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REGULAR ARTICLE

Plasminogen activator inhibitor-1 4G/5G polymorphism in breast cancer patients and its association with tissue PAI-1 levels and tumor severity

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Abstract

Background: The plasminogen activator inhibitor type 1 (PAI-1) 4G/5G polymorphism may have significance for PAI-1 expression. High levels of PAI-1 in breast cancer patients are associated with a poor prognosis. In this study, we analyzed the influence of the PAI-1 4G/5G polymorphism on tissue PAI-1 levels and its association with tumor severity in women with breast cancer.
Material and methods: We studied 104 women with breast carcinoma (patient group) and 104 healthy age-matched women (control group). In patients and controls, the PAI-1 4G/5G polymorphism was determined by PCR amplification using allele-specific primers. In patients, PAI-1 levels were quantified in breast cancer tissue by using an ELISA.
Results: The frequency of the PAI-1 4G allele tended to be higher in patients than in controls ($p=0.062$). The presence of the 4G allele (4G/5G plus 4G/4G genotypes) was significantly higher among patients with histological grade 3 tumors than among those with grade 1 tumors ($p=0.026$). Furthermore, patients with the 4G/4G genotype had significantly higher tissue PAI-1 levels than those with the 5G/5G genotype. Moreover, tissue PAI-1 antigen levels were significantly and positively correlated with tumor severity ($p=0.003$) and tumor size ($p=0.009$). However, no

Abbreviations: PAI-1, Plasminogen activator inhibitor type 1; uPA, urokinase type plasminogen activator; tPA, tissue type plasminogen activator.

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significant differences in PAI-1 level were observed in relation to menopause, hormone receptor or nodal status.
Conclusion: Tissue PAI-1 antigen levels and tumor severity seem to be associated with the PAI-1 4G/5G polymorphism. Further studies with a larger number of patients are needed to clarify the influence of this polymorphism in breast cancer.

Introduction

Tumor cell invasion and metastasis result from interactions between cell migration potential, cell adhesion properties, and extracellular matrix proteolysis [1]. Urokinase type plasminogen activator (uPA) is a serine protease that catalyzes the conversion of plasminogen to plasmin, an active enzyme that can degrade a variety of extracellular matrix proteins [1]. It is generally believed that uPA initiates a proteolytic cascade on the cell surface, which promotes tumor invasion and angiogenesis [2]. uPA is inhibited mainly by plasminogen activator inhibitor type 1 (PAI-1), but can also be inhibited by PAI-2 and PAI-3. All PAIs are members of the superfamily of serine protease inhibitors [3–7].

PAI-1, the primary inhibitor of the plasminogen activation system, inactivates tissue type plasminogen activator (tPA) and uPA [3] but also plays an important role in signal transduction, cell adherence, and migration. Indeed, studies of several types of cancers, including breast cancer, have paradoxically shown that increased uPA and PAI-1 levels are associated with aggressive tumor behavior and poor prognosis [8–10]. One might speculate that, since uPA promotes invasion and metastasis, increase in tumor tissue PAI-1 levels should produce a reduction in the local invasion and development of metastasis. However, several studies have shown, on the contrary, that PAI-1 actually promotes those aggressive behaviors [11–14]. Possible mechanisms by which PAI-1 contributes to cancer dissemination include prevention of excessive degradation of the extracellular matrix, modulation of cell adhesion [15,16], and stimulation of angiogenesis [17–19] and cell proliferation [20].

In vitro studies have shown that PAI-1 levels can be altered by cytokines, growth factors, and hormones [21,22], but the genetic and environmental determinants of PAI-1 expression are not fully understood. Changes in PAI-1 biosynthesis are usually preceded by changes in its gene transcription [23–25]. A guanosine insertion/deletion polymorphism in the promoter region of the PAI-1 gene at the −675 bp position, named 4G/5G, has been described [26]. In vitro studies suggest that the 4G allele has higher activity than the 5G allele because the 5G allele contains an additional binding site for a DNA-binding protein that acts as a transcriptional repressor [27,28]. Studies involving healthy subjects or patients with coronary artery disease or metabolic syndrome have reported that high plasma levels of PAI-1 are associated with a high prevalence of the 4G allele [27–30].

Results of studies on the association between the PAI-1 4G/5G polymorphism and the invasive behavior of cancer are contradictory. Although one study reported that there was no association between the polymorphism and cancer progression [25], another suggested that the 5G/5G genotype is associated with less aggressive cancer phenotypes [32].

In this study, we examined the influence of the PAI-1 4G/5G polymorphism on tissue PAI-1 levels and its association with tumor severity in women with breast cancer.

Materials and methods

Clinical groups

One hundred and four patients (mean age 60 years; range 24–83 years) with primary, operable, and unilateral breast cancer were included in our study.

Patients with distant metastasis or other malignancies at the time of diagnosis were excluded from the study, as were those that had been treated prior to surgery (neoadjuvant therapy) or had presented with synchronous bilateral breast cancer.

Age, menopausal status, tumor size and histological characteristics, axillary lymph node infiltration, and steroid receptor status of patients were recorded (Table 1). Tumor severity was scored according to the Scarf–Bloom–Richardson criteria (SBR histological grade) [33,34].

All cancer patients underwent surgery. Samples of tumor tissue were taken for protein analysis and DNA extraction, and were snap frozen in liquid nitrogen immediately after excision.

The control group was recruited from same geographical area as the patients and comprised

Table 1 Clinical characteristics of breast cancer patients

Parameter	Number of patients
Menopausal status	104
Pre/perimenoupausal	31
Postmenopausal	73
Estrogen receptor[a]	
Positive	84
Negative	20
Progesterone receptor[a]	
Positive	71
Negative	33
Tumor size	
T_1 (≤2cm)	55
T_2 (>2cm)	49
Nodal status	
N_0	56
N_1	48
SBR[b] *histological grade*	
1	45
2	42
3	17
Histological features	
Invasive ductal carcinoma	88
Invasive lobular carcinoma	7
Other types[c]	9

[a] Cutoff point = 15 fmol/mg protein.
[b] SBR: Scarf–Bloom–Richardson.
[c] Include tubular, cribiform, glucogen-rich and papillary carcinoma.

104 unrelated age-matched women (mean age 58 years; range 24–85 years). All the controls appeared to be healthy and those older than 50 years had undergone a clinical and mammographic examination every two years since the age of 50. All participants in the study gave informed consent before inclusion. The protocol was approved by the local ethics committee.

Methods

PAI-1 promoter 4G/5G polymorphism

DNA was extracted from whole blood collected into tubes containing EDTA (controls) or from breast tissue samples (patients) using the Genomic Purification System (Promega, Madison, WI) and following the manufacturer's protocol. The PAI-1 promoter 4G/5G polymorphism was analyzed using an allele-specific PCR technique modified from Falk et al. [35] as described previously [28]. An alternative forward primer [GTCTGGACACGTGGGGG for the 5G allele or GTCTGGACACGTGGGGA for the 4G allele] with a common reverse primer [GCTGTCCACCCGGTGCTCTG] (designed to minimize dimer-primer formation) and a control reverse upstream primer [AAGCTTTTACCATGGTAACCCCTGGT] were used. The PCR procedure included an initial hot-start step to avoid the production of dimer-primer artifacts. Electrophoresis was performed using 3% high-resolution agarose MS-8 (Pronadisa, Condalab, Madrid, Spain). Photographs of gels were taken after ethidium bromide staining.

Quantification of PAI-1 antigen and total protein

For PAI-1 antigen determination, frozen samples of tumor tissue were homogenized in 10 mmol/L Tris–HCl buffer, pH 7.4, containing 1.5 mmol/L EDTA and 10% glycerol. The suspension was centrifuged at 100,000 × *g* at 4 °C for 15 min, and aliquots of the supernatant (cytosol extract) were stored at −80 °C. The pelleted membranes were solubilized in 20 mmol/L Tris–HCl buffer containing 125 mM NaCl and 1% Triton X-100, incubated overnight at 4 °C, and centrifuged at 100,000 × *g* at 4 °C for 15 min. Aliquots of the supernatant (membrane extract) were stored at −80 °C.

PAI-1 antigen in cytosol and membrane extracts from breast cancer tissue was quantified using an ELISA (Tint Elize PAI-1, Biopool, Sweden). The assay detects active and latent (inactive) forms of PAI-1 and complexed PAI-1 with equal efficiency. The intra- and interassay variabilities were 3% and 7%, respectively.

Total protein concentration in cytosol and membrane extracts was determined using the BCA protein assay (Pierce, Rockford, IL). Fraction V bovine serum albumin (Sigma-Aldrich, St Louis, MO) was used for calibration. Samples and standards were analyzed in duplicate.

Estrogen and progesterone receptors

Estrogen and progesterone receptors were assayed by ELISA (ER-EIA Monoclonal and PgR-EIA Monoclonal, respectively, Abbott Laboratories, Chicago, IL). The cutoff point was set at 15 fmol/mg cytosolic protein.

Statistical analysis

The Chi-squared test was used to detect differences in allele and genotype frequencies between patients and controls and according to tumor severity in patients. The Kruskal–Wallis test was used to compare differences in PAI-1 levels between genotypes and tumor severity groups. Differences in PAI-1 levels between menopausal status, hormone receptor status, tumor size, and nodal status groups were determined using the Mann–Whitney test. Values of $p<0.05$ were considered to be statistically significant. All data were

Table 2 4G/5G PAI-1 polymorphism and allelic frequency by histological tumor grade (tumor severity) in patients

	Grade 1 ($n=45$)		Grade 2 ($n=42$)		Grade 3 ($n=17$)	
	n	Frequency	*n*	Frequency	*n*	Frequency
Allele						
5G	47	0.52	35	0.42	12	0.35
4G	43	0.48	49	0.58	22	0.65
Genotype[a]						
5G/5G	11	0.24	3	0.07	0	0
4G/4G or 4G/5G (presence of 4G allele)	34	0.76	39	0.93	17	1.00

[a] Statistical significance ($p=0.013$). Grade 3 vs. grade 1 ($p=0.026$); grade 2 vs. grade 1 ($p=0.040$).

analyzed with the statistical package, SPSS Release 10.0 for Windows (SPSS Inc., Chicago, IL).

Results

To determine whether the PAI-1 4G/5G polymorphism contributes to the level of PAI-1 antigen in breast cancer tissue, we genotyped 104 women with breast cancer and 104 age-matched control women. The PAI-1 4G allele frequency tended to be higher in patients (0.55) than in controls (0.45) ($p=0.062$).

The presence of the 4G allele (4G/5G plus 4G/4G genotypes) was significantly higher in the group of patients with histological grade 3 tumors than in the group with grade 1 tumors ($p=0.026$). It was also higher in the group with grade 2 tumors than in the group with grade 1 tumors ($p=0.040$) (Table 2).

Patients with the 4G allele (4G/5G plus 4G/4G genotypes) constituted a significantly higher percentage of those with a tumor size >2 cm (T_2) than of those with a tumor size ≤2cm (T_1) (96% and 78%, respectively) ($p=0.009$). However, no significant differences in the distribution of the 4G/5G genotype were observed in relation to the presence of hormone receptors and nodal status (data not shown).

We determined whether the PAI-1 4G/5G polymorphism modulates tissue PAI-1 levels (Table 3). We found that tissue antigen PAI-1 levels increased with the number of 4G alleles ($p=0.008$). Similarly, we compared tissue PAI-1 levels among three groups of tumor classed according to SBR (Table 3). We found that tissue PAI-1 levels increased as the histological tumor grade worsened ($p=0.003$).

Tissue PAI-1 levels were higher in the 49 patients with a tumor size >2 cm (T_2) than in the 55 patients with a tumor size ≤2 cm (T_1) (mean, median, range: 4.30, 3.9, 0.30–11.63 ng/mg vs. 3.02, 3.1, 0–10.37 ng/mg, respectively; $p=0.009$). However, no significant differences in PAI-1 level were observed in relation to menopause, hormone receptor or nodal status.

Discussion

In the present study, we found that tissue PAI-1 levels and the frequency of the PAI-1 4G allele were significantly increased in patients with less favorable tumor characteristics (histological grade and macroscopic size). Furthermore, patients with the 4G/4G genotype had tissue PAI-1 levels significantly higher than those with the 5G/5G genotype.

Several studies have shown that high tissue levels of PAI-1, u-PA and uPA:PAI-1 complex are associated with a poor prognosis in breast cancer [8–14,36,37]. Moreover, there is increasing evidence to support a role for PAI-1 in the growth, invasion, and metastasis of malignant tumors [38,39]. PAI-1 could exert these effects by regulating the pericellular function of the plasminogen activator system during migration or by protecting the extracellular matrix, which is necessary for cancer cells during migration. Indeed, higher tissue PAI-1 expression has been associated with aggressive tumors [38].

Table 3 Tissue PAI-1 levels according to 4G/5G PAI-1 polymorphism and histological tumor grade in breast cancer

	5G/5G ($n=14$)	4G/5G ($n=66$)	4G/4G ($n=24$)	Statistical significance
PAI-1ag (ng/mg)	2.14; 1.95 (0.75-4.37)	3.72; 3.32 (0-10.77)	4.21; 4.00 (0.73-11.63)	$p=0.008$
	Grade 1 (n=45)	Grade 2 (n=42)	Grade 3 (n=17)	Statistical significance
PAI-1ag (ng/mg)	2.93; 2.90 (0-8.8)	3.89; 3.71 (0.18-11.63)	4.78; 4.50 (2.2-10.14)	$p=0.003$

Data are expressed as mean; median and range (ng target protein /mg total protein).

As the presence of the 4G allele results in a higher PAI-1 transcription response to cytokines or growth factors than the 5G allele [27,39], the 4G/5G polymorphism may influence tissue PAI-1 levels in breast cancer patients through the action of cytokines released by tumor cells.

The 4G allele of the PAI-1 4G/5G polymorphism seems to be associated with increased plasma PAI-1 levels in vascular disease, but little is known about the possible role of the 4G/5G polymorphism in cancer. It has been reported that this polymorphism seems to be associated with different rates of uPA:PAI-1 complex accumulation in breast cancer and that patients with the 5G/5G genotype showed less aggressive tumor behavior [32]. However, a lack of association between this PAI-1 polymorphism and cancer progression has been observed [31].

In agreement with a previous study [31], we found no significant differences in allele frequencies between patients with breast cancer and controls. On the other hand, both tissue PAI-1 levels and the PAI-1 4G/5G polymorphism seem to be associated with tumor severity in women with breast cancer. This assertion is based on the observation that tissue PAI-1 levels and the number of 4G alleles were significantly higher in patients with histological grade 3 tumors than in those with grade 1 tumors. The PAI-1 4G/5G polymorphism was associated with tumor tissue PAI-1 levels. Thus, the tissue PAI-1 levels significantly increased with the number of 4G alleles. As high tissue PAI-1 levels seem to be correlated with a poor outcome in women with breast cancer [9–12], our results suggest that certain genetic characteristics, particularly the presence of 4G allele, may exert an unfavorable influence on the local behavior of tumors. However, the low number of patients included in our study does not permit to draw definitive conclusions regarding the association of PAI-1 polymorphism with breast cancer.

In conclusion, higher frequencies of the 4G allele are associated with less favorable local characteristics of breast cancer and with increased levels of tumor tissue PAI-1. Further studies with a greater number of patients are needed to clarify the role of the PAI-1 4G/5G polymorphism in breast cancer.

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