# Quantitative Real-Time Reverse Transcription-PCR Assay for Urokinase Plasminogen Activator, Plasminogen Activator Inhibitor Type 1, and Tissue Metalloproteinase Inhibitor Type 1 Gene Expressions in Primary Breast Cancer

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**Background:** The plasminogen activation system and matrix metalloproteinases (MMPs) play a key role in the degradation of basement membrane and extracellular matrix in tissue remodeling, cancer cell invasion, and metastasis.

**Methods:** Quantitative real-time reverse-transcription-PCR (RT-PCR) assays were developed to quantify urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor type 1 (PAI-1), and tissue metalloproteinase inhibitor type 1 (TIMP-1) mRNA in 54 breast cancer tissues. Gene fragments were amplified in a LightCycler real-time PCR system using gene-specific primers and SYBR Green I. The results were normalized to $\beta$-actin mRNA. We also quantified antigen and functional concentrations of these components.

**Results:** The intra- and interassay variabilities for mRNA quantification showed mean SDs for the crossing point of 0.12 and 0.15 cycles, respectively. PAI-1, uPA, and TIMP-1 mRNA and antigen concentrations and PAI-1 and uPA functional concentrations increased with tumor severity; the increase was statistically significant for PAI-1, uPA, and TIMP-1 mRNA and antigen concentrations and for uPA functional concentrations. Node-positive patients showed significantly higher PAI-1, uPA, and TIMP-1 mRNA and antigen concentrations than those who were node negative.

**Conclusions:** Quantitative real-time RT-PCR is a highly sensitive, reproducible, and fast method for measuring gene expression of PAI-1, uPA, and TIMP-1 in breast cancer. These components may be involved in breast cancer development, and increased mRNA expression may be associated with a worse prognosis.
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Tumor cell invasion and metastatic processes require the coordinated regulation of a series of adhesive, proteolytic, and migratory events *(1)*. The plasminogen activation system and matrix metalloproteinases (MMPs)[3] play a key role in the degradation of basement membrane and extracellular matrix in tissue remodeling, cancer cell invasion, and metastasis *(2)*.

Urokinase-type plasminogen activator (uPA) is a serine protease that catalyzes the conversion of plasminogen to plasmin, an active enzyme that is able to degrade various extracellular matrix proteins and to activate MMPs and growth factors *(3)*. Plasminogen activator inhibitor type-1 (PAI-1) is a multifaceted proteolytic inhibitor. It not only functions as a fibrinolytic inhibitor *(4)*, but also plays an important role in signal transduction, cell adherence, and cell migration *(5)*. There is clinical evidence implicating PAI-1 as a key factor for tumor invasion and metastasis. Both uPA and PAI-1 have been linked to poor prognosis in several cancers, including breast cancer *(6, 7)*.

On the other hand, MMPs are involved in several pathologic processes, including tumor invasion, in which

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degradation of the extracellular matrix is a key event (8). MMP activities are regulated by tissue inhibitors of metalloproteinases (TIMPs) (9). Four members of this family have been identified, among which TIMP-1 acts against all members of the collagenase, stromelysin, and gelatinase classes of MMPs (9). TIMP-1 is thus considered to inhibit carcinoma invasion and metastasis. However, recent reports have suggested that TIMP-1 may also posses a growth-promoting function (10).

The amount of protein produced represents only one aspect of altered gene expression in tumor cells. The relationship between the protein concentrations and the amount of mRNA is an important basic factor in understanding changes in gene expression. Previous studies have indicated that high antigenic concentrations of uPA (7, 11), PAI-1 (7, 12), and TIMP-1 (13) in extracts of primary breast cancer are associated with poor prognosis for the patients. However, the quantification of uPA, PAI-1, and TIMP-1 gene expression in primary breast cancer tissue by quantitative reverse transcription-PCR (RT-PCR), compared with protein concentrations, has not been studied. Quantitative RT-PCR is a potent method that efficiently and selectively measures mRNA quantities that are undetectable by other techniques. It is a more sensitive method to quantify gene expression than conventional methods used for mRNA evaluation, such as Northern blotting, dot-blot analysis, and the RNase protection assay. In practice, breast tumor biopsies available for RNA analysis are usually small, and the conventional methods require large amounts of starting material.

In the present study, a novel quantitative real-time RT-PCR was developed and validated for specific quantification of uPA, PAI-1, and TIMP-1 mRNAs in breast cancer. We compared the results with the corresponding protein concentrations and analyzed the relationship with cancer severity.

## Materials and Methods

### PATIENTS

The study group consisted of 54 breast cancer patients (mean age, 58 years; range, 34–78 years). Female patients were selected according to the following criteria: (a) primary unilateral breast cancer; (b) previously untreated and no evidence of metastatic disease or any other malignancy at the time of diagnosis; (c) complete clinical, histologic, and biological information; and (d) surgery as the first treatment.

The following information was recorded for each patient: age and diagnosis, menopausal status, clinical and macroscopic tumor size, pTNM grade, axillary lymph node status, and steroid receptor status. Histologic types included 44 ductal carcinomas not otherwise specified, 4 lobular tumors, and 6 others. All the tumors were graded according to pTNM in stages I (n = 20), IIA (n = 21), and IIB (n = 13). The patients were also classified into two groups according to axillary lymph node status: node-negative ($N_0$; n = 30) or node-positive ($N_1$; n = 24). Tumor

tissue samples were obtained in accordance with Helsinki Declaration.

### ISOLATION AND PURIFICATION OF TOTAL RNA

Tumor specimens were obtained at surgery, selected by the pathologist, and stored in liquid nitrogen. The tissue was cut into two pieces: one was used for RNA extraction, and the other for protein extraction. Total RNA was extracted from tumor samples according to the protocol of the RNeasy total RNA reagent set (Qiagen). The amount of RNA was measured spectrophotometrically by the absorbance at 260 nm. One microgram of RNA was incubated for 15 min at room temperature with DNase I (1 U/$\mu$g; Invitrogen), followed by thermal inactivation of the enzyme (65 °C for 10 min) in the presence of 2.5 mmol/L EDTA and a rapid cooling down to 4 °C. The purity of the RNA was estimated by the ratio of the absorbance at 260/280 ($A_{260/280}$). The RNA was stored at −80 °C until use.

### REVERSE TRANSCRIPTION

The reverse transcription reaction was carried out in a total volume of 20 $\mu$L of 1× reverse transcriptase buffer containing 10 mM dithiothreitol, 500 $\mu$M deoxynucleotide triphosphates, 3 $\mu$M oligo(dT)$_{15}$, 60 units of RNasin, and 200 U of Superscript RNase H$^-$ (Gibco). To this mixture, we added 1 $\mu$g of total RNA treated with DNase I. The reaction was allowed to proceed for 60 min at 42 °C, followed by 5 min of heating at 95 °C and rapid cooling on ice. The cDNA was stored at −20 °C until use.

### PREPARATION OF cDNA CALIBRATORS

cDNA calibrators were prepared by PCR amplification run to saturation (35 PCR cycles) with the appropriate primers. The resulting cDNAs were purified by column chromatography (high pure product purification reagent set; Roche Diagnostics) and eluted with Tris-EDTA (pH 8.0) buffer. The samples showed a unique band in agarose electrophoresis. The amount of DNA was determined by Pico Green fluorescence (Molecular Probes).

### REAL-TIME PCR

The primers used in the analysis of uPA, PAI-1, TIMP-1, and $\beta$-actin gene expression are given in Table 1. TIMP-1 and $\beta$-actin primers were obtained as described previously (14, 15). The uPA and PAI-1 primers were designed using specific primer analysis software (Oligo 4.0). and these sequences analyzed by FASTA in the EMBL database (http://www.embl-heidelberg.de/).

The PCR reactions were performed in a LightCycler apparatus using LC-Fast Start Reaction Mix SYBR Green I (Roche Diagnostics). Thermocycling was done in a final volume of 10 $\mu$L containing 1.5 $\mu$L of cDNA sample (diluted 1:10) or calibrator; 3 mM MgCl$_2$; 0.5 $\mu$M each of the uPA, PAI-1, and TIMP-1 primers or 0.3 $\mu$M each of the $\beta$-actin primers; 1 $\mu$L of LC-Fast Start Reaction Mix SYBR Green I; and 1 $\mu$L of LC-Fast Start DNA Master SYBR

## Table 1. Oligonucleotide PCR primers for human PAI-1, uPA, TIMP-1, and β-actin.

| Oligonucleotide name | Oligonucleotide primer sequence, 5'→3' | Fragment size, bp |
|---|---|---|
| PAI-1 | | |
|   Forward primer | TGC TGG TGA ATG CCC TCT ACT | 399 |
|   Reverse primer | CGG TCA TTC CCA GGT TCT CTA | |
| uPA | | |
|   Forward primer | CAC GCA AGG GGA GAT GAA | 341 |
|   Reverse primer | ACA GCA TTT TGG TGG TGA CTT | |
| TIMP-1 | | |
|   Forward primer | CTG TTG TTG CTG TGG CTG ATA | 481 |
|   Reverse primer | CCG TCC ACA AGC AAT GAG T | |
| β-actin | | |
|   Forward primer | CGT ACC ACT GGC ATC GTG AT | 452 |
|   Reverse primer | GTG TTG GCG TAC AGG TCT TTG | |

Green I/Enzyme (including *Taq* DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mixture). After 10 min at 95 °C to denature the cDNA and to activate the *Taq* DNA polymerase, the cycling conditions were as follows: 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C (for uPA, PAI-1, and TIMP-1) or at 62 °C (for β-actin) for 5 s, and extension at 72 °C for 18 s (for uPA, PAI-1, and TIMP-1) or for 15 s (for β-actin).

After PCR, a melting curve was constructed by increasing the temperature from 65 °C to 95 °C with a temperature transition rate of 0.1 °C/s. PAI-1, uPA, TIMP-1, and β-actin sequences were amplified in duplicate from the patient samples. The assay was completed in ~1 h. To ensure that the correct product was amplified in the reaction, all samples were separated by 2% agarose gel electrophoresis. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. The Second Derivative Maximum Method was used to determine the crossing point (Cp) automatically for the individual samples. This was achieved by a software algorithm (Ver. 3.5) that identifies the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve, which serves as the Cp. The LightCycler software constructed the calibration curve by plotting the Cp vs the logarithm of the number of copies for each calibrator. The numbers of copies in unknown samples were calculated by comparing their Cps with the calibration curve. To correct for differences in both RNA quality and quantity between samples, data were normalized using the ratio of the target cDNA concentration to that of β-actin.

TISSUE EXTRACTS FOR PAI-1, uPA, AND TIMP-1 ASSAYS
Tissue fragments was pulverized in 10 mmol/L Tris-HCl buffer containing 1.5 mmol/L EDTA and 100 mL/L glycerol. The suspension was centrifuged at 100 000g at 4 °C for 15 min, and the supernatant (cytosol extract) was aliquoted and stored at −80 °C. The pelleted membranes

were solubilized in 20 mmol/L Tris-HCl buffer containing 125 mmol/L NaCl and 10 mL/L Triton X-100, incubated overnight at 4 °C, and centrifuged at 100 000g at 4 °C for 15 min. The supernatant (membrane extracts) was aliquoted and stored at −80 °C.

Total protein in both cytosol and membrane extracts was determined with the BCA protein assay (Pierce). Bovine serum albumin, fraction V (Sigma), was used for calibration. Samples and calibrators were both assayed in duplicate.

PAI-1, uPA, AND TIMP-1 QUANTIFICATION
PAI-1 antigen was quantified by a commercially available ELISA (Tint Elize PAI-1; Biopool). The assay detects active and inactive forms of PAI-1, as well as tissue-type plasminogen activator/PAI and uPA/PAI complexes. The intra- and interassay CVs were 3% and 7%, respectively.

PAI-1 functional concentrations were determined as described previously *(16)*. One unit of PAI-1 activity was defined as the amount that inhibited 1 IU of single tissue-type plasminogen activator in 15 min at room temperature under the conditions used. The intra- and interassay CVs 6% and 10%, respectively.

The uPA antigen was quantified by a commercially available ELISA (Tint Elize uPA; Biopool). It measures single-chain uPA and a high-molecular-weight form of uPA with approximately the same efficiency. The intra- and interassay CVs were 4% and 10%, respectively.

The uPA and activatable single-chain uPA functional concentrations were determined by an immunosorbent activity assay (Chromolize uPA; Biopool). The intra- and interassay CVs were 6% and 11%, respectively.

TIMP-1 antigen was quantified by a commercially available ELISA assay (TIMP-1 ELISA; Oncogene). The intra- and interassay CVs were 4% and 7%, respectively.

The PAI-1, uPA, and TIMP-1 antigen concentrations were determined in both cytosol and membrane extracts. The PAI-1 and uPA functional concentrations were determined in cytosol extracts.

STATISTICAL ANALYSIS
Differences between the means of the analyzed variables observed in the patient subgroups were determined by the Student *t*-test and by the Mann–Whitney nonparametric *U*-test. Significance in correlations between tumor stage and variables was calculated by bivariate Pearson correlation, partial correlation, and multiple linear regression test. $P < 0.05$ (two-tailed) was considered significant. All these tests were performed using the statistical package SPSS Release 6.0 for Windows (SPSS Inc.).

## Results

We analyzed PAI-1, uPA, and TIMP-1 mRNA and antigen concentrations in 54 breast cancer tissue samples. The mRNA concentrations were determined after extensive optimization of PCR conditions, including primer and

MgCl$_2$ concentrations and reaction temperatures and times. This allowed us to obtain a highly sensitive, specific, and reproducible quantitative real-time RT-PCR for specific detection of these mRNAs.

## SENSITIVITY AND SPECIFICITY OF PCR

To test the sensitivity of the method based on Cp values, calibration curves were prepared for PAI-1, uPA, and TIMP-1 cDNAs from known quantities of cDNAs (each diluted 10-fold from $10^6$ to $10^2$ copies of cDNA per reaction mixture). All calibration curves showed correlation coefficients >0.99, indicating a precise log-linear relationship. The detection limit, using the specific primers showed in Table 1, was the same (10 copies of cDNA) for all three target genes (Fig. 1). The efficiencies for the dilution series for all target genes were very similar: 1.87 for PAI-1, 1.90 for uPA, and 1.88 for TIMP-1.

As shown Fig. 2, melting behavior, expressed as a plot of the first negative derivative ($-dF/dT$) of the fluorescence vs temperature, revealed the high purities and homogeneities of PCR products. Single and sharply defined melting curves with narrow peaks were obtained for PCR products of the analyzed genes. No fluorescence signal was observed with no-template samples or when reverse transcription was omitted, even after 40 PCR cycles. Bands visible after electrophoresis in 2% agarose gel and ethidium bromide staining correlated well with the obtained quantitative PCR results.

## REPRODUCIBILITY

PAI-1, uPA, and TIMP-1 mRNA intraassay variability was determined from triplicate samples of the 11 targets and 5 controls. The mean SDs for Cp were 0.12 cycles for PAI-1, 0.11 cycles for uPA, and 0.12 cycles for TIMP-1. The difference in absolute Cp values for each set of triplicates was <0.57 cycles. Interassay variability, calculated from triplicates of these samples assayed on 3 different work days, showed SDs of 0.15 cycles for PAI-1, 0.16 cycles for uPA, and 0.14 cycles for TIMP-1. The difference in Cp was never >1.2 cycles. The targets were obtained from the same mRNA preparation, and the calibrators were run on each day.

## QUANTIFICATION OF PAI-1, uPA, AND TIMP-1 mRNAS IN BREAST CANCER TISSUE

The PAI-1, uPA, and TIMP-1 mRNA values in breast cancer tissues were normalized to $\beta$-actin mRNA. Table 2 shows the tissue PAI-1, uPA, and TIMP-1 mRNA concentrations according to node status. Significant increases in all mRNA concentrations in node-positive ($N_1$) compared with node-negative ($N_0$) breast cancer patients were found. As shown in Table 3, PAI-1, uPA, and TIMP-1 expression increased with tumor severity, and the increases were significant between grades I and IIB for all mRNA concentrations. Furthermore, statistically significant correlations between PAI-1 mRNA ($r = 0.29$; $P$ <0.05), uPA mRNA ($r = 0.29$; $P$ <0.05), and TIMP-1
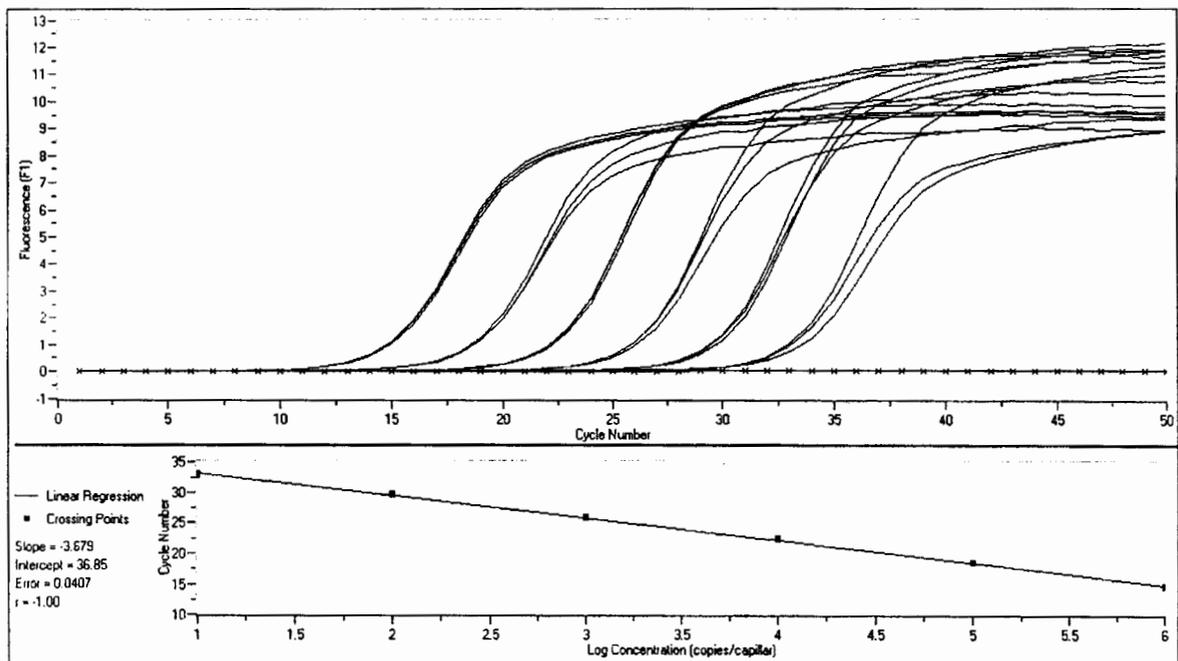


Fig. 1. Fluorescence data for PAI-1 calibrators (*top*) together with the resulting calibration curve generated by the LightCycler software (*bottom*).
Solid lines indicate PAI-1 diluted from $10^6$ to 10 copy numbers assayed in triplicate. No product was amplified in the no-template sample or when reverse transcription was omitted ($\times$). Similar results were obtained when PCR products of uPA and TIMP-1 were analyzed.
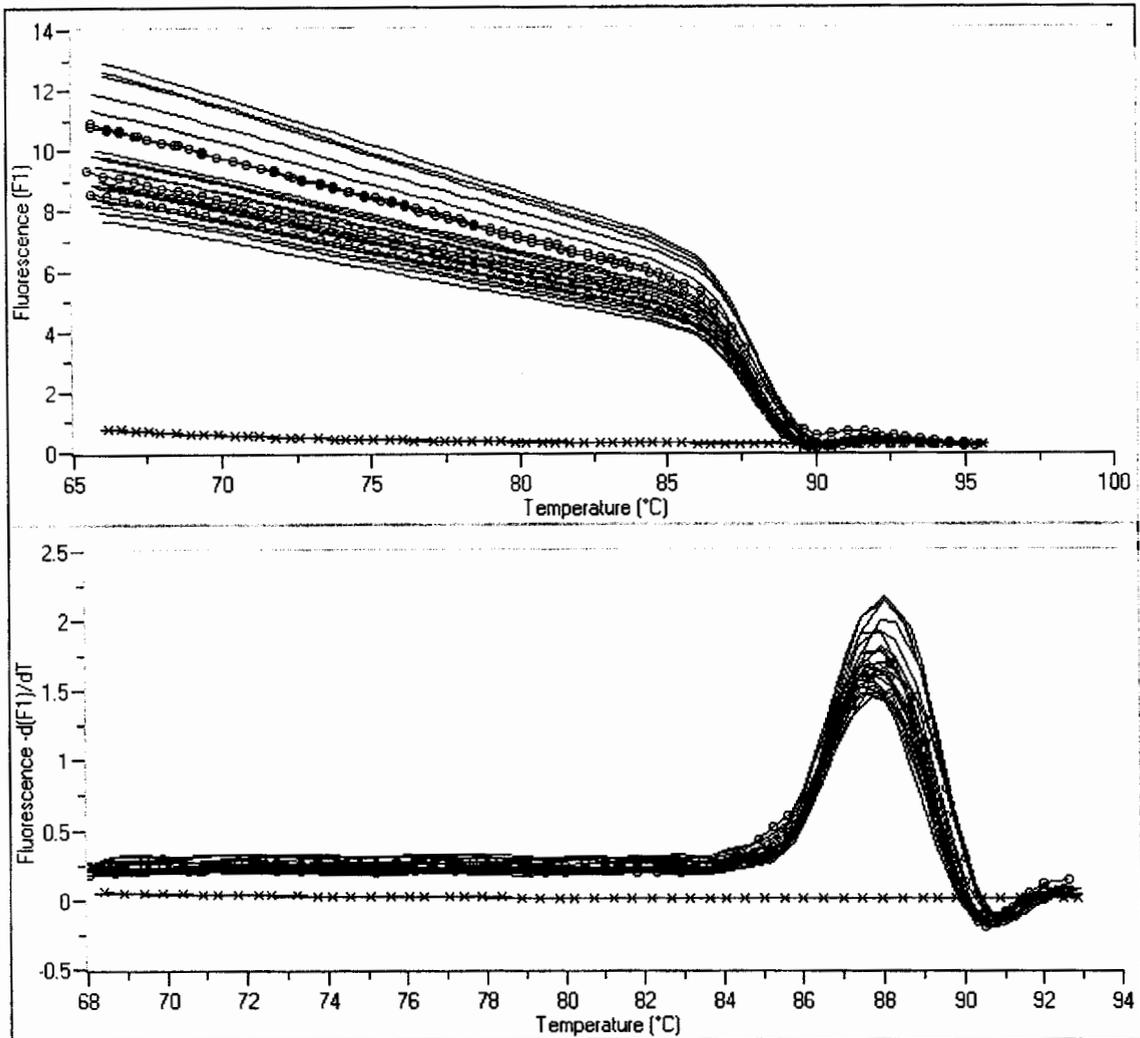
Fig. 2. Melting curves for all PCR products after uPA amplification.

Only one sharp peak for each sample [controls (O) and targets (*solid lines with no symbols*)] was observed. This indicates that only one product was amplified, and no product was observed in the no-template sample or when reverse transcription was omitted (✕). In a 2% agarose gel, only one band was observed in all samples, and no signal was found in the no-template sample or when reverse transcription was omitted. Similar results were obtained when PCR products of PAI-1 and TIMP-1 were analyzed.

mRNA ($r = 0.26$; $P < 0.05$) concentrations and tumor severity were observed.

### PAI-1, uPA, AND TIMP-1 ANTIGEN AND PAI-1 AND uPA FUNCTIONAL CONCENTRATIONS IN BREAST CANCER TISSUE

The PAI-1, uPA, and TIMP-1 antigen concentrations in tumor extracts (cytosol plus membrane extracts) and the tissue PAI-1 and uPA functional (cytosol extracts) concentrations of patients with ($N_1$) or without ($N_0$) positive nodes are shown in Table 2. Significant increases in PAI-1, uPA, and TIMP-1 antigen concentrations were observed in the $N_1$ group compared with the $N_0$ group (Table 2). Moreover, when patients were classified according to tumor severity (Table 3), a significant increase was observed in PAI-1, uPA, and TIMP-1 antigen and mRNA

concentrations and uPA functional concentrations in group IIB compared with group I. Furthermore, significant increases in uPA and TIMP-1 antigen concentrations in group IIA compared with group I were found. Significant correlations were observed between tumor severity and PAI-1 antigen ($r = 0.57$; $P < 0.001$), uPA antigen ($r = 0.49$; $P < 0.005$), TIMP-1 antigen ($r = 0.48$; $P < 0.001$), and uPA ($r = 0.27$; $P < 0.05$) functional concentrations.

Positive but nonsignificant correlations between PAI-1, uPA, and TIMP-1 antigen and mRNA concentrations were also observed in breast cancer tissues.

### Discussion

In the present study, we developed a quantitative real-time RT-PCR method to quantify, for the first time, PAI-1, uPA, and TIMP-1 mRNAs in breast cancer. The PAI-1,

**Table 2. PAI-1, uPA, and TIMP-1 antigen and mRNA concentrations and PAI-1 and uPA functional concentrations in patients with node-negative ($N_0$) or node-positive ($N_1$) breast cancer.**

| | Mean ± SE | | |
|---|---|---|---|
| | $N_0$ (n = 30) | $N_1$ (n = 24) | P |
| PAI-1 | | | |
| mRNA[a] | 0.51 ± 0.01 | 1.17 ± 0.20 | <0.01 |
| Ag,[b,c] ng/mg | 3.32 ± 0.54 | 5.60 ± 1.02 | <0.05 |
| Fc,[d] units/mg | 3.23 ± 1.06 | 4.69 ± 1.10 | NS |
| uPA | | | |
| mRNA[a] | 1.99 ± 0.20 | 2.96 ± 0.40 | <0.05 |
| Ag, ng/mg | 2.12 ± 0.26 | 3.73 ± 0.41 | <0.05 |
| Fc, ng/mg | 0.37 ± 0.10 | 0.50 ± 0.11 | NS |
| TIMP-1 | | | |
| mRNA[a] | 2.93 ± 0.30 | 3.99 ± 0.40 | <0.05 |
| Ag, ng/mg | 41.7 ± 4.1 | 58.2 ± 5.6 | <0.05 |

[a] Normalized mRNA = 100 × (target gene mRNA copies/β-actin mRNA copies).

[b] Ag, antigen; Fc, functional concentration; NS, not significant.

[c] PAI-1, uPA, and TIMP-1 antigen values represent cytosol extract plus membrane extract.

[d] PAI-1 and uPA functional concentrations were determined only in the cytosol extract.

uPA, and TIMP-1 mRNA concentrations increased with tumor severity.

The assay is a rapid *(17)* and sensitive method to quantify gene expression and requires smaller amounts of breast tissue than conventional methods, such as Northern blotting *(18)*. Quantitative real-time RT-PCR is an ideal method for studies when the amount of tissue is limited. Competitive RT-PCR is a PCR endpoint analysis that permits only semiquantitative analysis at a low sensitivity and requires multiple assays *(19)*. In contrast, in quantitative real-time RT-PCR assays quantification is performed during the exponential phase of amplification and permits accurate quantification without any post-PCR processing. Moreover, lower intra- and interassay variabilities are obtained in comparison with those published previously *(20)*, which is probably attributable to the differences in assay targets.

In the present study, PAI-1, uPA, and TIMP-1 mRNA and antigen concentrations increased with tumor severity. However, mRNA assays are faster, less expensive, and require smaller amounts of tissue than do protein assays. The mRNA assay could be used preferentially over the protein assay when the amount of tissue is limited because breast tumor tissues available for analysis are usually small.

The plasminogen activation system plays a role in cancer progression, presumably via extracellular matrix degradation and tumor cell migration *(3)*. It is generally believed that uPA at the cell surface initiates a proteinase cascade and promotes tumor invasion and angiogenesis *(21)*. uPA is frequently overexpressed in breast cancers and is a strong prognostic indicator for decreased patient survival rates *(22)*. In the present report, increased uPA mRNA concentrations were associated with breast cancer severity. To our knowledge, there have been only a few published studies concerning uPA mRNA expression in breast cancer. Our results using this quantitative real-time RT-PCR technique are in accordance with those obtained using semiquantitative techniques, such as Northern blotting *(23)*.

We also observed that PAI-1 mRNA and protein concentrations increased with tumor severity, and a good correlation with PAI-1 protein concentrations was obtained. Surprisingly, high concentrations of PAI-1 protein have been reported to correlate with cancer severity,

**Table 3. PAI-1, uPA, and TIMP-1 antigen and mRNA concentrations and PAI-1 and uPA functional activities according to the grade of malignancy of breast cancer tissues.[a]**

| | Grade | | | P | | | |
|---|---|---|---|---|---|---|---|
| | I (n = 20) | IIA (n = 21) | IIB (n = 13) | ANOVA | I-IIA | I-IIB | IIB-IIA |
| PAI-1 | | | | | | | |
| mRNA[b] | 0.53 ± 0.07 | 0.81 ± 0.15 | 1.18 ± 0.33 | <0.05 | NS[c] | <0.05 | NS |
| Ag,[d] ng/mg | 2.72 ± 0.60 | 5.02 ± 0.98 | 5.68 ± 1.28 | <0.05 | NS | <0.05 | NS |
| Fc,[e] units/mg | 2.29 ± 0.93 | 4.75 ± 1.34 | 4.91 ± 1.9 | NS | NS | NS | NS |
| uPA | | | | | | | |
| mRNA[b] | 1.92 ± 0.16 | 2.31 ± 0.28 | 3.30 ± 0.69 | <0.05 | NS | <0.05 | NS |
| Ag, ng/mg | 1.66 ± 0.25 | 3.17 ± 0.37 | 3.72 ± 0.57 | <0.05 | <0.05 | <0.05 | NS |
| Fc, ng/mg | 0.25 ± 0.08 | 0.46 ± 0.14 | 0.64 ± 0.18 | <0.05 | NS | <0.05 | NS |
| TIMP-1 | | | | | | | |
| mRNA[b] | 2.93 ± 0.31 | 3.37 ± 0.44 | 4.17 ± 0.47 | <0.05 | NS | <0.05 | NS |
| Ag, ng/mg | 33.0 ± 5.2 | 50.4 ± 4.7 | 66.2 ± 6.6 | <0.005 | <0.05 | <0.01 | NS |

[a] Data are expressed as means ± SE.

[b] Normalized mRNA = 100 × (target gene mRNA copies/β-actin mRNA copies).

[c] NS, not significant; Ag, antigen; Fc, functional concentration.

[d] PAI-1, uPA, and TIMP-1 antigen values represent cytosol extract plus membrane extract.

[e] PAI-1 and uPA functional concentrations were determined only in the cytosol extract.

despite the known ability of PAI-1 to inhibit uPA activity *(7, 12, 24)*. Furthermore, several studies have shown that high PAI-1 protein concentrations are associated with a poor prognosis in breast cancer *(7, 25)*. No explanation has been found for this apparent paradox. Recently, Bajou et al. *(26)* demonstrated that PAI-1 is essential for tumor angiogenesis by inhibiting proteolytic activity, suggesting that excessive plasmin proteolysis prevents assembly of tumor vessels. The authors suggested that a critical balance between proteases and PAI-1 concentrations is necessary for optimal invasion *(26)*.

MMPs are involved in degradation of basement membrane extracellular matrix; this degradation represents a key element in tumor invasion and metastasis *(27–29)*. TIMP-1 is an inhibitor of MMPs and is considered to inhibit carcinoma invasion *(30)*. However, increased TIMP-1 protein concentrations have been associated with tumor severity in breast cancer *(13)*. In the present study, an increase in TIMP-1 mRNA was correlated with tumor severity. An increase in expression of TIMP-1 in breast cancer has been also reported, in a study using a semiquantitative in situ hybridization technique *(31)*. Furthermore, high concentrations of TIMP-1 mRNA, analyzed using the Northern blot method, have been associated with metastasis in breast cancer *(32)*. These data are inconsistent with the concept of TIMP-1 being an inhibitor of MMPs. However, it has been suggested that TIMP-1 expression is a tumor-induced host response to extracellular matrix perturbation caused by invasion *(31, 33)*. Moreover, TIMP-1 may have a double function in tumor progression: *(a)* as a MMP inhibitor, it could prevent tumor dissemination, and *(b)* as a stimulator of vascular endothelial growth factor, it could enhance tumor growth *(34)*. It has also been reported that TIMP-1 stimulates the growth of human breast cancer cells and that the nuclear localization of this inhibitor can affect cellular proliferation *(35)*.

In conclusion, we have described for the first time a quantitative real-time RT-PCR assay for PAI-1, uPA, and TIMP-1 mRNA in breast cancer. The method is highly sensitive, reproducible, and rapidly measures gene expression. PAI-1, uPA, and TIMP-1 may be involved in breast cancer development, and increased protein and mRNA concentrations of these components would lead to a poorer prognosis for patients. We believe that this quantitative real-time RT-PCR assay is rapid and easy and may be a valuable tool for further investigations of plasminogen activation and MMP systems in cancer.

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