>87 (0.18)</td>
<td>0.011</td>
<td>0.70 (0.41–0.89)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of individuals (frequency).
<sup>b</sup> Pearson’s chi-square test; DR: with diabetic retinopathy; DWR: without diabetic retinopathy.

All patients were subjected to ophthalmological examination, which included corrected visual acuity, funduscopy examination and photography, and slit-lamp microscopy examination with and without a preset lens. DR was defined as at least one microaneurysm, haemorrhage or exudate in either eye, and was subdivided into less severe non-proliferative diabetic retinopathy (NPR), based on fluid leakage from blood vessels into the retina leading to blurred vision, and the more severe proliferative diabetic retinopathy (PR), associated with neovascularization of the eye, haemorrhage and retinal scarring. Fluorescein angiography was performed on some patients to confirm the funduscopy findings.

### 2.2. PAI-1 genotyping

The −844G/A (rs2227631) polymorphism was screened for by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP), using the following primers: forward, 5′-CAGGGCTCTCTTGTGTCAAC-3′; and reverse, 5′-GAGGGCTCTCTTGTGTCAAC-3′. Amplified products were digested by XhoI and separated on 2% agarose gel; the −844G allele was visualized as 314 + 146 bp bands, and the −844A allele as the 510-bp fragment. The 4G/5G insertion-deletion polymorphism (rs1799889) was determined by single-specific primer (SSP)–PCR using the following primers: (forward; 4G), 5′-GTCTGGACACGTGGGGA-3′; and (common reverse) 5′-TGCAAGCAGCCACGTGGATTGTCTAG-3′. Amplified fragments underwent electrophoresis on 2.5% agarose gel. Quality-control measures comprised genotyping the participants’ clinical characteristics are shown in Table 1. There were 473 DWR and 383 DR patients. Mean age, body mass index (BMI), age of onset and duration of diabetes were comparable between the two groups. Mean systolic – but not diastolic – BP was significantly higher among DR patients. Serum glucose, HbA1c, LDL and HDL cholesterol, and PAI-1 antigen levels were comparable between DR and DWR patients, although cholesterol and triglyceride concentrations were both significantly higher in DR patients. While initial diabetes management was comparable between both T2D groups, a significantly higher proportion of DR than DWR patients required later (> 5 years after initial diagnosis) supplementation with insulin (46.8% vs 20.7%, respectively; P < 0.001).

### 3. Results

#### 3.1. Clinical characteristics of study subjects

Statistical analyses were performed using SPSS version 13.0 software. Data were expressed as means ± SD (continuous variables) or as percent totals (categorical variables). Allele frequencies were calculated by the gene-counting method. Pearson’s χ<sup>2</sup> or Fisher’s exact test were used to assess intergroup significance, and Student’s t-test was used to determine differences in means. Both PAI-1 polymorphisms were tested for the Hardy–Weinberg equilibrium, using the χ<sup>2</sup> goodness-of-fit test and HPlus 2.5 software. All analyses were conducted assuming an additive genetic effect, and the overall power (79.7%) was calculated as the average power over the two SNP genotyped (Genetic Power Calculator; SGDP Statistical Genetics Group).

PAI-1 haplotype estimation was done by the expectation maximization method using HPlus 2.5 software, whereby the sum of the probability estimates for all four possible haplotypes = 1.0. Where haplotype assignment was uncertain (heterozygous carriers), the haplotype assignment probability estimate was used to determine the individual’s contribution to that haplotype. Univariate and multivariate regression analyses were also performed (using HPlus 2.5 and HAPStat software), with results expressed as P values, odds ratio (OR) and 95% confidence intervals (CI).

#### 3.2. PAI-1 antigen assay

Within 20 min of blood collection, plasma samples were separated by centrifugation for 15 min at 3500 g, and stored in aliquots at −80°C, pending analysis. PAI-1 antigen was measured twice by quantitative ELISA, using the Asserachrom kit according to the manufacturer’s specifications (Diagnostica Stago, Asnières sur Seine, France). The detection limit was 1.0 ng/mL, with a working range of 2.5–115 ng/mL.
3.2. PAI-1 4G/5G and −844G/A genotype analysis

Genotype distributions of 4G/5G (χ² = 0.337, P = 0.562) and −844G/A (χ² = 0.552, P = 0.458) polymorphisms did not deviate from the Hardy-Weinberg equilibrium model among healthy subjects. However, significantly higher frequencies of −844G/G and 4G/4G genotypes, and lower frequencies of −844G/A and −444A/A genotypes, and the −844A allele, were seen in DR patients (Table 2). In contrast, comparable distributions of 4G/5G and −844G/A alleles and genotypes were seen in PR and NPR patients (data not shown). Although not significantly different between DR and DWR groups, higher plasma PAI-1 levels were seen in the 4G/4G genotype carriers. Significant increases in PAI-1 plasma levels were seen in DR patients only in the −844G/G genotype carriers (33.4 ± 20.1 ng/mL vs 25.6 ± 16.1 ng/mL; P = 4.2 × 10⁻⁴), but were otherwise generally comparable among the other PAI-1 genotype carriers.

3.3. Haplotype distributions

The two-locus PAI-1 haplotype analysis, stratified by study groups, is shown in Table 3. A lower frequency of the 4G/−844A haplotype (P = 0.003) and a higher frequency of the 4G/−844G haplotype (P = 6.3 × 10⁻⁵) were seen in the DR patients, conferring disease preventative and susceptibility aspects, respectively, to these haplotypes. Comparable distributions of the four PAI-1 haplotypes were seen in the PR and NPR patients, indicating that none of the haplotypes was linked to DR severity. Higher plasma PAI-1 was seen in the 4G-containing haplotypes, which was even more pronounced in DR patients. Higher plasma PAI-1 levels were also seen in the 4G/−844A (66.66 ± 22.19 ng/mL vs 52.79 ± 17.72 ng/mL; P < 0.001), 4G/−844G (56.44 ± 17.96 ng/mL vs 44.56 ± 17.85 ng/mL; P < 0.001), and 5G/−844A (24.88 ± 11.86 ng/mL vs 21.15 ± 10.49 ng/mL; P = 0.032) haplotype carriers among DR patients. On contrast, PAI-1 plasma levels were similar between PR and NPR patients, irrespective of the PAI-1 haplotype (data not shown).

3.4. Regression analyses

The association of PAI-1 haplotypes with DR was examined by univariate and multivariate analyses. Taking the 5G/−844G haplotype as reference, univariate analyses identified the 4G/−844A haplotype as being negatively associated (P = 0.02; OR = 0.68; 95% CI = 0.49–0.95) and the 4G/−844G haplotype as being positively associated (P < 0.001; OR = 1.96; 95% CI = 1.45–2.66) with DR (Table 4). Multivariate analyses confirmed the association of 4G/−844A (P = 0.04; OR = 0.69; 95% CI = 0.48–0.98) and 4G/−844G (P < 0.001; OR = 1.98; 95% CI = 1.45–2.70) haplotypes with DR after adjusting for the following covariates: age; gender; age of disease onset; HbA1c; hypertension; PAI-1 antigen; creatinine; disease duration; and total cholesterol concentrations (Table 4). None of the four PAI-1 haplotypes was linked with DR severity (PR vs NPR) at either univariate or multivariate levels (Table 4).

4. Discussion

Previous studies investigating the contribution of PAI-1 polymorphisms and changes in PAI-1 activity to DR pathogenesis have reported inconsistent results. In the present study, specific
associations between the 4G/5G and −844G/A alleles and genotypes, and DR, were seen, highlighted by the significantly increased prevalence of the −844G allele, and −844G/G and 4G/4G genotypes, among DR patients. This association was confirmed by identifying susceptibility (4G/−844G) and protective (4G/−844A) haplotypes, indicating a role for PAI-1 polymorphisms in DR pathogenesis. However, neither PAI-1 variations nor changes in PAI-1 levels were linked to DR severity, as comparable 4G/5G and −844G/A allele, genotype and haplotype distribution, and PAI-1 plasma levels, were seen in PR and NPR patients.

As DWR and DR patients were matched according to a number of DR risk factors – including age at onset, duration of diabetes and HbA1c – this rules out the possibility that patients were prone to DR because of longer exposure to hyperglycaemia and poor glycaemic control [23,24]. Our DR patients had elevated systolic BP, which is consistent with other findings that link hypertension and DR development [25], and also had raised total cholesterol and triglycerides, similar to the findings of the CURES Eye Study-2 [26]. The PAI-1 4G/5G insertion-deletion polymorphism was significantly associated with DR, as reflected by the increased prevalence of the 4G/4G genotype in our DR patients.

Our results are in agreement with studies of Austrian [5], Pima Indian [9] and Turkish [4] T2D patients, but are inconsistent with other studies that failed to demonstrate any links between the 4G/5G polymorphism and DR [10,18,21,22]. It has also been suggested that PAI-1 4G/4G is associated with DR only in patients positive for the angiotensin-converting enzyme D/D genotype [18], or in those with elevated fibrinogen [22], thereby prompting speculation that the contribution of 4G/5G to DR might be dependent on the presence of additional disease-modulating factors. However, these conflicting findings may be reconciled by differences in ethnicity [10,18,21,22], sample size [10,21], and the failure to control for confounding factors (gender, HbA1c levels, obesity and duration of diabetes) in some studies.

In our study, specific DR-susceptible and -protective PAI-1 haplotypes were identified, and confirmed by multivariate analysis, thereby pointing to possible interactions between 4G/5G and −844G/A variants in influencing disease susceptibility. It is also intriguing that the disease susceptibility or not of the strong 4G allele is dependent on the presence of the −844G and −844A alleles, respectively. While this may be explained by direct epistatic interactions between both variants, the contribution of other disease-modulating factors cannot be excluded at this time.

While pronounced elevations of PAI-1 antigen levels were seen in 4G carriers [27,28], PAI-1 antigen levels were not significantly different in unselected DR and DWR cases. According to the regression analysis model we employed, PAI-1 antigen levels were not significant predictors of DR, not even after controlling for several DR-associated covariates. This is in agreement with the study in Pima Indians in which the 4G/4G genotype – but not PAI-1 antigen levels – was predictive of DR [9], but inconsistent with studies in Caucasian T2D patients [18,22]. While differences in ethnic backgrounds, sample size and patient selection may partly explain these discrepancies, the contribution of non-genetic factors that reportedly can precipitate elevated PAI-1 activity – including the renin-angiotensin system, dyslipidaemia and underlying endothelial dysfunction – should not be overlooked.

Compared with other PAI-1 variants, the most significant variation in PAI-1 expression resides in the PAI-1 4G/5G alleles. Unlike the 5G allele that binds a transcription repressor protein, resulting in low PAI-1 expression, the 4G allele does not bind a transcription repressor, thus conferring a ‘high PAI-1 expressor’ nature to the allele [29]. The mechanism(s) by which PAI-1 influences DR pathogenesis remain to be elucidated. It is known that the retinal microvessels in T2D patients contain higher quantities of PAI-1 compared with age-matched non-diabetic controls [19,30], and that overexpression of PAI-1 in the retinal microvasculature of PAI-1 transgenic mice leads to retinal disease [31]. Potential mechanisms include induction of systemic inflammation, endothelial dysfunction, formation of abnormal degradation-resistant clots and increased blood volume or hormonal changes [32]. However, because of its antifibrinolytic properties, it is suggested that PAI-1 may, on the contrary, protect against retinal damage in experimental mouse models [33,34]. Nevertheless, as similar findings have not been reported in humans, the applicability of this animal finding is open to doubt.

PAI-1 is located on chromosome 7 (7q21.3-q22) in close proximity to cytokine (IL-6) and metabolic genes, including the glucokinase (GCK), glyceral-3-phosphate transporter (SLC37A3) and transcription factor 7-like 2 (TCF7L2) [35] genes. The International HapMap Project has identified several SNP in linkage disequilibrium (LD) with the 4G/5G (rs1799889) within the 100-kb region encompassing the PAI-1 locus, including rs34857375, rs1799762 and rs1799768 (http://www.hapmap.org/cgi-perl/gbrowse/hapmap3). This raises the possibility that epistasis between these variants might explain the variance in PAI-1 level loci and DR susceptibility [15].

In conclusion, PAI-1 gene polymorphisms, but not PAI-1 levels, represent an independent risk factor for DR pathogenesis, but not DR severity. The strengths of the present study include the sample size and specific ethnic group studied (North African Tunisian Arabs). However, these findings need to be confirmed through additional investigations using larger populations and additional polymorphisms in LD with the PAI-1 variants studied here to be sufficiently powered to reduce the probability of false-positive findings. A limitation of our study is that it was limited to Tunisian Arabs, thus requiring follow-up studies in T2D patients with DR from other ethnic groups. Elucidating the association of the PAI-1 gene variants with DR susceptibility will boost our understanding of the link between the fibrinolytic system and DR pathogenesis.

References


