Markers of the uPA System and Common Prognostic Factors in Breast Cancer

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Abstract

The urokinase plasminogen activator (uPA) system includes uPA and plasminogen activator inhibitor types 1 (PAI-1) and 2 that mainly act by regulating extracellular matrix degradation, and it is involved in tumor progression. The –675 4G/5G polymorphism of the PAI-1 gene regulates PAI-1 activity in serum. We aimed at studying the –675 4G/5G polymorphism of the PAI-1 gene and uPA, PAI-1, and cyclooxygenase-2 (COX-2) immunohistochemical expression in a series of breast cancer cases. Homozygosity for the 4G allele of the PAI-1 gene was associated with node-positive breast cancer (P = .02). We showed a direct correlation between uPA and estrogen receptor expression (P = .03); negative uPA expression was associated with negative hormonal expression, high tumor grade, and high proliferation index (P < .05). A direct correlation was seen between uPA and PAI-1, uPA and COX-2, and PAI-1 and COX-2 expression (P < .05). Interaction between uPA and COX-2 systems in breast cancer deserves further study.

Tumor invasion and metastasis require proteolytic enzymes that destroy the extracellular matrix and enhance the formation of novel blood vessels. The urokinase plasminogen activator (uPA) system1 acts by modulating plasminogen activation and has a critical role in tumor progression, modulating extracellular matrix degradation and cell proliferation, adhesion, and migration.2 The uPA system includes the serine protease uPA, its 2 serpin inhibitors known as plasminogen activator inhibitor (PAI)-1 and PAI-2, and its membrane-linked receptor (uPAR). After the binding of the inactive precursor pro-uPA to uPAR, the formation of active uPA leads to conversion of plasminogen to plasmin with consequent degradation of matrix components, enhancing cell migration and invasion.1

Notably, uPA and its principal inhibitor are secreted by stromal and breast cancer cells.3 PAI-1, by forming a stable complex with active uPA, determines a negative feedback control with consequent inhibition of plasmin formation.

Several research groups have consistently demonstrated that determination of uPA and PAI-1 levels by the commercially available and quality controlled enzyme-linked immunosorbent assay (ELISA) has strong prognostic value in primary breast cancer.4,5 Duffy et al6 published the first report on the association between high enzymatic activity of uPA and shorter disease-free survival (DFS) in patients with breast cancer. Subsequently, high levels of PAI-1 were unexpectedly reported to be predictive of poor outcome.7 A possible explanation of this apparent paradox was found in the PAI-1 involvement in processes such as cell migration, invasion, and angiogenesis.8 The independent prognostic value of uPA and PAI-1 was widely demonstrated,9-11 and further confirmation was obtained by a pooled analysis by the European Organization for Research and Treatment of Cancer that
involved 8,377 patients with breast cancer with a median follow-up of 79 months. Multivariate analysis showed that high levels of uPA and PAI-1 were associated with shorter DFS and overall survival. Furthermore, in the subgroup analysis of 3,362 patients with node-negative disease who did not receive systemic treatment, uPA and PAI-1 showed the strongest prognostic significance. Thus, according to the demonstrated level I evidence and clinical relevance, uPA and PAI-1 largely satisfied the criteria of the tumor marker utility grading system to be used as prognostic factors in daily practice.

Previous reports have suggested that the insertion (5G)/deletion (4G) polymorphism at position –675 of the PAI-1 gene could sensibly influence the amount of PAI-1 synthesis. In fact, owing to its location at the promoter region, a major impact on the transcription of the gene was shown. Patients with prevalence of 4G polymorphism showed higher PAI-1 plasma levels, whereas 5G/5G homozygosity was associated with lower levels of the inhibitor. More recently, Castelló et al analyzed the PAI-1 genotype distribution in a case-control study comparing 104 women with breast carcinoma and 104 healthy age-matched women. The frequency of the PAI-1 4G allele tended to be higher in patients than in control subjects (P = .062). In addition, after quantification of PAI-1 tumor tissue levels by ELISA, patients with the 4G/4G genotype showed higher levels than those with the 5G/5G genotype.

Cyclooxygenase-2 (COX-2) is an inducible enzyme that is overexpressed in many malignant tumor types and is involved in tumor development and progression. COX-2 may have a role in tumor angiogenesis, inhibition of apoptosis, growth factor signal transduction, and metalloproteinase activation. Some studies have shown that in breast cancer, COX-2 expression is associated with indicators of poor prognosis. It has been argued that COX-2 may interplay with the uPA system in modulating tumor invasion. Accordingly, shorter DFS and overall survival were observed in patients with breast cancer with tumors overexpressing COX-2.

To better understand the role of the uPA system and COX-2 expression in the biology of breast cancer, the present study investigated the relationships of immunohistochemical expression of uPA, PAI-1, COX-2, and other relevant prognostic factors in a series of patients with breast cancer. In addition, the same series was analyzed for the –675 4G/5G polymorphism in the promoter region of the PAI-1 gene.

Materials and Methods

The study cohort included patients with breast cancer who were consecutively referred to the Department of Clinical Oncology, University of Udine, Udine, Italy, between June 2003 and June 2004. All patients were informed about the experimental design of the study and gave written informed consent. All subjects expressly consented to DNA extraction and genotype determination. The study was carried out in conformity with the Helsinki Declaration.

For each patient, tumor samples were reviewed for histologic diagnosis, nodal status, histologic grade, immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PR), MIB-1, HER-2, uPA, PAI-1, and COX-2. Blood samples were obtained from all participants to extract genomic DNA for the genotype determination.

Determination of the 4G/5G PAI-1 Gene Polymorphism

Genotype Determination

Genomic DNA was extracted from 5 mL of venous blood, and the status of the polymorphism –675 4G/5G of the PAI-1 gene was evaluated by polymerase chain reaction (PCR), using an allele-specific PCR procedure. Oligonucleotides used as primers were as follows: 5’-GTCTGGACACGTGGGGA-3’ as the forward primer to detect the 4G allele; 5’-GTCTGGA-CACGTGGGGG-3’ as the forward primer to detect the 5G allele; and 5’-TTTTCCCCAGGGCTGTCCA-3’ as the reverse primer.

The PCR parameters were the following: denaturation at 95°C for 45 seconds, annealing at 62°C for 1 minute, and elongation at 72°C for 1.5 minutes for 38 cycles. After the PCR reaction, samples were directly loaded in a 2.5% agarose gel prestained with ethidium bromide.
Immunohistochemical Analysis of uPA, PAI-1, and COX-2

Immunohistochemical analysis of uPA, PAI-1, and COX-2 was performed on 5-µm sections of paraffin-embedded, formalin-fixed tumor samples. After blocking the endogenous peroxidase with 3% hydrogen peroxide in phosphate-buffered saline for 15 minutes, antigen retrieval for uPA was carried out in a 5-mol/L urea solution at 98°C and for COX-2 was carried out in citrate buffer at 98°C.

Sections were separately incubated overnight at 4°C with antibody to uPA (mouse monoclonal uPA [Ab-1], Calbiochem, San Diego, CA), 2 hours with antibody to PAI-1 (mouse monoclonal NCL-PAI-1, Novocastra, Newcastle upon Tyne, England), and overnight with antiserum to COX-2 (rabbit polyclonal SC-7951, Santa Cruz Biotechnology, Santa Cruz, CA). Subsequent incubations were at room temperature with biotin-labeled appropriate antibody for 30 minutes and avidin-biotin peroxidase complex for 30 minutes. Negative control experiments were carried out by replacing the primary antiserum with appropriate immunoglobulin. The tissue sections were first surveyed at ×100; uPA and PAI-1 immunostaining in tumor cells was scored as 0 (absent), 1 (weak), or 2+ (moderate or strong) and COX-2 immunostaining was scored as 0 (absent), 1+ (weak), 2+ (moderate), or 3+ (strong). The final score was expressed by combining an estimate of the percentage of immunoreactive tumor cells (quantity score) with an estimate of the staining intensity (staining intensity score) in the following formula:

\[ \Sigma (\% \text{ Positive Cells}) \times (\text{Staining Score}) \times 100 \]

Positivity or negativity of fibroblasts in tumor stroma was also analyzed (positive vs negative).

The immunoreactivity of MIB-1, HER-2, ER, and PR was evaluated as previously reported.28 Histologic grading for infiltrating carcinoma was done according to the criteria described by Elston and Ellis.29

Statistical Methods

The \( \chi^2 \) and Fisher exact tests were used to assess the association between categorical variables. The nonparametric Mann-Whitney and Kruskal-Wallis tests were used, as appropriate, to evaluate the presence of statistically significant differences in continuous variables between groups of interest (eg, PAI-1 genotypes). The Spearman correlation was
performed to investigate the strength of the relationship between pairs of variables. \( P \) values less than .05 were considered statistically significant.

## Results

A total of 199 patients with breast cancer were studied; all were white, and the median age was 55 years. Because some patients had bilateral or multifocal/multicentric tumors, the total number of neoplastic lesions was 211. Infiltrating ductal carcinoma and lobular carcinoma were present in 154 (72.9%) and 22 (10.4%) tumors, respectively, with other histologic types in the remaining cases. Hormone receptor–positive tumors (ER and/or PR >0%) were the great majority of the study sample (165 [78.2%]). Nodal status at diagnosis was assessed in 157 patients, and it was negative in 104 of them.

The PAI-1 promoter region polymorphism was assessed in 193 patients with breast cancer: 56 (29.0%) had 4G/4G homozygosity, 52 (26.9%) had 5G/5G homozygosity, and 85 (44.0%) had the 4G/5G genotype, with frequencies of 0.51 and 0.49 for alleles 4G and 5G, respectively. In a control population of 142 unaffected women, 39 (27.5%), 35 (24.6%), and 68 (47.9%) had the 4G/4G, 5G/5G, and 4G/5G genotypes, respectively. The allelic frequencies in the control population were 0.51 and 0.49 for the 4G and 5G alleles, respectively. Therefore, in terms of genotype and allele frequency, no significant difference exists between control subjects and patients. Control and breast cancer populations were in Hardy-Weinberg equilibrium.

4G/4G homozygosity was associated with positive nodes at diagnosis (\( P = .03; \chi^2 \) test; odds ratio, 1.63; 95% confidence interval, 1.02-2.62) [\textit{Table 1}]. No statistically significant associations were found between \textit{PAI}-1 promoter region polymorphism genotypes and \textit{PAI}-1 protein expression in tumor or in stroma cells; no association of genotype with other tumor characteristics was evident.

Median scores of immunohistochemical expression for uPA, \textit{PAI}-1, and \textit{COX}-2 were 100, 70, and 100, respectively; 8% of samples were negative for uPA, 9% for \textit{PAI}-1, and 3% for \textit{COX}-2 expression. Correlations in immunohistochemical expression scores for uPA, \textit{PAI}-1, and \textit{COX}-2 are reported in [\textit{Table 2}].

Positive correlations were found between uPA and \textit{PAI}-1 expression, between uPA and \textit{COX}-2 expression, and between \textit{PAI}-1 and \textit{COX}-2 expression. We showed a direct correlation between uPA and ER expression (Spearman \( \rho = 0.2; P = .03 \)). Absence of uPA expression in cancer cells was associated with negative hormone receptor status (\( P < .05; \) Fisher exact test), higher histologic grade (\( P < .05; \) Fisher exact test), and higher proliferative activity, as determined by MIB-1 immunoreactivity (23.9% vs 8.9%; \( P = .005; \) Mann-Whitney \( U \) test). In addition, intense expression of HER-2 (score 3+) was significantly associated with low expression of uPA (\( P = .03; \) Mann-Whitney \( U \) test).

Positive expression of uPA in tumor cells was associated with positive expression in tumor stroma fibroblasts (\( P < .05; \chi^2 \) test). Similarly, there was also an association between tumor cells and fibroblast expression of \textit{PAI}-1 (\( P < .05; \chi^2 \) test).

### [\textit{Table 1}]

**Association Between \textit{PAI}-1 Genotype and Node Status**

<table>
<thead>
<tr>
<th>Node Status</th>
<th>Positive ((n = 53))</th>
<th>Negative ((n = 99))</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G/4G</td>
<td>19 ((36))</td>
<td>20 ((20))</td>
</tr>
<tr>
<td>4G/5G and 5G/5G( ^{†} )</td>
<td>34 ((64))</td>
<td>79 ((80))</td>
</tr>
</tbody>
</table>

\( ^{†} \) \textit{PAI}-1, plasminogen activator inhibitor type 1.

\* Data are given as number (percentage). All associations were significant \( (P = .03; \chi^2 \) test). \n
4G/5G cases, 23 positive and 49 negative; 5G/5G cases, 11 positive and 30 negative.

### [\textit{Table 2}]

**Correlation of uPA, \textit{PAI}-1, and \textit{COX}-2 Immunohistochemical Expression**

<table>
<thead>
<tr>
<th>( \rho ) *</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA/\textit{PAI}-1</td>
<td>0.23</td>
</tr>
<tr>
<td>uPA/\textit{COX}-2</td>
<td>0.30</td>
</tr>
<tr>
<td>\textit{PAI}-1/\textit{COX}-2</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\* Spearman correlation test.

COX-2, cyclooxygenase-2; \textit{PAI}-1, plasminogen activator inhibitor type 1; uPA, urokinase plasminogen activator.
High COX-2 expression was associated with younger age at diagnosis (Spearman $\rho = -0.2; P = 0.03$) and intense HER-2 expression (score $3+ vs < 3+; P = 0.003$; Mann-Whitney $U$ test).

**Discussion**

Previous studies aiming to investigate the influence of genetic variability on PAI-1 synthesis reported that the 4G allele of PAI-1 promoter region is associated with higher levels of PAI-1 in serum or in tumor tissues. However, this observation was not consistent in all published works. Our study failed to demonstrate an association between PAI-1 promoter region polymorphism and PAI-1 immunohistochemical expression in breast cancer tumors. However, we observed an association between 4G/4G PAI-1 promoter region polymorphism and PAI-1 immunohistochemical expression and positive nodes in patients with breast cancer. This discrepancy could have different explanations. First, our immunohistochemical method might not be sensitive enough to detect changes in PAI-1 expression. Accordingly, most studies indicating the effect of the 4G/5G genotype on PAI-1 expression have been performed by ELISA. If this explanation is the correct one, it is tempting to hypothesize that higher PAI-1 activity levels, although not detected by immunohistochemical analysis, could promote cell migration by modulation of the extracellular matrix degradation and tumor invasion with consequent higher risk of lymph node metastases. A second explanation could be the linkage disequilibrium between the 4G allele and a different genetic determinant that has a direct role in determining the node positivity.

In our series, PAI-1 immunohistochemical expression was not associated with other classical predictive and prognostic factors in breast cancer. We found positive correlation in PAI-1, uPA, and COX-2 expression.

The absence of uPA immunohistochemical expression was more common in endocrine nonresponsive, poorly differentiated, and highly proliferative tumors; in addition, more intense HER-2 expression was associated with lower uPA scores. Although the observation that negative or low expression of uPA in neoplastic cells is associated with a more aggressive tumor phenotype could seem paradoxical, some explanation could be hypothesized. The knowledge that stromal cells rather than the epithelial cells of the tumor produce the majority of uPA and PAI-1 may suggest that the separate study of the different compartments of the tumor environment by immunohistochemical analysis could not give information about the functional interaction among cells.

Of note, Dublin et al$^{33}$ reported that uPA expression in breast tumor cells and fibroblasts was associated with high histologic grade and was more common in invasive than in intraductal carcinoma. Similarly, strong expression of PAI-1 in neoplastic cells and fibroblasts was associated with high histologic grade; the patients with strong fibroblastic expression of uPA showed a trend toward a worse prognosis. Dublin et al$^{33}$ concluded that fibroblastic expression rather than tumor cell expression of the uPA system molecules could influence breast cancer outcome.

Our study also showed a positive correlation between the uPA system and COX-2 expression. This finding supports previous observations suggesting the existence of a link between the uPA- and metalloproteinase-mediated tumor invasion activity and the functional role of COX-2 in the metastatic process. In particular, it has been demonstrated that COX-2 up-regulates matrix metalloproteinases (MMPs). In a corneal model, COX-2 increased uPA, MMP-1, and MMP-9 levels. Similarly, colorectal tumor cells treated with COX-2 inhibitor (NS398) showed a decrease in uPA messenger RNA and proliferation. COX-2 increased cellular migration and invasion in breast cancer cells through enhancement of pro-uPA levels. Sivula et al$^{37}$ evaluated COX-2 and MMP-2 expression in a series of breast carcinomas. A significant association between MMP-2 and COX-2 was found, and high expression of both markers resulted in decreased disease-specific survival. New insights of mechanisms of tumor invasion and metastasis are eagerly awaited. In breast cancer, a better understanding of the relationship between expression of the uPA system and COX-2 is of valuable biologic importance and could translate into new therapeutic opportunities.$^{38}$

**References**


