

Original article

The –675 4G/5G plasminogen activator inhibitor-1 promoter polymorphism in house dust mite-sensitive allergic asthma patients

Background: Plasminogen activator inhibitor (PAI)-1 plays an important role in inflammation and tissue remodeling. Recently, the –675 4G/5G PAI-1 polymorphism has been linked with asthma.

Objective: This study was undertaken to evaluate associations of the –675 4G/5G PAI-1 polymorphism with functional and immunologic parameters of newly diagnosed house dust mite-sensitive allergic asthmatics (HDM-AAs).

Methods: This study was performed in 127 HDM-AAs, who responded with at least 20% fall of forced expiratory volume during the first second (FEV₁) to a bronchial challenge with *Dermatophagoides pteronyssinus* allergen and during the follow up observation fulfilled GINA criteria for mild–moderate asthma. About 89 healthy control nonatopic subjects (HCs) were used as controls.

Results: The frequency of 4G allele was greater in HDM-AAs (0.69; 95% CI: 0.62–0.76) than in HCs (0.55; 95% CI: 0.48–0.62; $P = 0.0034$). The PAI-1 polymorphism was associated with an increased risk of HDM-AA; adjusted for sex and age odds ratio was 2.62; (95% CI: 1.16–5.92) for 4G/5G genotype and 3.48 (95% CI: 1.54–7.89) for 4G/4G genotype compared with 5G/5G genotype. Total serum immunoglobulin E (tsIgE) level in 4G/4G homozygotes (557 ± 343 kU/l) was significantly greater than in 5G/5G homozygotes (241 ± 288 kU/l; $P < 0.001$). Both nonspecific and allergen-specific bronchial reactivities were greater in 4G/4G homozygotes than in 5G/5G homozygotes. 4G/4G genotype was associated with significantly higher morning plasma PAI-1 concentration in HDM-AAs and HCs. Morning plasma PAI-1 concentration correlated significantly with log_{PC20} ($r = -0.39$; $P = 0.0001$) and with log_{tsIgE} ($r = 0.247$; $P = 0.0117$).

Conclusion: These results support the hypothesis linking the 4G/4G PAI-1 genotype with an increased risk of allergic asthma, bronchial hyperreactivity, and increased tsIgE levels.

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Even in newly diagnosed asthma patients signs of bronchial wall remodeling can be unequivocally demonstrated (1). Progression of these structural changes is associated with increased bronchial hyperreactivity to nonspecific stimuli such as histamine or metacholine (2, 3). An imbalance between proteases and their inhibitors was implicated in the pathologic processes of tissue repair and remodeling (4–6). The plasminogen activator system (PAS) plays an important role in regulating

extracellular matrix (ECM) proteolysis, both directly through plasmin formation and indirectly through plasmin-mediated activation of matrix metalloproteinases (MMPs) (7). Plasminogen activator inhibitor-1 (PAI-1) has been recently involved in the pathogenesis of asthma, by inhibition of both fibrinolysis and the MMP system (8). The PAI-1, indeed, promotes ECM deposition in the airways of a murine model of chronic asthma (9) and its expression was increased in lung mast cells from asthmatic patients (10).

Recently, a single base insertion/deletion 4G/5G polymorphism in the promoter region of PAI-1 gene, located 675 bp upstream from the transcriptional start site, has been associated with asthma (11, 12). 4G homozygote cells produce more PAI-1 than cells derived from 5G homozygotes, either spontaneously or after stimulation (13). Similarly, 4G homozygotes have higher plasma PAI-1 levels than 5G homozygotes (14).

Abbreviations: PAI-1, plasminogen activator inhibitor-1; ECM, extracellular matrix; PAS, plasminogen activator system; Dp, *Dermatophagoides pteronyssinus*; FEV₁, forced expiratory volume during the first second; PC₂₀, concentration of a bronchoconstricting agent causing 20% fall of FEV₁; PD₂₀, allergen dose causing 20% fall of FEV₁; HDM-AAs, house dust mite allergic asthmatic patients; tsIgE, total serum immunoglobulin E; DpIgE, anti-Dp IgE; PCR, polymerase chain reaction; SBE, standardized biologic unit.

Genome-wide screening for susceptibility to allergy demonstrated that the region of chromosome 7q, which contains the PAI-1 gene, is linked to total serum immunoglobulin E (tIgE) concentration in Dutch families (15). Unfortunately, no such an association was demonstrated in studies evaluating the -675 4G/5G PAI-1 polymorphism in allergy or asthma patients (11, 12). The lack of association may result from heterogeneity of asthma and allergic diseases. As both bronchial reactivity and serum IgE concentration are influenced by many environmental factors these parameters change during the course of asthma (16, 17). Therefore, we decided to evaluate a possible association between the -675 4G/5G PAI-1 polymorphism and lung function parameters as well as tIgE and specific serum IgE in a well defined population of newly diagnosed house dust mite allergic asthmatic patients (HDM-AAs) from northeastern Poland.

Materials and methods

The study was performed in 216 Caucasian nonsmoking subjects from the population of northeastern Poland: 127 asthmatics and 89 healthy controls. The HDM-AAs, who at the time of initial evaluation gave a history of recent episodes of wheezing upon dust exposure (<6 months), had positive skin prick test results to *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df), demonstrated significant bronchial reactivity to histamine and significant bronchial response to Dp allergen challenge were selected for the study. Moreover, during the subsequent 1-year follow up period these patients fulfilled GINA criteria for mild-moderate persistent asthma (18). All patients who received allergen immunotherapy or any antiasthma medication, except short-acting inhaled β -agonists before the initial visit were not included in the study. Other exclusion criteria included respiratory tract infection within 3 months before the initial visit, smoking, diabetes, hypercholesterolemia or any systemic disease which might influence PAI-1 plasma concentration.

Eighty-nine nonsmoking healthy subjects with no symptoms of asthma or rhinitis and with negative skin prick test results with a panel of common aeroallergens were used as healthy controls (HCs). The study was approved by the local Ethics Committee. All participants provided written informed consent.

Skin prick tests

All persons were skin tested using prick methodology with a screening panel of aeroallergens (Allergopharma, Reinbek, Germany) as described before (19).

Bronchial challenge

Histamine bronchial challenge was performed as described earlier (19). Briefly, all patients inhaled doubling concentrations of histamine starting from a concentration of 0.62 mg/ml. The procedure was continued until either at least 20% fall of forced expiratory volume during the first second (FEV₁) or a histamine concentration of 32 mg/ml was reached. Nonspecific bronchial reactivity was expressed as histamine concentration causing 20% fall of FEV₁ (PC₂₀).

Bronchial provocation test with aqueous Dp extracts (Allergopharma) was performed as described earlier (19). Allergen inhalations were continued until either at least 20% fall of FEV₁ or a cumulative dose 5000 standardized biologic unit (SBE) was reached. Specific bronchial reactivity was expressed as allergen dose causing 20% fall of FEV₁ (PD₂₀). Bronchial challenge with Dp allergen extract was performed in all HDM-sensitive patients.

Blood samples

Citrate anticoagulated blood samples and serum samples were collected between 7 and 9 AM. Immediately after centrifugation at 1500 g for 20 min at 4°C plasma supernatants were aliquoted and were frozen at -80°C until testing. Cellular pellets were stored at -80°C until used for DNA extraction.

Biochemical and immunologic assays

The tIgE and specific anti-DpIgE (DpIgE) in serum were measured using UniCap system (Pharmacia, Sweden).

Plasma PAI-1 antigen (PAI-Ag) concentration was evaluated using enzyme-linked immunosorbent assay (Asserachrom, Diagnostica Stago, Stanford, CT, USA) according to the manufacturer's instruction. The testing range was 4–43 ng/ml. All samples were run in duplicates. Whenever PAI-Ag was above the testing range the evaluation was repeated using appropriate dilutions. All measurements were performed in a blinded fashion.

DNA extraction and 4G/5G promoter polymorphism detection

Genomic DNA was extracted from whole blood cellular pellets using DNA purification kit (Promega Corporation, Madison, WI, USA).

The PAI-1 promoter 4G/5G genotypes were analyzed with an allele-specific polymerase chain reaction (PCR) (14). Briefly, genomic DNA samples were amplified using the following allele-specific primers: 5'-GTC TGG ACA CGT GGG GG-3' for the insertion allele and 5'-GTC TGG ACA CGT GGG GA-3' for the deletion allele, each in a separate reaction, together with the common downstream primer 5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3'. A fourth primer (5'-AAG CTT TTA CCA TGG TAA CCC CTG GT-3') located upstream of the polymorphic region was used as a positive control of the PCR. The thermal cycling conditions were the following: denaturation (45 s at 94°C), annealing (45 s at 65°C), extension (75 s at 72°C), repeated for 30 step cycles. About 25 μ l of PCR contained 100 ng genomic DNA, 100 ng of each appropriate forward and reverse primers, 1.5 mM MgCl₂, 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 0.2 mM dNTPs and 1 unit of *Taq* Polymerase (Promega Corporation).

The PCR products were run by electrophoresis in a 3% agarose gel and visualized directly by ethidium bromide staining. An internal quality control of DNA with known genotype for PAI-1, evaluated by direct sequencing, was used. Moreover, 5% of DNA samples were re-tested. A correspondence of 100% among the genotypes was obtained.

Statistical analysis

Calculated sample size of about 100 cases and 100 controls is necessary to detect an increase of 150% in the relative risk for carriers of the 4G allele in comparison with 5G/5G homozygotes, with a power of 80% ($\beta = 0.20$) and the level of type I error ($\alpha = 0.05$). Continuous

variables were compared using the Kruskal–Wallis test. Chi-square test (or Fisher exact test for small frequencies) was used to compare discrete parameters. Allele and genotype frequencies were determined by genotype count and compared with the values predicted by the assumption of Hardy–Weinberg equilibrium performing the chi-squared test. Odds ratios (ORs), were calculated using unconditional logistic regression analysis. Adjusted ORs were obtained in multivariate logistic regression analysis with adjustment for age and sex. Data for continuous variables were expressed as mean $\pm$ SD; a two-tailed *P*-value of <0.05 was chosen as the level of significance. All computations were carried out using the SAS statistical package (Version 8.2 for Windows; SAS Institute Inc., Cary, NC, USA).

Results

There were no differences in gender distribution and age between the groups (Table 1). The genotype distribution of -675 4G/5G PAI-1 was in Hardy–Weinberg equilibrium in both studied groups (Table 1). The prevalence of the 4G allele in HDM-AAs was higher than in HCs and

the frequency of 4G/4G homozygotes was higher in HDM-AAs, when compared with HCs (Table 1). In univariate analysis, 4G/4G and 4G/5G genotypes were significantly associated with allergic asthma. The OR for 4G/4G genotype was 3.33 (95% CI: 1.48–7.49), while for 4G/5G genotype OR was 2.49 (95% CI: 1.11–5.58). Adjustment for sex and age did not modify the association OR = 3.48 (95% CI: 1.54–7.89) for 4G/4G genotype and OR = 2.62 (95% CI: 1.16–5.92) for 4G/5G genotype. Mean plasma level of PAI-Ag in HDM-AAs was significantly higher than HCs (Table 2).

Further adjustment for plasma PAI-Ag levels completely abolished the association between PAI-1 genotypes and allergic asthma (OR = 1.00; 95% CI: 0.30–3.40 for 4G/4G genotype and OR = 1.20; 95% CI: 0.40–3.80 for 4G/5G genotype) strongly suggesting that the association between PAI genotypes and asthma is mediated by increased PAI-1 production.

There was a strong genetic regulation of plasma PAI-Ag concentration both in HCs and in HDM-AAs, being 4G/4G and 4G/5G genotypes associated to the same extent with increased levels of PAI-1 (Table 2).

The mean tIgE concentration in HDM-AAs was 475 ± 363 kU/l and the tIgE level was associated with PAI-1 polymorphisms, being higher in 4G/4G homozygotes than in 5G/5G homozygotes. Heterozygotes showed intermediate, although not significantly different levels of tIgE (Table 3). In HDM-AAs a significant correlation was found between $\log_{10}$ tIgE concentration and plasma PAI-1 concentration (Table 4). No difference in serum DpIgE concentration was found according to genotypes of asthmatic patients (Table 3). Similarly no correlation was observed between $\log_{10}$ DpIgE serum concentration and plasma PAI-Ag concentration (Table 4).

In asthmatic patients mean baseline FEV₁ was significantly greater in 5G/5G homozygotes than in 4G/4G homozygotes but did not differ from that in 4G/5G heterozygotes (Table 3). Bronchial reactivity to histamine was greater in 4G/4G homozygotes than in 5G/5G homozygotes and 4G/5G heterozygotes (Table 3). Bronchial reactivity to allergen challenge was significantly greater in 4G/4G homozygotes than in 5G/5G homozygotes but did not reach statistical significance when compared with that in 4G/5G heterozygotes (Table 3). A significant inverse correlation was found between $\log_{10}$ PC₂₀

Table 1. Age, sex, and genotype distribution of -675 4G/5G polymorphism of plasminogen activator inhibitor (PAI)-1 gene in patients and controls

	Healthy controls (<i>n</i> = 89)	Allergic asthma (<i>n</i> = 127)	Comparison vs controls (<i>P</i> -value)
Age (years)	26 $\pm$ 5	25 $\pm$ 9	0.34
Sex (female/male)	42/47	52/75	0.404
4G/4G (%)	31 (35)	61 (48)	
4G/5G (%)	36 (40)	53 (42)	
5G/5G (%)	22 (25)	13 (10)	
Frequency of 4G allele	0.55 (0.48–0.62)	0.69 (0.62–0.76)	0.0034

Table 2. Plasminogen activator inhibitor (PAI)-1 levels in controls and patients according to the -675 4G/5G polymorphism of PAI-1 gene

	Controls	Allergic asthma
All	26.0 $\pm$ 11.4	49.7 $\pm$ 26.6*
5G/5G	19.3 $\pm$ 9.7	43.7 $\pm$ 30.3*
4G/5G	27.3 $\pm$ 11.6	40.4 $\pm$ 23.1*
4G/4G	29.3 $\pm$ 10.8	57.4 $\pm$ 26.2*

**P* < 0.0001 vs controls.

Genotype effect: in controls, *P* = 0.0029; in asthma patients, *P* = 0.0074.

Table 3. Characteristics of asthmatic patients in relation to -675 4G/5G PAI-1 gene polymorphism

	4G/4G	4G/5G	5G/5G	4G/4G vs 4G/5G (<i>P</i> -value)	4G/4G vs 5G/5G (<i>P</i> -value)
Symptom duration (months)	4.53 $\pm$ 0.65	4.28 $\pm$ 0.76	4.36 $\pm$ 0.9	0.06	0.46
FEV ₁ (% predicted)	88 $\pm$ 12	91 $\pm$ 12	97.5 $\pm$ 11.7	0.15	0.011
PC ₂₀ (mg/ml)	2.88 $\pm$ 4.48	5.73 $\pm$ 6.36	8.16 $\pm$ 6.35	0.012	0.0002
tIgE (kU/l)	557 $\pm$ 343	432 $\pm$ 394	241 $\pm$ 288	0.0728	0.0028
PD ₂₀ (SBE)	1198 $\pm$ 1018	1578 $\pm$ 871	1950 $\pm$ 780	0.073	0.008
DpIgE (kU/l)	25.1 $\pm$ 27.2	19.4 $\pm$ 24.1	15.9 $\pm$ 24.3	0.27	0.23

PAI, plasminogen activator inhibitor; FEV₁, forced expiratory volume during the first second; tIgE, total serum immunoglobulin E.

Table 4. Correlation between morning PAI-1 plasma concentration and selected functional and immunologic parameters of HDM-AAs

PAI-1 vs	FEV ₁	log _{tsIgE}	log _{DpIgE}	log _{PC20}	log _{PD20}
Correlation coefficient (r)	0.014	0.247	0.158	-0.391	-0.129
P-value	0.905	0.011	0.16	0.0001	0.214

PAI, plasminogen activator inhibitor; FEV₁, forced expiratory volume during the first second; HDM-AA, house dust mite-sensitive allergic asthmatic patients.

and plasma PAI-Ag levels while no correlation was found between plasma PAI-Ag levels and log_{PD20} (Table 4).

Discussion

A primary involvement of PAI-1 in the pathogenesis of asthma has been recently hypothesized (8). We provide support for this hypothesis studying a population of well defined newly diagnosed HDM-AAs. Our findings are consistent with data obtained in unselected asthmatic populations showing the preferential transmission of the 4G allele to asthmatic children in UK (11) and a higher frequency of 4G allele in allergic asthmatics in the Czech population (12). For the first time we were able to demonstrate an association of the -675 4G/5G PAI-1 promoter polymorphism with resting FEV₁, bronchial reactivity to histamine and tsIgE. The lack of association between -675 4G/5G PAI-1 promoter polymorphism and lung function test results, tsIgE or bronchial reactivity in the previous studies may be explained by heterogeneity of asthmatic populations, different disease duration and medications used (11, 12). Asthma is a heterogeneous disease and both bronchial reactivity and tsIgE levels can be influenced by genetic and environmental factors. Moreover, lung function test results, bronchial reactivity and tsIgE levels change with time as disease progresses and are also influenced by medications (16, 17, 20). Our approach to study a subpopulation of HDM-AAs was selected because sensitization to HDM is an independent risk factor for asthma, is prevalent in allergic asthmatics and allows selection of HDM-AAs early in the course of the disease (21, 22). This evaluation of bronchial reactivity early in the course of asthma diminishes the potential effect of environmental factors. The association between the PAI-1 polymorphism and HDM-sensitive asthma was further supported by evaluation of plasma PAI-Ag concentration. Adjustment for morning PAI-Ag plasma concentration, which abolished the association between the PAI-1 genotypes and HDM-sensitive asthma indicates that PAI-1 production at the cellular level is responsible for the observed association.

The strongest association was observed between the -675 4G/5G polymorphism and nonspecific bronchial reactivity suggesting that the effect of PAI-1 on bronchial reactivity may be the predominant effect in asthma. It has

been demonstrated that airway inflammation and remodeling of the bronchial wall are major factors leading to bronchial hyperreactivity (3, 20). Resolving the role of PAI-1 in these two processes may therefore, help to understand a possible mechanism in which this protease inhibitor may affect bronchial responsiveness and development of asthma. Experimental studies indicated that PAI-1 could potentially influence both inflammatory response and tissue remodeling. *In vitro* PAI-1 inhibits cell migration by blocking binding of integrin $\alpha_v\beta_3$ to vitronectin (23) but in experimental models of ovalbumin induced asthma and bleomycin induced pulmonary fibrosis cellular infiltrations and cellular composition of bronchoalveolar fluid were similar in PAI-1 knockout and wild-type mice (9, 24). These observations indicate that other interactions of PAI-1 may play an important role in chronic pulmonary diseases including asthma. In fact, main differences seen in experimental asthma model in PAI-1 knockout mice are decreased ECM deposition and increased MMP-9 activity (9). Our results indirectly support these observations showing in humans the strongest correlation between plasma PAI-Ag concentration and bronchial reactivity to histamine. As airway remodeling is a stronger predictor of nonspecific bronchial response than airway inflammation our results indicate that it should be remodeling that is predominantly affected by -675 4G/5G PAI-1 polymorphism and PAI-1 production at cellular level (25). However, our observations do not clarify whether PAI-1 is the primary factor involved in tissue remodeling or is induced by other mediators such as interleukin (IL)-13 or transforming growth factor (TGF)- β . Although the association of PAI-1 genotype with the risk of allergic asthma seems completely explained by its increasing effect on PAI-Ag levels, the high concentration of PAI-Ag seen in allergic patients cannot be explained solely by PAI-1 genetics. Some other factors are possibly involved in the regulation of PAI-1 production in atopic patients. Moreover, the polymorphism studied does not extensively cover the whole PAI-1 gene, therefore the possibility that other variants may show association with allergic asthma cannot be excluded.

An association between 4G/5G PAI-1 promoter polymorphism and tsIgE concentration in HDM-AAs is further supported by demonstration of a significant correlation between plasma PAI-Ag concentration and tsIgE concentration. Human PAI-1 gene has been mapped to chromosome 7 q21-q22 where also a locus linked to IgE levels has been found (15). Moreover, tsIgE concentration correlates with nonspecific bronchial reactivity (16), which suggests that common mechanisms may operate in controlling IgE synthesis, PAI-1 synthesis and development of bronchial hyperreactivity. Alternatively, bronchial hyperreactivity is influenced by mediators released in an IgE-dependent process, which also exert an effect on PAI-1 synthesis. Finally, PAI-1 may be

involved in the processes leading to tissue remodeling and also affecting IgE synthesis (7).

In conclusion, the present study shows that allergic HDM-induced asthma is strongly influenced by genetic predisposition and that PAI-1 could play a primary pathogenetic role in the disease, possibly by increasing bronchial hyperreactivity. Further studies are warranted to reveal the mechanisms in which PAI-1 participate in airway inflammation and remodeling in asthma.

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