

Tumor Tissue Concentrations of the Proteinase Inhibitors Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) and Plasminogen Activator Inhibitor Type 1 (PAI-1) Are Complementary in Determining Prognosis in Primary Breast Cancer*

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The purpose of this study was to investigate the association between tumor tissue levels of total tissue inhibitor of metalloproteinases-1 (TIMP-1) and prognosis in patients with primary breast cancer and to analyze whether measurement of TIMP-1 in tumor extracts added prognostic information to that obtained from measurements of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 (PAI-1). An established sandwich enzyme-linked immunosorbent assay was thoroughly validated for the measurement of total TIMP-1 in tumor tissue extracts and used to determine levels of total TIMP-1 in 341 detergent-extracted tumor tissue samples from patients with primary breast cancer. The median age of the patients was 56 years (range, 29–75 years), and 164 were lymph node-negative, and 177 were lymph node-positive. The median follow-up time of the patients was 8.5 years (range, 7.3–11.3 years), and during follow-up 153 patients experienced recurrence of disease, and 136 patients died. In univariate survival analysis, we found a significant association between tumor tissue TIMP-1 level and both shorter recurrence-free survival ($p = 0.0004$) and shorter overall survival ($p = 0.03$). In multivariate survival analysis, higher tumor tissue TIMP-1 levels significantly and independently predicted shorter recurrence-free sur-

vival ($p < 0.05$, hazard ratios > 1 , comparing quartiles II–IV with I). In addition, we found that measurement of TIMP-1 levels added prognostic information to that obtained from measurement of PAI-1. In conclusion, high levels of TIMP-1 in tumor tissue extracts are significantly associated with a poor prognosis in patients with primary breast cancer. Furthermore TIMP-1 adds prognostic information to that obtained from PAI-1. However, further validation in independent data sets is needed. *Molecular & Cellular Proteomics* 2:164–172, 2003.

The present use of adjuvant systemic therapy in patients with primary breast cancer is based on classical prognostic parameters (nodal status, tumor size, grade of malignancy, receptor status, and age) (1). However, a small proportion of patients who are not offered therapy will still experience recurrence of disease, *i.e.* these patients are undertreated. In addition, a large proportion of the patients who are offered systemic therapy would have remained free of recurrence even without that therapy, *i.e.* these patients are overtreated. To optimize the use of adjuvant systemic therapy an improvement in the methods for identification of patients at risk of developing recurrence is needed.

Proteolytic enzymes, their receptors, and their inhibitors are all important in the processes of cancer invasion and metastasis. In the urokinase-type plasminogen activator (uPA)¹ system, the serine protease uPA mediates localized activation of plasminogen with subsequent generation of plasmin. Proteolysis mediated by plasmin then is involved in several pro-

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¹ The abbreviations used are: uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; DBCG, Danish Breast Cancer Cooperative Group; CI, confidence interval; HR, hazard ratio; ELISA, enzyme-linked immunosorbent assay.

cesses such as cell migration and invasion (2). Both uPA and its principal inhibitor plasminogen activator inhibitor type 1 (PAI-1) are now well recognized as prognostic markers in patients with primary breast cancer as high levels of uPA and/or PAI-1 are related to poor prognosis (3), and these two markers have recently reached the level of evidence 1 for clinical markers (3–5). That high tumor tissue levels of PAI-1 are associated with shorter survival was recently explained by the demonstration of a proangiogenic effect (6) as well as an antiapoptotic function of this molecule (7).

However, a classification of patients based on measurements of uPA and PAI-1 leaves a small group of patients that, although classified as being at high risk based on uPA and PAI-1 measurements, do not experience recurrence of disease. Thus, additional markers are needed as a complete prognostic stratification is not possible based on these markers. As the uPA system is only one of several extracellular proteolytic systems, we looked to other proteolytic systems for alternative marker candidates. One such system is the one of matrix metalloproteinases (MMPs) for which several interactions with the uPA system have been established. For instance, MMP-3 is capable of activating pro-uPA (8), and also MMP-3-mediated cleavage of uPA separates the receptor-binding domain from the enzymatically active part of uPA (9). Furthermore MMP-3 is capable of inactivating PAI-1 by means of proteolytic cleavage (10). In addition, several MMPs are capable of activating plasminogen, and similarly serine proteases can activate MMPs (11).

The MMPs make up a family of zinc-dependent extracellular endopeptidases cooperatively capable of degrading most components of the extracellular matrix (12). The MMPs interact non-covalently with members of the tissue inhibitor of metalloproteinases (TIMP) family of endogenous metalloproteinase inhibitors, thus inhibiting proteolytic activity (13). Both the MMPs and the TIMPs have been assigned functions in several steps of the process of cancer cell invasion and metastasis, thus influencing cancer progression (12).

TIMP-1 is one of the four known TIMPs (14). It is expressed by a range of different cell types and is present in a variety of body fluids and tissues (15). In addition to the MMP-inhibitory function, several other functions have been suggested for TIMP-1. These include antiapoptotic, growth-promoting, and possibly both proangiogenic and antiangiogenic effects (16–20). TIMP-1 and PAI-1 may thus have similar biological functions in cancer. Having recognized these functions of TIMP-1 in addition to the MMP-inhibitory function, a more complex role of TIMP-1 in the growth and dissemination of cancer cells can be expected. Especially the idea of a purely protective role for TIMP-1, as could be anticipated from the MMP-inhibitory function, should be abandoned in favor of a multifunctional one.

In breast cancer tissue, TIMP-1 mRNA transcripts have been detected in tumor cells as well as in stromal cells with a predominance in the peritumoral stroma of invasive carcino-

mas (21). In a study of 53 samples of cancerous and non-cancerous tissue of the breast, higher levels of TIMP-1 mRNA were found in the cancerous than in the non-cancerous tissue (22). Additionally the possible value of TIMP-1 as a prognostic marker in breast cancer has been investigated in a number of studies. In a study of breast cancer tissue from 34 patients, high levels of TIMP-1 mRNA were associated with lymph node-positive disease, development of distant metastases, and a poorer 5-year survival (23). Furthermore, in a study of 139 primary breast carcinomas, an association between high levels of TIMP-1 protein and both shorter disease-free survival and shorter overall survival was demonstrated using an optimized cut-off point (24).

In the present study, we investigated TIMP-1 protein levels in tumor tissue extracts from 341 breast cancer patients and related these levels to patient outcome. In addition, we investigated the correlation between levels of TIMP-1 and levels of uPA and PAI-1 as well as with several clinicopathological parameters (lymph node status, hormone receptor status, tumor size, grade of malignancy, menopausal status, and age). In particular, we wanted to investigate whether TIMP-1 added prognostic information to that obtained by uPA/PAI-1 measurements.

EXPERIMENTAL PROCEDURES

Patients—341 patients were included in this study. All patients underwent surgery in the period 1989–1993 in Denmark for histologically verified primary breast cancer and were included in the treatment protocols of the Danish Breast Cancer Cooperative Group, DBCG-82 or DBCG-89 (25, 26). Patients with distant metastases at the time of diagnosis or with previous malignancies were excluded from the treatment protocols. A total of 10,918 patients were registered by the DBCG during this period, and patients were included in the present study provided that unfixed frozen tumor tissue was available. The present group was found to be representative of the total group with slight exceptions regarding age and tumor size; younger patients and patients with medium-sized tumors were slightly over-represented in this group (27). The median age of patients included in the study was 56 years (range, 29–75 years), and the median follow-up time was 8.5 years (range, 7.3–11.3 years). 177 patients were lymph node-positive, and 164 were lymph node-negative; 109 were premenopausal, and 232 were postmenopausal. 260 patients were hormone receptor-positive; tumors were classified as hormone receptor-positive if estrogen and/or progesterone receptor analysis was positive, *i.e.* >10 fmol/mg of cytosol protein by biochemistry or >10% stained cells by immunohistochemistry. During the follow-up period 153 patients experienced recurrence of disease, and 136 patients died. Recurrence was defined as the appearance of new breast cancer lesions after primary surgery confirmed by biopsy and/or other relevant diagnostic procedures. During follow-up, patients were seen for clinical examination every 3 months for the 1st year, then every 6 months for years 2–5, and after that once a year until 10 years after primary surgery (25). Recording of survival was based on death from all causes; these data were obtained from the Danish Death Registry. Clinicopathological data registered for the patients were provided by the DBCG.

Primary surgical procedures included mastectomy with partial axillary dissection or breast-conserving lumpectomy with partial axillary dissection. Patients who underwent breast-conserving lumpectomy also received local radiotherapy. Low risk patients did not receive

systemic adjuvant therapy, whereas high risk patients were randomized to systemic adjuvant therapy consisting of chemotherapy and/or endocrine therapy as described in the protocols (26). High risk patients included those with axillary lymph node metastases (lymph node-positive patients), patients with a tumor larger than 5 cm, patients in the DBCG-82 protocol with tumors invading skin or the chest wall, and premenopausal patients in the DBCG-89 protocol with a ductal carcinoma showing grade II or III of malignancy irrespective of size and nodal status. A total of 199 patients (58%) received adjuvant therapy.

33 of the total patient number (341) were included in the DBCG-82 protocol, and 21 of these received adjuvant therapy. The remaining 308 patients were included in the DBCG-89 protocol, 178 of whom received adjuvant therapy.

Tissue—Frozen tissue was extracted as described previously (27). In brief, frozen tumor tissue stored at -80°C was mechanically pulverized, suspended in ice-cold, low pH, detergent-containing extraction buffer (28), and centrifuged at $105,000 \times g$ for 1 h at 4°C . The supernatant was stored at -80°C . Before performing the assay, the samples were thawed at 37°C . Total protein content, uPA and PAI-1 concentrations (27), and the TIMP-1 concentration were determined in each tissue extract.

Assays—All assays were performed without knowledge of patient outcome or of any clinicopathological data. Total protein concentrations were determined using the Bradford method (29) with bovine serum albumin as a standard (27). A tumor tissue extract pool was made from 271 individual breast tumor tissue extracts for use as an internal standard. In addition, pools of plasma with high and low TIMP-1 levels, respectively, were used as references on each ELISA plate.

An established TIMP-1 ELISA (30) was used for measurement of TIMP-1 in the tumor tissue extracts. In brief, immunoassay plates were coated with a sheep polyclonal anti-TIMP-1 antibody (31), and bound TIMP-1 was detected using a murine monoclonal anti-TIMP-1 antibody (MAC 15) that binds both free TIMP-1 and TIMP-1 in complex with MMPs (32, 33) followed by an alkaline phosphatase-conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark). Readings of color development were taken every 10 min for 60 min at 405 nm. On every assay plate, serial dilutions of duplicates of recombinant TIMP-1 were included, allowing determination of the TIMP-1 concentration in individual tumor tissue extracts. In addition, duplicates of the control tumor tissue extract pool and plasma pools were included on every assay plate as internal controls. Tissue extracts were diluted 1:101 in sample dilution buffer (30). KinetiCalc II software (Bio-Tek Instruments, Winooski, VT) was used for calculation of the concentrations.

TIMP-1 ELISA Validation—The TIMP-1 assay that was used was not previously validated for tumor tissue extracts, and therefore the assay was thoroughly validated for measurement of TIMP-1 in this matrix. The limit of detection for tissue extracts was determined as the concentration corresponding to the signal 3 standard deviations above the mean for TIMP-1 blank (sample dilution buffer only). The linearity of signal as a function of dilution was investigated in a dilution series of the control tumor tissue extract pool in sample dilution buffer ranging from 5% (1:20) to 0.04% (1:2560).

For determination of recovery of TIMP-1 signal in the control tumor tissue extract pool, recombinant TIMP-1 protein was added to 1:200 dilutions of the tissue pool. Recovery was calculated by comparing the slope of the line illustrating the TIMP-1 signal in the diluted tissue extract as a function of concentration with the slope of a similar line representing dilution of recombinant TIMP-1 in pure sample buffer.

Intra-assay variation was determined for 32 duplicates of 1:101 dilutions of the control tumor tissue extract pool on the same assay plate. The interassay variation was determined for duplicates of 1:101

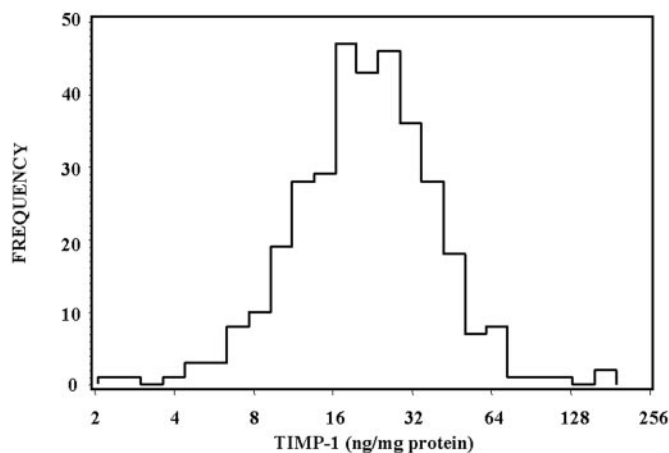


FIG. 1. Histogram showing the distribution of tumor tissue TIMP-1 concentrations (ng/mg of protein) in the patient group. Median = 21.65 ng/mg of protein; range, 2.06–190.32 ng/mg of protein.

dilutions of the control tumor tissue extract pool on individual plates run on 14 different days. Tumor tissue concentrations of uPA and PAI-1 were described previously (27).

Statistical Analysis—The SAS[®] software package (version 8.2, SAS Institute, Cary, NC) was used to manage patient data and for statistical analysis. TIMP-1 concentrations were normalized by total protein concentrations and were scored using the quartiles. The end points for survival analysis were recurrence and death of any cause. The Kaplan-Meier method was used to estimate survival probabilities, and the log rank test was used to test for equality of strata. The Cox proportional hazard model was used for multivariate analysis. Model validation was done graphically and by using Schoenfeld and Martingale residuals as described in Harrell *et al.* (34). Rank statistics were used to calculate correlation coefficients and to test hypothesis on location. *p* values less than 5% were considered significant.

RESULTS

Using the control tumor tissue extract pool, the assay was thoroughly validated for measurements of TIMP-1 in tumor tissue extracts treated with a detergent-containing buffer. In detergent-extracted tissue, the limit of detection of the assay was 67 pg/ml. Furthermore the control tissue extract pool gave good linearity of signal as a function of dilution down to 0.08% (1:1280) dilution in sample buffer. Moreover recovery of TIMP-1 signal in the control tumor tissue extract pool was 102%. Looking at variation, we found that the intra-assay variation for the control tumor tissue extract pool (32 duplicates) was 7.3% and that interassay variation (14 duplicates) was 14.4%.

All tumor tissue extracts contained measurable levels of TIMP-1, and the TIMP-1 concentration of each sample was normalized by the total protein concentration of that sample. The mean TIMP-1 level (\pm S.D.) of the control tumor tissue extract pool was 17 (\pm 1.23) ng/mg of protein. The mean level (\pm S.D.) of TIMP-1 in the 341 tumor tissue extracts was 25.66 (\pm 18.97) ng/mg of protein, and the median TIMP-1 concentration was 21.65 ng/mg of protein (range, 2.06–190.32 ng/mg of protein). The distribution of tumor tissue TIMP-1

TABLE I
Patient characteristics and comparisons of studied parameters

	<i>n</i>	%	Median TIMP-1 (range) ng/mg protein	<i>p</i> value ^a
Lymph node status				
Positive	177	52	21 (3–107)	0.6
Negative	164	48	22 (2–190)	
Hormone receptor status ^b				
Positive	260	79	22 (5–113)	0.6
Negative	71	21	21 (3–190)	
Tumor size				
0–20 mm	100	29	21 (5–163)	0.08
20–50 mm	193	57	23 (3–190)	
>50 mm	48	14	20 (2–110)	
Tumor grade ^c				
Grade I	82	30	22 (5–70)	0.9
Grade II	122	44	22 (2–107)	
Grade III	72	26	20 (3–190)	
Menopausal status				
Premenopausal	109	32	19 (6–58)	0.02
Postmenopausal	232	68	23 (2–190)	
Age				
≤40 years	21	6	19 (8–41)	0.01
41–50 years	85	25	21 (6–190)	
51–60 years	96	28	19 (2–164)	
61–70 years	105	31	23 (4–113)	
≥71 years	34	10	30 (5–107)	

^a Kruskal-Wallis comparison of TIMP-1 in ng/mg of protein; *p* values.

^b Data available for 331 patients only. Tumors were classified as hormone receptor-positive if estrogen and/or progesterone receptor analysis was positive (*i.e.* >10 fmol/mg of cytosol protein by biochemistry or >10% stained cells by immunohistochemistry).

^c Ductal carcinomas only; 276 patients.

concentrations in the patient group is illustrated in Fig. 1.

A significant correlation was found between TIMP-1 and menopausal status (Kruskal-Wallis test, $p = 0.02$) and between TIMP-1 and age (Kruskal-Wallis test, $p = 0.01$) as shown in Table I. However, no significant association was found between TIMP-1 and other clinicopathological parameters; in particular, no association with lymph node status was found. Moreover weak but significant correlations between TIMP-1 levels and levels of uPA and PAI-1 were observed (Fig. 2, A and B). A significant positive correlation has previously also been demonstrated between uPA and PAI-1 (27).

The significance of the classical clinicopathological prognostic parameters in predicting recurrence-free and overall survival has been investigated in a previous study within this patient group (27). That study also demonstrated the prognostic power of uPA and PAI-1. In addition, it was found that large tumor size, positive lymph node status, and a high grade of malignancy significantly predicted both shorter recurrence-free survival and shorter overall survival. Furthermore positive hormone receptor status significantly predicted a longer recurrence-free and overall survival.

For univariate survival analysis, patients were divided into

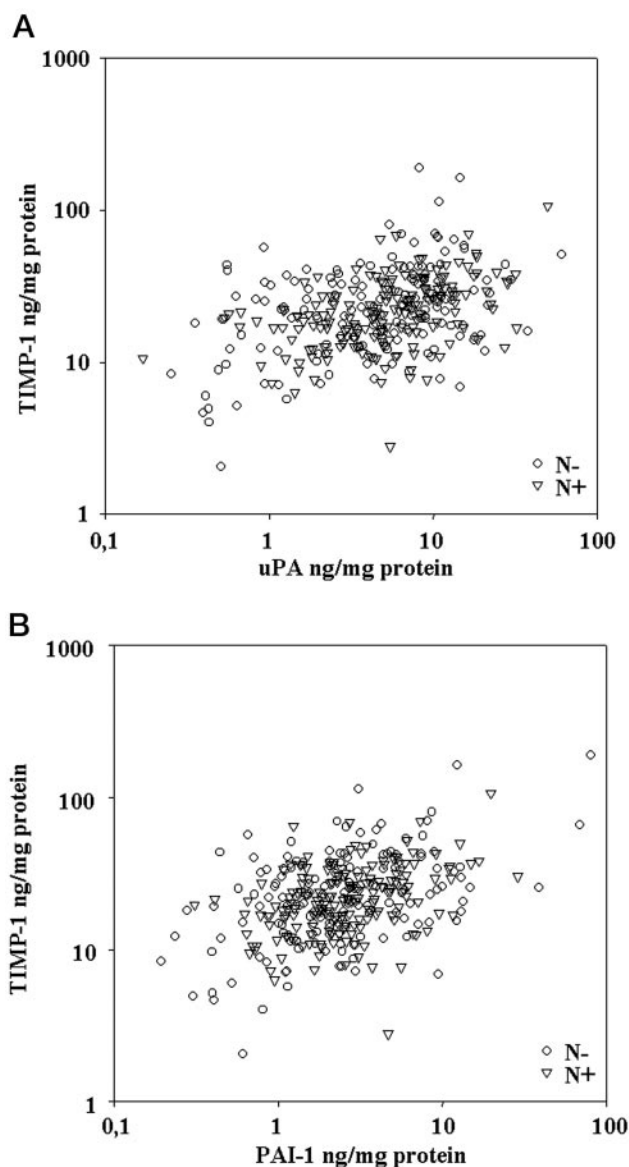
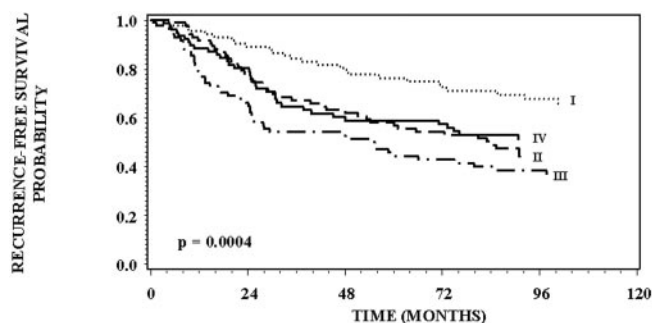


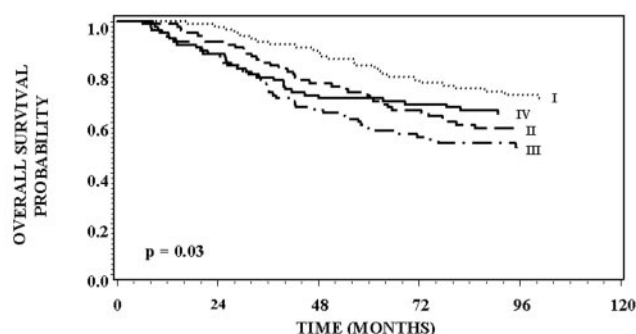
Fig. 2. A, scatter plot of tumor tissue uPA levels (ng/mg of protein) versus tumor tissue TIMP-1 levels (ng/mg of protein). Spearman correlation coefficient = 0.38 ($p < 0.0001$). Circles represent lymph node-negative patients; triangles represent lymph node-positive patients. B, scatter plot of tumor tissue PAI-1 levels (ng/mg of protein) versus tumor tissue TIMP-1 levels (ng/mg of protein). Spearman correlation coefficient = 0.36 ($p < 0.0001$). Circles represent lymph node-negative patients; triangles represent lymph node-positive patients.

four groups (quartiles, groups I–IV) based on increasing tumor tissue TIMP-1 levels, each group consisting of the same number of patients. As illustrated in Fig. 3, TIMP-1 levels were significantly associated with recurrence-free survival ($p = 0.0004$, log rank test); patients with higher tumor tissue TIMP-1 levels had significantly shorter recurrence-free survival than patients with lower TIMP-1 values. However, for the group of patients having the highest tumor tissue TIMP-1 levels (group



EVENTS	PATIENTS AT RISK				
26	86	71	61	55	33
44	85	62	50	41	20
48	85	94	37	29	17
35	85	57	40	39	23

FIG. 3. Survival curves showing the association between tumor tissue TIMP-1 and recurrence-free survival. Patients were divided into four groups of an equal number of patients: I (for tumor tissue TIMP-1 <15 ng/mg of protein), II (for tumor tissue TIMP-1 >15 and <21 ng/mg of protein), III (for tumor tissue TIMP-1 >21 and <32 ng/mg of protein), and IV (for tumor tissue TIMP-1 >32 ng/mg of protein). The numbers of events in each group during the period and the numbers of patients at risk after each 24-month interval are shown. *p* values were calculated using the log rank test.



EVENTS	PATIENTS AT RISK				
26	86	84	76	66	42
37	85	78	64	55	36
42	85	74	55	46	27
31	85	74	59	57	40

FIG. 4. Survival curves showing the association between tumor tissue TIMP-1 and overall survival. Patients were divided into four groups of an equal number of patients: I (for tumor tissue TIMP-1 <15 ng/mg of protein), II (for tumor tissue TIMP-1 >15 and <21 ng/mg of protein), III (for tumor tissue TIMP-1 >21 and <32 ng/mg of protein), and IV (for tumor tissue TIMP-1 >32 ng/mg of protein). The numbers of events in each group during the period and the numbers of patients at risk after each 24-month interval are shown. *p* values were calculated using the log rank test.

IV), a somewhat different pattern was observed as this group had a prognosis similar to that observed for the group having low intermediate tumor tissue TIMP-1 levels (group II).

High tumor tissue TIMP-1 levels were also significantly associated with shorter overall survival (*p* = 0.03, log rank test; Fig. 4). High TIMP-1 levels predicted a poor prognosis except when looking at the group having the highest tumor tissue TIMP-1 levels (group IV) as this group had an overall survival similar to the one predicted for the group with low intermediate tumor tissue TIMP-1 levels (group II).

Separate univariate survival analyses were performed on lymph node-negative and lymph node-positive patients, respectively (quartiles, data not shown). When looking at the lymph node-positive patients (177 patients, 105 recurrences), increases in tumor tissue TIMP-1 levels were associated with a significantly shorter recurrence-free survival (*p* = 0.002, log rank test). Still, however, the group having the highest tumor tissue TIMP-1 levels had a prognosis similar to the prognosis for patients with intermediate tumor tissue TIMP-levels. In the lymph node-negative subgroup (164 patients, 48 recurrences) a similar although non-significant association was found.

We performed multivariate analysis with respect to all registered clinicopathological parameters (Table II). Information about hormone receptor status was available for 331 patients, and tumor grade of malignancy was only known for 276 patients (ductal carcinomas only).

In the multivariate analysis, TIMP-1 was treated as a discrete variable as the patients were divided into four groups of increasing tumor tissue TIMP-1 values (quartiles, groups I-IV). Using group I as the base line, it was found that in groups II,

TABLE II

Multivariate analysis for studied parameters (recurrence-free survival)

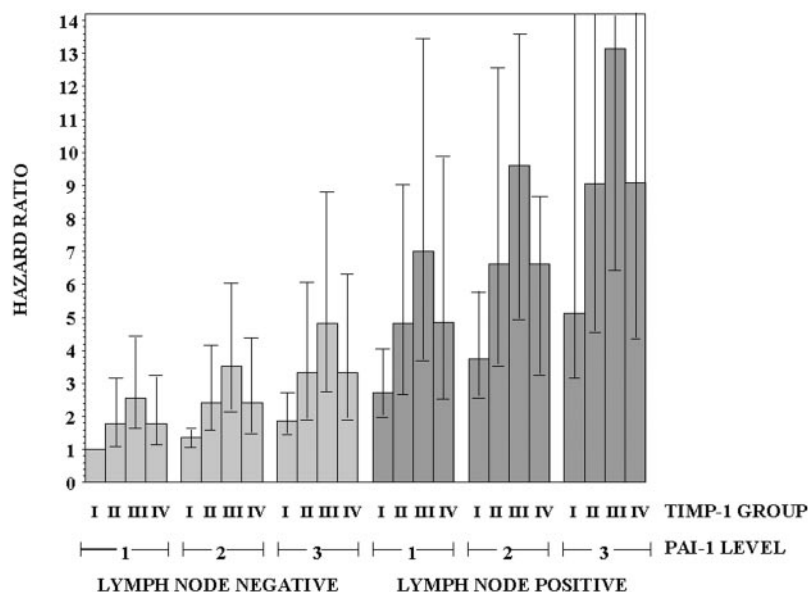
Parameter	<i>p</i>	HR	95% CI
Node-positive vs. -negative	<0.0001	2.7	1.9-3.9
Pre- vs. postmenopausal	0.63	1.1	0.7-1.9
Age in years	0.35	0.99	0.97-1.01
Tumor size			
<20 mm vs. 20-50 mm	0.76	1.1	0.7-1.6
>50 mm vs. 20-50 mm	0.09	1.5	0.9-2.3
log PAI-1	0.0002	1.7	1.3-2.3
log uPA	0.19	0.8	0.6-1.1
Receptor-positive vs. -negative ^a	0.04	0.6	0.4-1.0
Malignancy ^b			
Tumor grade II + III vs. I	0.02	1.7	1.1-2.8
TIMP-1			
Group II vs. I	0.02	1.8	1.1-2.9
Group III vs. I	0.0003	2.6	1.5-4.3
Group IV vs. I	0.04	1.8	1.0-3.1

^a Estrogen and/or progesterone receptor analysis; 331 patients, 151 recurrences. Grade of malignancy not included in this model.

^b Ductal carcinomas only; 276 patients, 125 recurrences. Receptor status not included in this model.

III, and IV TIMP-1 significantly and independently predicted shorter recurrence-free survival. The HRs were 1.8 (CI, 1.1-2.9; *p* = 0.02), 2.6 (CI, 1.5-4.3; *p* = 0.0003), and 1.8 (CI, 1.0-3.1; *p* = 0.04), respectively. When looking at the group of 331 patients with known receptor status, TIMP-1 retained significance in predicting shorter recurrence-free survival when comparing groups II, III, and IV with group I (data not shown). Furthermore when looking at the group for which tumor grade of malignancy is known (276 patients) signifi-

FIG. 5. Plot of hazard ratios, including 95% confidence limits, for different combinations of nodal status, PAI-1 levels, and TIMP-1 levels adjusted for the other covariates in the multivariate model. Patients were divided into four groups of an equal number of patients: I (for tumor tissue TIMP-1 <15 ng/mg of protein), II (for tumor tissue TIMP-1 >15 and <21 ng/mg of protein), III (for tumor tissue TIMP-1 >21 and <32 ng/mg of protein), and IV (for tumor tissue TIMP-1 >32 ng/mg of protein). 1 = first quartile of tumor tissue PAI-1 (1.36 ng/mg of protein); 2 = median tumor tissue PAI-1 (2.41 ng/mg of protein); 3 = third quartile of tumor tissue PAI-1 (4.27 ng/mg of protein). Light bars represent lymph node-negative patients; dark bars represent lymph node-positive patients.



cance was retained for the comparison of group III with group I (data not shown).

Other parameters that independently and significantly predicted shorter recurrence-free survival were positive lymph node status ($p < 0.0001$; HR = 2.7; CI, 1.9–3.9) and high tumor tissue PAI-1 levels ($p = 0.0002$; HR = 1.7; CI, 1.3–2.3), while uPA did not reach significance in this model. In addition, when looking at grade of malignancy, grade II and III carcinomas were significantly and independently associated with a shorter recurrence-free survival ($p = 0.02$; HR = 1.7; CI, 1.1–2.8) when compared with grade I carcinomas. Also hormone receptor-positive tumors were associated with a significantly better prognosis than hormone receptor-negative tumors ($p = 0.04$; HR = 0.6; CI, 0.4–1.0). When looking at overall survival in multivariate analysis, a significant association between higher TIMP-1 levels and shorter survival was found only when comparing group III with group I (data not shown).

Based on the final multivariate model, the hazard ratios including 95% confidence intervals of increasing TIMP-1 levels combined with PAI-1 levels and the nodal status have been calculated; these are shown in Fig. 5. The impact of different tumor tissue TIMP-1 levels is shown for patients with low, intermediate, and high PAI-1 with negative or positive lymph node status, respectively. As base line, we chose the group of lymph node-negative patients with low PAI-1 and low TIMP-1. As illustrated in Fig. 5, an increase in tumor tissue TIMP-1 levels is associated with an increase in HR. This applies to all groups except the groups having the highest TIMP-1 level (IV) as these groups have HRs similar to those for groups with low intermediate tumor tissue TIMP-1 levels (II). When dividing the patients into groups with similar PAI-1 levels and similar lymph node status, additional prognostic stratification was obtained from tumor tissue TIMP-1 levels.

DISCUSSION

This study demonstrates that the total tumor tissue TIMP-1 level is an independent prognostic marker in patients with primary breast cancer and that TIMP-1 is additive to PAI-1 in predicting prognosis in these patients. In the present study, tumor tissue TIMP-1 levels were measured using an established and well characterized ELISA developed for use in plasma samples (30). We rigorously tested and validated this ELISA for use in tumor tissue extracts to assure reliable measurements. The precision of the assay in tissue extracts was confirmed through the demonstration of acceptably low inter- and intra-assay variations. Moreover a reliable range of measurement was identified with good recovery of TIMP-1 signal in a tissue pool and with a linear relationship between TIMP-1 concentration and the signal obtained in the ELISA. In addition, sensitivity of the assay was determined. Also it was assured that tumor tissue extracts were diluted to a suitable concentration giving a signal in the reliable range of measurement, and tumor tissue extract and plasma controls were included on every assay plate to monitor stability of the assay.

In our study, TIMP-1 levels were measured in low pH, detergent-extracted tumor tissue samples. However, it has not been determined whether a low pH, detergent-containing buffer is the most appropriate for extracts to be used for TIMP-1 measurements. The prognostic value of a marker may be influenced by the extraction procedure. For example, when comparing detergent-extracted tissue extracts with cytosol fractions from the same tissue samples, Jänicke *et al.* (35) demonstrated a difference in prognostic power of uPA as higher prognostic power was obtained when using detergent-extracted tissue samples. It was speculated that the use of detergent in the extraction procedure might free uPA bound to, for example, the uPA receptor. Since cell surface binding

has been demonstrated for TIMP-1 (36) a similar relationship could be expected for TIMP-1. Furthermore, when comparing TIMP-1 levels of detergent-extracted tumor tissue with cytosolic fractions, a difference is seen as higher TIMP-1 levels are found in detergent-treated tissue (not normalized by total protein concentration).² Therefore, for future studies a comparison of different extraction methods is warranted. Moreover an agreement on assay methods must be reached, and sufficient quality control must be established.

Analyzing the association between TIMP-1 and prognosis, significant associations between high TIMP-1 levels and both shorter recurrence-free survival and overall survival were found in univariate survival analysis. Among clinicopathological parameters included in the study, menopausal status and age were associated with TIMP-1. No association between TIMP-1 and nodal status was observed. In multivariate survival analysis, we found a significantly longer recurrence-free survival for the group with the lowest tumor tissue TIMP-1 levels compared with those with higher TIMP-1 levels. In multivariate as well as in univariate analysis, we found an unexpected tendency for very high TIMP-1 levels to be associated with a prognosis similar to the one associated with low intermediate tumor tissue TIMP-1 levels.

Measurements of TIMP-1 in tumor tissue added prognostic information to that obtained from measurements of PAI-1. The additive effect of TIMP-1 was seen in groups of lymph node-negative as well as lymph node-positive patients and at low as well as at high PAI-1 values. Thus, TIMP-1 may improve prognostic stratification of both groups, and in particular, it is possible that a better identification of lymph node-negative patients with a good prognosis could be achieved using TIMP-1 as a marker in combination with PAI-1.

Being an MMP inhibitor, TIMP-1 is capable of protecting against excessive degradation of the extracellular matrix consequently rendering the dissemination of cancer cells difficult. Thus, one would expect high levels of TIMP-1 to be associated with a good prognosis. However, the association between high TIMP-1 levels and poor prognosis in breast cancer has previously been demonstrated in a number of other studies (23, 24), and several explanations for this are possible. It has to be considered that the increased expression of TIMP-1 could simply be associated with an increased level of proteolysis during tumor growth and dissemination, thus representing a host response to increased levels of extracellular matrix degradation. However, several activities in addition to MMP inhibition have been suggested for TIMP-1 including anti-apoptotic, growth-promoting, and angiogenesis-regulating effects. A regulatory role for TIMP-1 in these processes supports the view that TIMP-1 could influence the growth of the tumor directly.

First, TIMP-1 has been shown to stimulate growth in several cell types including, among others, skin epithelial cells, lym-

phoblasts, aortic smooth muscle cells, and breast adenocarcinoma cells (37). Moreover the TIMP-1-mediated protection of cells from apoptosis further contributes to the adverse regulation of tumor growth. Protection of human breast epithelial cells from apoptosis has been demonstrated (16), and this effect proved to be independent of TIMP-1-mediated MMP-inhibition. However, it cannot be ruled out that in some cases MMP inhibition further contributes to the protection of cells from apoptosis by stabilizing interactions between cells and extracellular matrix. Furthermore a regulatory role on angiogenesis has also been suggested for TIMP-1. Several MMPs are capable of cleaving plasminogen thereby generating angiostatin, which inhibits the proliferation of endothelial cells (20). By inhibiting MMPs, TIMP-1 could inhibit this pathway of angiostatin production, thus disinhibiting endothelial cell proliferation and, eventually, facilitating angiogenesis. It has, however, been demonstrated that TIMP-1 is also capable of decreasing endothelial cell migration by inhibiting proteolytic activity (19).

Finally the interplay of the MMP-TIMP system with other proteolytic systems should also be taken into account when considering the effect of TIMP-1 on tumorigenesis, tumor growth, and cancer cell dissemination. In particular, several interactions between the MMP-TIMP system and the uPA-PAI-1 system have been demonstrated (8–11). Some of these studies indicate a possible regulatory role for the MMP-TIMP system in controlling the activities of the uPA-PAI-1 system as well as a similar role for the uPA-PAI-1 system in controlling the MMP-TIMP system. As both proteolytic systems have been assigned several roles in the growth and dissemination of cancer cells, mutual regulation of that kind would have extensive consequences.

The diverse functions suggested for TIMP-1 clearly support the view of TIMP-1 as a multifunctional protein in tumor growth and cancer cell dissemination. Furthermore it was recently suggested that the balance between the anti-MMP tumor-suppressing and the tumor-protecting activities of TIMP-1 is regulated by the amount of TIMP-1 available in the tumor environment (38). High TIMP-1 levels in the tumor environment then would have a predominantly tumor-suppressing effect, whereas tumor-promoting activities would dominate at lower tumor tissue TIMP-1 concentrations. Our results support this view as we find that the group of patients having the highest tumor tissue TIMP-1 levels has a prognosis similar to that of the groups with intermediate levels.

Additionally it should be considered that the net effect of TIMP-1 could be influenced by other parameters, and it has been suggested that both the time of TIMP-1 expression as well as the presence of a putative TIMP-1 receptor on the tumor cells might be determinative of the net effect of TIMP-1 on tumor growth (38). Notably the possible influence of time on the effect of TIMP-1 on tumor growth has been demonstrated in a study of lymphomas (39). In this study, a biphasic effect of TIMP-1 overexpression on tumor growth was ob-

² A.-S. Schrohl, unpublished observations.

served with a growth-promoting effect dominating during tumor onset and a growth-suppressing effect dominating at later stages of tumor development.

Our study suggests that tumor tissue TIMP-1 measurements add prognostic information to that obtained from measurements of PAI-1. However, to use this information in a clinical setting, a large confirmatory study must be executed. Also several aspects of methodological and statistical importance should be addressed, including identification of an appropriate cut-off value for separation of patients.

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