Measurement of the Uncomplexed Fraction of Tissue Inhibitor of Metalloproteinases-1 in the Prognostic Evaluation of Primary Breast Cancer Patients*

Sidse Ørnbjerg Würtz‡§, Ib Jarle Christensen¶, Anne-Sofie Schrohl‡, Henning Mouridsen‖, Ulrik Lademann‡, Vibeke Jensen‡, and Nils Brünnер‡

Several studies have demonstrated an association between high tumor tissue levels of total tissue inhibitor of metalloproteinases-1 (TIMP-1) and a poor prognosis of primary breast cancer patients. In the present study we investigated whether measurements of the uncomplexed fraction of TIMP-1 added prognostic information to that already obtained from total TIMP-1. We measured the uncomplexed fraction of TIMP-1, using a thoroughly validated ELISA specific for this fraction, in 341 tumor tissue extracts obtained from patients with primary breast cancer. These measurements were related to previously performed measurements of total TIMP-1 as well as to patient outcome. The observation time was 8.3 years (range, 7.3–11.3 years). During this period 136 patients died, and 153 patients experienced recurrence of disease. Cox regression analysis of recurrence-free survival (RFS) suggested that a score based on both uncomplexed and total TIMP-1, reflecting the tumor level of TIMP-1/MMP complexes, would be a more precise estimate of prognosis than total TIMP-1 alone. Univariate survival analysis showed a highly significant relationship between high values of the score and poor outcomes for RFS (p = 0.0002; hazard ratio = 2.7; 95% confidence interval, 1.5–4.8). Similar results were found for overall survival (p = 0.0001; hazard ratio = 3.3; 95% confidence interval, 1.8–6.3). Multivariate analysis of RFS and overall survival demonstrated that the score was significant including the classical prognostic factors used in breast cancer (p < 0.0001). The present study raises the hypothesis that it is the tumor level of TIMP-1/MMP complexes (i.e. activated matrix metalloproteinases) rather than TIMP-1 itself that determines prognosis, supporting the use of the combined score and not only total TIMP-1 in stratification of breast cancer patients. Molecular & Cellular Proteomics 4:483–491, 2005.

From the ‡Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Ridebanevej 9, DK-1870 Frederiksberg C, ¶Department of Surgical Gastroenterology, Hvidovre Hospital, DK-2650 Hvidovre, and ‖Department of Oncology, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark

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1 DBCG guidelines, www.dbcg.dk.
2 The abbreviations used are: MMP, matrix metalloproteinase; CI, confidence interval; DBCG, The Danish Breast Cancer Cooperative Group; HR, hazard ratio; OS, overall survival; RFS, recurrence-free survival; TIMP, tissue inhibitor of metalloproteinases; Temed, N,N,N’-tetramethylmethylenediamine.
and basement membrane, which facilitates the penetration of cancer cells through the barriers normally maintaining the integrity of the tissues. Several studies have demonstrated that the expression of various MMPs is increased in breast cancer, and high levels of various MMPs (MMP-2, MMP-9, and MMP-11) have been associated with a poor prognosis in breast cancer patients (6–10).

Tissue inhibitor of metalloproteinases type-1 (TIMP-1) is a 28.5-kDa glycoprotein that forms non-covalent 1:1 stoichiometric complexes with MMPs thereby inhibiting the proteolytic activity of these enzymes. It is a member of the TIMP family currently comprising four members (TIMP-1, -2, -3, and -4), is expressed by a wide range of cells, and is present in most tissues and body fluids (11, 12). High levels of TIMP-1 mRNA as well as TIMP-1 protein have been found in several types of cancers including breast cancer, and this has been associated with a poor prognosis (13–17). That high levels of TIMP-1 predict a poor prognosis has been explained by at least two different models (4). First, the up-regulation of TIMP-1 in cancer being associated with a poor prognosis could reflect the fact that the balance between expression of MMPs and TIMP-1, although still favoring MMPs, is at a higher overall level than under non-cancerous conditions. High TIMP-1 levels would therefore be associated with a poor prognosis but would not be the actual cause of it. Second, several lines of evidence have recently implied that TIMP-1 is a multifunctional protein that in addition to its MMP inhibitory effect also possesses distinct tumor stimulatory functions such as inhibition of apoptosis (18–21), stimulation of proliferation (22), and both pro- and antiangiogenic functions (23–25). In this case, high levels of TIMP-1 form part of the malignant progression and could actually be the cause of the poor outcome.

TIMP-1 exists in different molecular forms, i.e. TIMP-1 in complex with pro-MMP-9 (26) and with various activated MMPs and as uncomplexed TIMP-1. So far the prognostic value of only the total amount of TIMP-1 (uncomplexed TIMP-1 plus TIMP-1/MMP complexes) has been evaluated (13–17). In the case of other markers such as prostate-specific antigen it has been shown that specific fractions of the antigen carry useful clinical information (27), and we raised the hypothesis that this could also be the case for TIMP-1. Therefore, the present study, in contrast to previous studies, addresses the possible prognostic value of the specific fraction of uncomplexed TIMP-1 and investigates whether measuring this fraction adds prognostic information to that already obtained from total TIMP-1. In practice, we thoroughly validated an established ELISA, which is specific for the uncomplexed fraction of TIMP-1 (28–30), and by using this ELISA we measured uncomplexed TIMP-1 in 341 tumor tissue extracts obtained from patients with primary breast cancer. In a previous study including the same 341 tumor tissue extracts, the levels of total TIMP-1 were determined, and in that study an association was demonstrated between high levels of total TIMP-1 and poor prognosis (16). By relating the measurements performed in the present study with the measurements of total TIMP-1 from the previous study we evaluated whether uncomplexed TIMP-1 in combination with total TIMP-1 improved prognostic stratification.

**EXPERIMENTAL PROCEDURES**

**Patients**—Patient characteristics have been described previously (16, 31). In brief, the present study included 341 patients who underwent surgery in Denmark in the period 1989–1993 for histologically verified primary breast cancer. The Danish Breast Cancer Cooperative Group (DBCG) registered a total of 10,918 patients in this period. Patients were included in the present study provided that frozen tumor tissue was accessible and that they were included in the treatment protocols DBCG-82 or DBCG-89 (32). In addition, patients with distant metastases and/or previous malignancies were omitted from the study. The group of patients entered in the study was shown to be representative of the total number of patients registered besides minor exceptions related to patient age and tumor size, i.e. no patients included were older than 75 years and medium sized tumors were slightly over-represented (31).

Clinicopathological data concerning the patients were registered by the DBCG. According to these, 164 of the patients were lymph node-negative, and 177 were lymph node-positive. The median patient age at the time of surgery was 56 years (range, 29–75 years). 109 women were premenopausal, and 232 were postmenopausal. 260 patients were stated hormone receptor-positive, and 71 patients were hormone receptor-negative (tumors were considered hormone receptor-positive if estrogen and/or progesterone analysis was positive, i.e. ≥10 fmol/mg of cytosol protein by biochemistry or ≥10% stained cells by immunohistochemistry).

Surgical procedures included breast-conserving lumpectomy or modified radical mastectomy followed by partial axillary lymph node dissection. Patients who underwent breast-conserving lumpectomy were also treated with local radiotherapy. High risk patients received adjuvant systemic treatment, consisting of chemotherapy and/or endocrine treatment, following surgery. These patients comprise all lymph node-positive patients as well as lymph node-negative patients with tumors greater than 5 cm, patients in the DBCG-82 protocol with skin or thoracic wall invasion, and premenopausal patients in the DBCG-89 program having tumors showing grade II or III of malignancy. A total of 199 patients (58%) received adjuvant therapy. Low risk patients did not receive any systemic adjuvant treatment following surgery but were observed only.

The median follow-up time was 8.3 years (range, 7.3–11.3 years). During the follow-up period 136 patients died, and 153 patients experienced recurrence of disease. Recording of survival was based on death from all causes. Recurrence was defined as the appearance of new breast cancer lesions after primary surgery as determined by biopsy and/or other relevant diagnostic procedures.

**Tumor Tissue Extracts**—Following surgery and histopathological procedures remaining tissue was stored in sealed containers at −80 °C until tissue extraction. Tissue extracts were prepared as described previously (31). In brief, the frozen tissue was mechanically pulverized with a dry ice-cold powder pistol. Tissue powder was suspended in ice-cold acetate-detergent low pH extraction buffer (33). Subsequently the solution was centrifuged at 105,000 × g for 1 h at 4 °C. The supernatant was isolated and stored in aliquots at −80 °C until use.

**Protein Measurements**—The total protein content (31) as well as the total amount of TIMP-1 (16) in each tumor tissue extract was

determined previously. All protein assays were performed without knowledge of patient outcome or clinicopathological data.

Immediately before measurements, extracts were thawed rapidly at 37 °C and diluted. The total protein concentration in the extracts was determined using the Bradford method for protein analysis (34). Bovine serum albumin was used as a standard. The total amount of TIMP-1 was measured using a TIMP-1 ELISA thoroughly validated for tumor tissue extracts (16, 35). In this assay TIMP-1 was detected using a murine monoclonal anti-TIMP-1 antibody (MAC 15), which binds uncomplexed TIMP-1 as well as TIMP-1 in complex with MMPs (28, 30), thus measuring the total amount of TIMP-1.

In the present study, the uncomplexed fraction of TIMP-1 was determined in each tissue extract using an established TIMP-1 ELISA specific for uncomplexed TIMP-1 (28–30). In brief, 96-well microtiter plates were coated overnight at 4 °C with a sheep polyclonal anti-TIMP-1 antibody (36). Wells were incubated with extract samples (diluted 1:101 in sample dilution buffer). All samples were run in duplicate. Bound TIMP-1 was detected using a murine monoclonal antibody (MAC 19), which specifically detects the uncomplexed fraction of TIMP-1 (28, 30), followed by an alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dakocytomations D0314). p-Nitrophenyl phosphate substrate solution was added, and readings of color development were taken every 10 min at 405 nm for 1 h. KinetiCalc II software (Bio-Tek Instruments, Winooski, VT) was used for calculation of uncomplexed TIMP-1 concentrations. To determine the concentrations of uncomplexed TIMP-1 in individual tumor tissue samples a calibration curve was constructed for every assay plate by including recombinant free TIMP-1 in serial dilutions run in duplicate. Furthermore, on every plate duplicates of a control tumor tissue extract pool made from 271 individual breast tumor tissue extracts and two plasma pools containing high or low TIMP-1 levels, respectively, were included as internal controls. All incubations were performed in volumes of 100 μl at 30 °C. Details regarding buffers, wash steps, and other reagents have been published previously (35).

Validation—The uncomplexed TIMP-1 ELISA was previously used for plasma samples (30). For this reason, it was necessary to validate the assay for measurements in tumor tissue samples. To cover the concept of ELISA performance, assay accuracy and precision were investigated. Accuracy was covered by investigation of assay specificity and recovery. Precision was determined by investigation of intra- and interassay variation as well as by assessment of the precision profile of the assay. In addition, the linearity of TIMP-1 signal as a function of dilution was investigated in a dilution series of tumor tissue extracts (pool) ranging from 1:20 to 1:2560. Furthermore, assay sensitivity, i.e., the lower limit of detection, was calculated as the mean TIMP-1 concentration in zero dose samples (TIMP-1 blanks) plus 3 times the standard deviation.

To ensure that the uncomplexed TIMP-1 ELISA specifically measured the uncomplexed fraction of TIMP-1, and not TIMP-1 in complex with MMPs, an ELISA experiment was conducted in which the polyclonal anti-TIMP-1 antibody was substituted with a polyclonal anti-MMP-9 antibody (diluted 1:5000, kindly supplied by Professor Niels Borregaard, Rigshospitalet, Copenhagen, Denmark). Plates were incubated with TIMP-1/MMP-9 complex (16 and 8 μg/liter) or negative controls comprising recombinant TIMP-1/MMP-9 complex (16 and 8 μg/liter), MMP-9, or dilution buffer only (background). All samples were run in duplicates. Detection was performed using MAC 19 and the alkaline phosphatase-conjugated rabbit anti-mouse antibody as described above. For the generation of TIMP-1/MMP9 complexes recombinant TIMP-1 (in PBS) was incubated with recombinant purified MMP-9 (in PBS) (Niels Borregaard, Rigshospitalet, Copenhagen, Denmark) in a 1:2 molar ratio for 1 h at 37 °C. As a positive control an identical experiment was conducted in which MAC 19 was replaced with MAC 15.

Recovery of the TIMP-1 signal in the control tissue extract pool was determined by adding increasing concentrations of recombinant TIMP-1 to 0.5 and 0.1% dilutions of the control tissue pool, respectively, followed by measurement of the recovery of the TIMP-1 signal using the free TIMP-1 ELISA. Recovery was then calculated by comparing the slope of the line representing the TIMP-1 signal in the diluted tissue extract as a function of the TIMP-1 concentration with the slope of a similar line representing recovery of recombinant TIMP-1 diluted in sample dilution buffer only (representing 100% recovery).

For determination of intra-assay variation, 32 identical samples of the tumor tissue extract pool diluted 1:101 in dilution buffer were measured in duplicate on the same plate. Likewise, interassay variation was determined by including duplicates of 1:101 dilutions of control tissue extract pool on every plate measured (n = 11). The degree to which precision is dose-dependent was determined by constructing a precision profile. This was done by determination of the coefficient of variation as a function of dilution of the control tumor tissue pool (1:20–1:2560).

Finally, to assure and to visualize the presence of TIMP-1 in the tumor tissue extracts, Western blotting of two tumor tissue extract pools containing high and low TIMP-1 levels, respectively, was performed. SDS-gel electrophoresis of 12 μl of tumor tissue extracts was carried out using a 12% acrylamide gel (375 mm Tris (pH 8.9), 0.1% ammonium persulfate solution, 12% acrylamide, double distilled H2O, Temed). 3 μl of molecular marker, 10 μl of TIMP-1 recombinant protein standard (1 ng/μl), and 5 μl of media from MCF-7 cells (secreting high amounts of TIMP-1) were also run on the gel. Before loading on the gel, all samples were diluted in 4× Laemmli sample buffer, boiled, and centrifuged shortly. Following electrophoresis, proteins were transferred electrophoretically from the gel into a nitrocellulose membrane. Subsequently, the membrane was incubated with 5% dried nonfat milk for 1 h. Following 3 × 10 min of washing (washing buffer: 0.1% Tween in PBS), the membrane was treated with MAC 15 (diluted 1:10,000 in 1% dried nonfat milk) or MAC 19 (diluted 1:6000 in 1% nonfat dried milk) overnight at 4 °C. After another round of washes, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (purchased from Dakocytomations, diluted 1:4000 in 1% nonfat dried milk) for 1 h. Finally, the membrane was washed 3 × 10 min in washing buffer, and the membrane was developed according to the manufacturer's instructions (Western blot ECL plus detection kit, Amersham Biosciences).

Statistical Analysis—The SAS® software package (version 8.2; SAS Institute, Cary, NC) was used for data management and statistical calculations. For analysis all concentrations of the uncomplexed fraction of TIMP-1 were normalized by the total protein concentrations in individual samples. The uncomplexed concentrations of TIMP-1 are presented by the median and range. Hypothesis tests on location were done using the Kruskal-Wallis rank sum test. In the association analyses between total TIMP-1 and uncomplexed TIMP-1 the variables were estimated by the Spearman rank correlation. Estimates of recurrence-free survival (RFS) as well as overall survival (OS) were done using the Kaplan-Meier method. The Cox proportional hazards model was used for univariate analysis and multivariate analysis of both endpoints. The covariates included in the model are the lymph node status, receptor status, tumor size, menopausal status, tumor grade, and age as well as the total and uncomplexed levels of TIMP-1. The TIMP-1 levels were entered into the model as the log of the actual level. All p values less than 5% were considered significant.

RESULTS

Validation—The uncomplexed TIMP-1 ELISA was validated using the control tumor tissue extract pool. The limit of detection of the assay was 48.7 pg/ml, and the TIMP-1 signal...
obtained with the uncomplexed TIMP-1 ELISA showed good linearity as a function of dilution of TIMP-1 in the entire range of dilutions tested (1:20–1:1260, Fig. 1A).

Acceptable assay accuracy was demonstrated as recovery of TIMP-1 in the 0.5% control tissue extract pool was 99.6% (Fig. 1B), and in the 0.1% control tissue pool it was 95% (data not shown), confirming that the assay was resistant to possible interfering molecules in the sample matrix. In addition, it was demonstrated that the MAC 19 anti-TIMP-1 antibody used as detection antibody in the uncomplexed TIMP-1 ELISA specifically detected the uncomplexed fraction of TIMP-1 as it did not recognize TIMP-1/MMP-9 complexes (TIMP-1/MMP-9 complexes generated signals at or below background in the specificity experiment). In contrast, when using MAC 15 instead of MAC 19 TIMP-1/MMP-9 complexes were detected (13.23 and 8.46 milliabsorbance units/min at complex concentrations of 16 and 8 μg/liter, respectively, compared with background samples, which generated a signal of 0.1 milliabsorbance unit/min). In support of the specificity of MAC 19 for uncomplexed TIMP-1, Cooksley et al. (28) have previously demonstrated that MAC 19 does not recognize other TIMP-1/MMP complexes such as TIMP-1/MMP-1 and TIMP-1/MMP-3 as well as the closely related molecules TIMP-2, -3, and -4. These results also confirm what has been reported previously (29) that when using MAC 15 as detection antibody the total amount of TIMP-1 is measured as this antibody binds both uncomplexed TIMP-1 and TIMP-1 in complex with MMPs. Finally it was demonstrated that the assay showed good precision exhibiting an interassay variation of 13.1% for the control tumor tissue extract and an intra-assay variation of 6.1%. The precision profile of the assay demonstrated that coefficients of variation were below 10% in the entire range of concentrations analyzed; this was considered satisfactory (Fig. 1C).

Unfortunately MAC 19 did not perform well in Western blotting. In contrast, using MAC 15, the Western blots of tumor tissue samples containing high and low levels of TIMP-1, respectively, both showed a band of ∼28 kDa (Fig. 1D, lanes 3 and 4), corresponding to uncomplexed TIMP-1. In addition, the quantitative difference between the two tumor tissue extracts was evident from the blot.

Measurements of Uncomplexed TIMP-1—All tumor tissue extracts contained measurable levels of uncomplexed TIMP-1. The mean concentration was 27.22 ± 20.24 ng/mg of protein, and the median concentration was 22.52 (range, 3.49–195.02) ng/mg of protein. Correlation analysis between uncomplexed TIMP-1 and classical prognostic parameters is given in Table I. The uncomplexed fraction significantly correlated with hormone receptor status (p < 0.04), menopausal status (p = 0.006), and age (p = 0.002), whereas no significant correlation was seen with nodal status, tumor size, and tumor grade.

Cox regression analysis of total TIMP-1 (log-transformed) showed a significant association to RFS (p = 0.008; hazard ratio (HR) = 1.4; 95% CI, 1.1–1.8). Adding uncomplexed TIMP-1 (log-transformed) to this model significantly improved the model fit (x² difference, 5.0) (p = 0.02; HR = 0.5; 95% CI, 0.3–0.9). The regression coefficients are 0.99 (S.E. 0.32) and −0.70 (S.E. 0.31) for total and uncomplexed TIMP-1, respectively. This model can be respecified as a combination of the total TIMP-1 level and the ratio of uncomplexed TIMP-1 to...
The results from the present study demonstrate that inclusion of TIMP-1 in stratification of breast cancer patients cannot be based on measurements of total TIMP-1 alone as this is not the sole determinant of prognosis; one would have to include information on how much of this TIMP-1 is uncomplexed (i.e. the ratio of uncomplexed/total TIMP-1).

**DISCUSSION**

The results of the present study demonstrate that inclusion of TIMP-1 in stratification of breast cancer patients cannot be based on measurements of total TIMP-1 alone as this is not the sole determinant of prognosis. It seems necessary to include information on the proportion of this TIMP-1 being uncomplexed (the uncomplexed/total TIMP-1 ratio) as this fraction provides important additional prognostic information.

Several studies have suggested that tumor tissue levels of total TIMP-1 carry prognostic information in breast cancer: high levels of total TIMP-1 are associated with a poor prognosis (13–17). In previous studies performed in our laboratories using a thoroughly validated ELISA the total levels of TIMP-1 were measured in tumor tissue extracts from patients suffering from primary breast cancer (16, 17). These studies supported the findings that high levels of total TIMP-1 predict a poor prognosis. In the present study, we measured the
specific fraction of uncomplexed TIMP-1 in 341 tumor tissue extracts, which have previously been subjected to measurements of total TIMP-1 (16), and investigated whether measuring this fraction would add prognostic information to that already provided by measurements of total TIMP-1. We used an ELISA specific for the uncomplexed fraction of TIMP-1 that we validated rigorously for measurements in tumor tissue extracts, and it was demonstrated that requirements concerning sensitivity, specificity, recovery, and precision were fulfilled. Furthermore, the presence of TIMP-1 in the tumor extracts was confirmed by Western blotting.

Multivariate analysis of RFS including the previously measured levels of total TIMP-1 and the presently determined levels of uncomplexed TIMP-1 demonstrated that uncomplexed TIMP-1 provided additional prognostic information and thereby improved prediction of prognosis compared with when total TIMP-1 was solely used for prediction. This suggests that the combination of the two fractions into a single prognostic score would be a more precise estimate of prognosis. The score tested included information from both the total level of TIMP-1 and the ratio of uncomplexed/total TIMP-1.

Univariate survival analysis based on the score showed that this parameter was highly significantly associated with both RFS as well as OS. Patients having high values of the score had significantly shorter RFS and OS compared with patients having low scores. In addition, multivariate analysis of RFS and OS demonstrated that the score significantly and independently predicted both endpoints when all the classical prognostic parameters in breast cancer were included in the model. Thus, the score added prognostic information to that provided by the classical prognostic parameters, and accordingly, the score should be considered as a candidate for

Fig. 2. A, Kaplan-Meier curves showing the association between the score and recurrence-free survival. B, Kaplan-Meier curves showing the association between the score and overall survival. The number of events (RFS and OS) and patients at risk at different time points during the observation period for each of the three groups (1–3) are given below the figure.

Fig. 3. Illustrations of the events leading to increasing values of the score. A, decreasing ratio of uncomplexed/total TIMP-1. B, increasing levels of total TIMP-1. C, A and B in combination. Note that increasing scores do not necessarily reflect increasing levels of total TIMP-1.
improving prognostic stratification in combination with prognostic factors already used in breast cancer.

In general, as can be deduced when analyzing the score, the score is a measure of the level of TIMP-1/MMP complexes in the tumor tissue extracts. Thus, what characterizes increasing values of the score is increasing levels of TIMP-1/MMP complexes and not necessarily increasing levels of total TIMP-1 (Fig. 3). Therefore, it could be hypothesized that it is the level of TIMP-1/MMP complexes that is the real determinant of poor prognosis. As can be seen from Fig. 4, A and B, high levels of total TIMP-1 are not necessarily associated with poor prognosis; it depends on the ratio of uncomplexed/total TIMP-1. Patients with high levels of total TIMP-1 can have prognoses more favorable than patients with lower levels of total TIMP-1 if the patients having these high levels at the same time have high ratios of uncomplexed/total TIMP-1, which correspond to low levels of TIMP-1/MMP complexes. In addition, patients having the exact same levels of total TIMP-1

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<th>Parameter</th>
<th>Recurrence-free survival</th>
<th>Overall survival</th>
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<td></td>
<td>p value</td>
<td>Hazard ratio</td>
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<tr>
<td>Nodal status (node-positive vs. node-negative)</td>
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<td>2.73</td>
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<tr>
<td>Menopausal status (post- vs. premenopausal)</td>
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<td>1.26</td>
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<tr>
<td>Age (yr)</td>
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<td>Tumor size</td>
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<td>&lt;20 vs. 20–50 mm</td>
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<tr>
<td>&gt;50 vs. 20–50 mm</td>
<td>&lt;0.0001</td>
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**Fig. 4.** HRs of different combinations of the ratio of uncomplexed/total TIMP-1 and total TIMP-1 for recurrence-free survival (A) and overall survival (B) compared with the base line.
may have different prognoses depending on the ratio of uncomplexed/total TIMP-1; patients with the highest ratios, which correspond to the lowest levels of TIMP-1/MMP complexes, have the most favorable prognoses. This supports the hypothesis that the level of TIMP-1/MMP complexes is the real determinant of prognosis rather than TIMP-1 itself. Therefore, the fact that the score, as opposed to total TIMP-1, is a direct measure of the level of TIMP-1/MMP complexes may explain why it is a more precise estimate of prognosis. High levels of TIMP-1/MMP complexes presumably reflect the presence of high tumor levels of the invasive promoters, i.e. the activated MMPs, and it could be speculated that these were the actual cause of the unfavorable prognosis of patients with high values of the score. In support of this, various MMPs (MMP-2, MMP-9, and MMP-11) have previously been demonstrated to carry prognostic information in breast cancer (6–10). That several studies have demonstrated an association between high levels of total TIMP-1 and a poor prognosis could be explained by the fact that high levels of total TIMP-1 in most cases also reflect high levels of MMPs. This study, however, demonstrates that this is not always the case. It also explains a surprising finding from our previous study, namely that patients with the highest tumor tissue levels of total TIMP-1 had prognoses similar to patients with intermediate levels (16). These patients were in the present study shown to have high ratios of uncomplexed/total TIMP-1 (data not shown) explaining their favorable prognosis. A major advantage of the score as a prognostic marker as compared with, for example, direct measurements of single MMPs is that the score reflects the level of all TIMP-1/MMP complexes, i.e. TIMP-1 can form complexes with most of the existing activated MMPs, and the score therefore reflects the level of several MMPs instead of only a single type.

From the above discussion it is evident that the results from the present study support the first model from the Introduction, i.e. the prognostic value of TIMP-1 is connected with the fact that the level of TIMP-1 indirectly reflects the level of MMPs in the tumor. It does not appear that high levels of TIMP-1 in itself result in a poor outcome as long as TIMP-1 is uncomplexed, thus speaking against the second model. However, it should be emphasized that this model cannot be rejected. For example, we do not know whether the uncomplexed fraction measured is represented by a biologically unfractional TIMP-1 or whether complexed TIMP-1 had a tumor stimulating function before forming complexes with MMPs.

It is worth noticing that in some cases the measured levels of the uncomplexed TIMP-1 are above the measured level of total TIMP-1. This can probably be explained by differences in affinities of the polyclonal anti-TIMP-1 coating antibody used in both of the assays toward uncomplexed TIMP-1 and TIMP-1/MMP complexes, respectively. The affinity constant of the antibody for uncomplexed TIMP-1 is $2.9 \times 10^8$ liters/mol, and for TIMP-1/MMP complexes it is $0.93 \times 10^9$ liters/mol.

Therefore, it could be speculated that in tumor extracts with high ratios of uncomplexed/total TIMP-1 the measurements obtained from the two different assays will be almost identical, thus making it highly possible for uncomplexed TIMP-1 to be overestimated because of experimental imprecision. However, this does not change the fact that in these cases uncomplexed TIMP-1 constitutes a major proportion of total TIMP-1 and accordingly that the level of TIMP-1/MMP complexes is low. Therefore, the results and conclusions from the present study are therefore not challenged.

In conclusion, the present study demonstrates that the score including information from both the total level of TIMP-1 and the ratio of uncomplexed/total TIMP-1 is a more precise estimate of prognosis in primary breast cancer as compared with total TIMP-1 alone. This is probably connected with the fact that the score more precisely than total TIMP-1 reflects the tumor levels of TIMP-1/MMP complexes, i.e. activated MMPs, which based on this study seem to be the primary determinants of prognosis. Future studies should be aimed at validating the present findings in larger and independent patient populations. Furthermore the clinical relevance of the score should be addressed. Clinical relevance is attained if the number of patients misclassified by using total TIMP-1 in stratification is unacceptable making inclusion of the uncomplexed fraction necessary. Finally, if the score is found to be useful in the clinical setting, it should be addressed whether measurements of TIMP-1 fractions carry prognostic information in other more easily attainable specimens such as blood.

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† To whom correspondence should be addressed. Tel.: 4535283749; Fax: 4535353514; E-mail: suw@kvl.dk.

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