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Tissue Inhibitor of Metalloproteinase-4 Is Elevated in Early-Stage Breast Cancers with Accelerated Progression and Poor Clinical Course

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An increasing number of breast cancer patients are diagnosed with small, localized, early-stage tumors. These patients are typically thought to have a good prognosis for long-term disease-free survival, but epidemiological studies indicate that up to 30% may have a recurrence within 3 to 5 years of diagnosis. Identifying patients with a high risk of recurrence and/or progression is important because they could be more aggressively treated at diagnosis to improve their chances for disease-free survival. Recent evidence suggests that elevated levels of the matrix metalloproteinase inhibitor, tissue inhibitor of metalloproteinase (TIMP)-4, are associated with malignant progression of ductal carcinoma *in situ*, a precancerous lesion. To examine the association of TIMP-4 with survival outcomes, we conducted a retrospective immunohistochemical analysis of 314 cases from patients with early-stage disease, defined as tumors smaller than 2 cm and no spread to lymph nodes (tumor-node-metastasis staging: T1N0MX). We found that tumors with elevated levels of TIMP-4 were correlated with a reduced probability of long-term disease-free survival, especially in patients with estrogen receptor-negative tumors. Our findings prompt further evaluation of TIMP-4 as a simple prognostic marker that may help identify patients with early-stage breast cancer who could benefit from more aggressive treatment at diagnosis. *(Am J Pathol 2009, 175:940–946; DOI: 10.2353/ajpath.2009.081094)*

Tissue inhibitor of metalloproteinase (TIMP)-4 is one of four members of the family of TIMPs, which modify the breakdown of extracellular matrix by the matrix metalloproteinases. Studies of the TIMPs in mammalian organisms have revealed distinctions in structure, biochemical properties, and tissue-specific expression patterns, suggesting unique roles in normal physiology.[1–3] Because of their key roles in cell motility and tissue organization, the interactions between TIMPs and matrix metalloproteinases have been widely studied in cancer research. Until recently, TIMPs were recognized primarily only as matrix metalloproteinase inhibitors. However, recent work has made it increasingly clear that TIMPs have non-matrix metalloproteinase-associated functions in cancer,[4] including roles in growth promotion,[5–7] apoptosis,[8–10] and angiogenesis.[10,11] In the mammary gland, the importance of TIMPs has been demonstrated during normal physiological processes such as glandular development and involution during pregnancy.[12] Although TIMPs have been studied in breast cancer, TIMP-4 has received limited attention and there is conflicting information about its significance. In one study, human breast cancer cells engineered to ectopically express TIMP-4 displayed a reduction in growth and metastasis after implantation in mice.[13] In contrast, another study showed that a gene therapy strategy to express TIMP-4 promoted mammary tumor formation.[14] Notably, in human breast cancer, TIMP-4 has been associated with the transition of ductal carcinoma *in situ* into invasive, infiltrating ductal carcinoma (IDC).[15] With an increasing number of patients being diagnosed with small early-stage tumors (<20 mm

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in diameter) and no signs of spread to lymph nodes, the majority of patients are predicted to have a favorable prognosis for long-term disease-free survival according to traditional tumor-node-metastases (TNM) staging. Nevertheless, epidemiological studies indicate that 20 to 30% of these patients will have a recurrence of their breast cancer within 3 to 5 years of diagnosis.[16,17] Markers that could identify this subgroup of patients who are at higher risk of relapse and/or malignant progression would be useful to stratify them for more aggressive treatment that might improve their chances for long-term disease-free survival. In this study, we tested the hypothesis that TIMP-4 expression correlates inversely with disease-free survival for patients with early-stage disease.

Materials and Methods

Breast Tumor Specimens

Two collections of archival formalin-fixed and paraffin-embedded breast cancer specimens were used in this retrospective study. The use of de-identified archival material was approved by the institutional review boards at Lankenau Hospital and Basel University Hospital. All personal identifiers were removed from the clinical and histopathological information that was stored in the pathology database before transfer to the research laboratory. The collection used as a pilot group for hypothesis testing was obtained from the archives of the Department of Pathology of Bryn Mawr Hospital (Bryn Mawr, PA). Specimens were collected from 183 consecutive, consenting breast cancer patients who had undergone breast cancer resection during 1990 to 1996 at Bryn Mawr Hospital. For the pilot study to establish whether TIMP-4 is associated with stage or survival phenotype, we used the 67 cases of infiltrating ductal carcinoma smaller than 20 mm in diameter, as determined by the pathologist, that were node-negative (T1N0MX). The histological data of the pilot cohort, which had been used previously for studies of other biomarkers,[18] is shown in Table 1. The collection used as an experimental group was obtained from a large collection of cases arrayed in a tissue microarray (TMA) made available from TriStar, Inc. (Bethesda, MD). The arrays consisted of one core from each of 2518 tissue blocks obtained from distinct patients, including control normal tissues from various organs. The total number of cored breast cancer specimens on the TMA was 2197 cases and of these 460 cores were from tumors smaller than 20 mm (T1), with 314 cases of the T1 tumors also being node-negative (T1N0MX). The staining results of these 314 cores evaluated by the pathologist were used in analysis of T1N0 IDC. Estrogen receptor (ER) status was determined by immunohistochemical analysis and scored according to Allred et al,[19] with all tumors showing at least weak staining in at least 10% of tumor cells being regarded as positive for ER expression. Analysis of estrogen receptor status demonstrated that 156 of the 314 T1N0 IDC cores also lacked expression of the estrogen receptor (ie, were ER-negative). Histological subtype, pathological stage, tumor diameter, nodal status, and histological grade according to Elston and Ellis (BRE) were provided with the TMA. The histological description for the breast cancer specimens is summarized in Table 2.

Table 1. Characteristics of Exploratory Pilot (Bryn Mawr) Specimens

	No. of patients	Value	%	Comments
Continuous variables				
Age at diagnosis, years				
Median		64.3		
Range		26–89		
No. of patients	178			
Follow-up time (months)				
Median		72		
Range		6–132		
No. of patients	183			
Tumor diameter (mm)				
Median		20		
Range		1–100		
No. of patients	168			
Discrete variables				
Lymph node status				
0		70	53.8	
1		30	23.1	
2		30	23.1	
Unknown		26		
Tumor grade				
I		23	14.9	
II		93	60.4	
III		38	24.7	
Unknown		2		
ER status				
Positive		96	62.3	
Negative		58	37.7	
Unknown		2		
Histological type				
IDC	156			
Other	27			
IDC tumor size				
≤20 mm	88			
≥20 mm	57			
Unknown	11			
Node status in IDC ≤20 mm (T1)				
N0	67			Used in pilot study
N1–N3	19			
Unknown	2			

Antibodies

The primary antibody used was a rabbit polyclonal anti-human TIMP-4 antibody (Chemicon International, Temecula, CA) selected for its ability to stain formalin-fixed, paraffin-embedded specimens. To ensure the use of the same batch of antibody throughout the work, a large number of vials were purchased, and the antibody solution was pooled. An aliquot of the pooled TIMP-4 antibody was used for staining of the TMAs (shipped on ice to the laboratory of G.S.).

Immunohistochemical Staining

Tissue sections from the pilot group were deparaffinized and rehydrated essentially as described previously.[18] Steam-based antigen retrieval was performed, and endogenous peroxidase activity was blocked by incubating slides in 0.3% H_2O_2 in water before blocking with the

Table 2. Characteristics of Experimental Test (TMA) Specimens

	No. of patients	Values	%	Comments
Continuous variables				
Age at diagnosis (years)				
Median		63		
Range		26–101		
No. of patients	1884			
Follow-up time (months)				
Median		62		
Range		1–176		
No. of patients	2221			
Tumor diameter (mm)				
Median		24		
Range		2–140		
No. of patients	2173			
Discrete variables				
Lymph node status				
0		503	46.1	
1		511	46.9	
2		76	7.0	
Unknown		204		
Tumor grade				
I		276	23.2	
II		449	37.7	
III		466	39.1	
Unknown		103		
TIMP-4 status				
0		635	49.1	
I		437	33.8	
II		197	15.2	
III		25	1.9	
ER status				
Positive		972	77.3	
Negative		285	22.7	
Unknown		37		
Histological type				
IDC	1294			Analyzable
Other	489			
IDC tumor size				
≤20 mm	460			
≥20 mm	834			
Node status in IDC ≤20 mm (T1)				Used in study of all T1N0
N0	314			
N1–N3	146			
ER status among T1N0				
ER-positive	158			
ER-negative	156			Used in study of ER-negative T1N0

ABC Elite kit (Vector Laboratories, Burlingame, CA). A rabbit polyclonal anti-human TIMP-4 antibody was added (10 μg/ml), and the mixture was incubated overnight in a moist chamber at +4°C. After extensive washes, the secondary biotin-conjugated antibody was added before addition of the avidin-peroxidase complex (ABC Elite kit). The diluted chromogen 3,3′-diaminobenzidine was added for 5 minutes after which specimens were thoroughly rinsed and counterstained with hematoxylin for 8 seconds. Coverslips were mounted with PermaMount (Vector Laboratories) on dry slides. The TMA slides from the experimental group were deparaffinized and rehydrated in a descending ethanol series followed by a wash in standard PBS. Pretreatments included epitope retrieval by autoclaving 5 minutes in citrate buffer (pH 6.0) followed by quenching of endogenous peroxidase activity by incubating slides in 0.3% H_2O_2/methanol. After blocking in normal horse serum (S-2000, Vector Laboratories), the primary TIMP-4 antibody was added (2.2 μg/ml) in PBS for 2 hours at 21°C in a moist chamber. After extensive washes, the horseradish peroxidase-conjugated secondary antibody was added (K4003 EnVision, anti-rabbit, Dako, Carpenteria, CA). The chromogen 3,3′-diaminobenzidine was added for 6 minutes followed by washes and counterstaining for 20 seconds with hematoxylin (Harris hematoxylin HTX 31000, Medite GmbH, Burgdorf, Germany). After drying in an ascending ethanol series and xylene, TMA slides were covered and examined.

As controls for specific staining in the pilot study, we used formalin-fixed and paraffin-embedded cell pellets of MDA-MB-231, a TIMP-4-expressing human breast carcinoma cell line (American Type Culture Collection, Manassas, VA), and MDA-MB-435, a TIMP-4-nonexpressing human breast carcinoma cell line (American Type Culture Collection) for quality control. All cases were also stained with a purified rabbit IgG immunoglobulin isotype standard serum as a specificity control. All controls were run in parallel with each case.

On the TMA, tissue cores from 18 organ types were present. These included colon, lung, heart, endometrium, pancreas, skin, and spleen.

Before the TMA was stained, smaller test arrays were used to establish staining conditions. In these tests, regular antibody, pre-immune serum, and pre-absorbed antibody were used to ensure that specific staining could be detected.

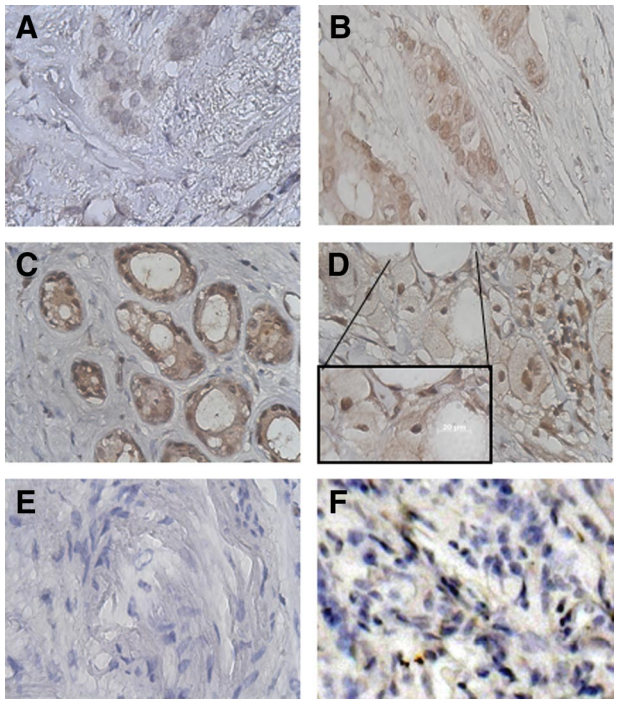

Figure 1. Immunohistochemical detection of TIMP-4 in formalin-fixed, paraffin-embedded breast cancer tissues. Absence (**A**) or presence (**B–D**) of TIMP-4 stained infiltrating ductal carcinoma. **B–D:** Polyclonal antibody staining showed cytoplasmic and nuclear staining of three levels of intensity. **E:** Representative control staining with isotype rabbit IgG. **F:** Antibody preabsorbed with recombinant human TIMP-4.

Table 3. TIMP-4 Expression in Various Histological Types of Breast Cancer

Histological type	On array	Analyzable	TIMP-4 score 0 (%)	TIMP-4 score 1, 2, or 3 (%)
All	2197	1783	52.3	47.7
Ductal carcinoma	1531	1294	49.1	50.9
Lobular carcinoma	311	192	67.7	32.3
Mucinous carcinoma	69	53	58.5	41.5
Cribriform carcinoma	64	55	58.2	41.8
Medullary carcinoma	57	49	40.8	59.2
Tubular carcinoma	56	49	52.3	47.7
Papillary carcinoma	30	26	65.4	34.6
Other carcinoma	79	70	62.9	37.1

Statistical Analysis

TMA staining data were analyzed with GraphPad (version 5.0, GraphPad Software Inc., San Diego, CA) and SAS (version 9.2, SAS Institute, Cary, NC). Early-stage IDC samples were analyzed for staining correlations to overall survival by constructing Kaplan-Meier curves,[20,21] and differences between curves were evaluated by a log-rank test. Multivariate and univariate hazard ratios were calculated by applying a Cox proportional hazards regression model (SAS phreg) on the results from the TMA. Because of incomplete or missing information on relapse, adjuvant and hormone therapy, progesterone receptor, and Her-2/*neu* status, these parameters were not included in the analysis.

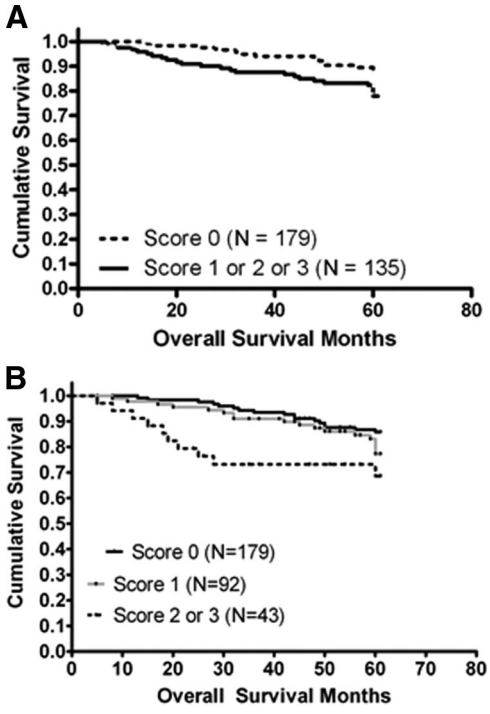

Figure 2. Kaplan-Meier survival graph for early-stage IDC. **A:** Overall survival analysis according to TIMP-4 status in patients from the TMA with infiltrating ductal carcinomas smaller than 20 mm in diameter without spread to local lymph nodes ($N = 314$) (log-rank $P = 0.0254$). **B:** Survival for the same patients group stratified according to undetectable (score 0) versus detectable levels of low (score 1) or intermediate/high (scores 2 and 3, respectively) of TIMP-4 in the tumor material.

Results

TIMP-4 Protein Is Present in Breast Cancer Specimens of All Histological Types

Immunohistochemical analysis of infiltrating ductal carcinoma demonstrated TIMP-4 staining associated with tumor epithelial cells. Staining results were obtained from 1783 of the total of 2197 (81%) tissue samples on the TMA. Reasons for failure among the nonstaining tissues included lack of tumor cells in the sample ($N = 295$; 13%) or missing sample spots on the TMA ($N = 119$; 6%). The relative levels of immunohistochemical expression of TIMP-4 on the TMA core specimens were evaluated by one pathologist, and the staining intensity was estimated by visual inspection in a four-step scale (score 0, 1, 2, and 3). Both nuclear and cytoplasmic patterns of staining were evident in the specimens (Figure 1, A–D). Using a proprietary threshold finder software tool (developed by G.S.), the following optimal scoring criteria were found. Cases for which no positive staining was observed were given the score 0, representing TIMP-4-negative tumors. Cases in which tumor cells had weak (light color) or ≤30% of tumor cells had a moderate staining intensity were given the score 1. The intermediate score 2 was used when >30% of tumor cells had moderate or <60% of tumor cells had strong staining intensity. Cases in which ≥60% of cells had a strong staining intensity (dark brown/black color) were assigned the score 3.

Cytoplasmic costaining was present in approximately 10% of breast cancers with nuclear positive staining and was recorded but scored separately. These patterns dif-

Table 4. Univariate Analyses of Early-Stage Breast Cancer Survival to Prognostic Factors in Breast Cancer

Parameter	Range or category	Hazard ratio (95% confidence interval)
BRE	1–3	0.876 (0.726–1.057)
TIMP-4 score	Positive vs. negative	1.389 (1.012–1.907)
Tumor size	5–20 mm	0.984 (0.943–1.029)
ER	Positive vs. negative	1.104 (0.690–1.687)

Statistical analyses were performed by the χ^2 test (Wald method) in Cox proportional hazards regression analysis to evaluate the prognostic power of factors in a multivariate manner.

Table 5. Multivariate Analyses of Early-Stage Breast Cancer Survival to Prognostic Factors in Breast Cancer

Parameter	Range or category	Estimate	SE	χ^2 statistic	*P*	Hazard ratio (95% confidence interval)
BRE grade	1–3	−0.13086	0.09909	1.7442	0.1866	0.877 (0.723–1.066)
TIMP-4 score	Positive vs. negative	0.35253	0.16323	4.6643	0.0308	1.423 (1.032–1.959)
Tumor size	5–20 mm	−0.01434	0.02324	0.3809	0.5286	0.985 (0.942–1.032)

Statistical analyses were performed by the χ^2 test (Wald method) in Cox proportional hazards regression analysis to evaluate the prognostic power of factors in a multivariate manner.

fered from those obtained with an isotype rabbit antibody or with primary antibody pre-absorbed with purified recombinant human TIMP-4 protein (Figure 1, E and F, respectively). Results from the TMA indicated that 50.9% of all IDC ($N = 890$) and 51.9% of the early-stage IDC ($N = 343$) stained positive for TIMP-4 protein. The results from the TMA also indicated that TIMP-4 expression is found in histological types of breast cancer other than IDC (Table 3).

Survival Analysis of Test Specimens

Because lymph node status is regarded as the single most critical prognostic factor for breast cancer, and there is a lack of simple immunohistochemical markers to predict prognosis of patients with node-negative breast cancer, we specifically analyzed the ability of TIMP-4 expression to predict prognosis of node-negative patients.[22] Kaplan-Meier analyses of overall survival were performed on results from only IDC specimens on the TMA.

No other histological subtype had sufficient data information to perform such analysis. The results from all early-stage IDC (T1N0MX) ($N = 314$) on the TMA indicated that the presence of TIMP-4 (scores 1, 2, and 3) was associated with decreased survival expectancy relative to the presence of no TIMP-4 in the tumor material (score 0) ($P = 0.0254$ by log-rank analysis) (Figure 2A).

To assess the ability of TIMP-4 to predict disease progression for early stage, node-negative IDC, breast cancer specimens were analyzed separately by univariate and multivariate analysis. By univariate analysis, the presence of TIMP-4 was associated with a significant risk of disease progression. Of the four markers examined (BRE, size, ER status, and TIMP-4), TIMP-4 was the only predictor with good prognostic capability (Table 4). Multivariate analysis using the Cox proportional hazards regression method,[23,24] to take several prognostic factors into account (BRE, size, and TIMP-4 score), showed that the presence of TIMP-4 remained an independent prognostic factor for survival (hazard ratio, 1.423; $P = 0.0308$) (Table 5). At the 5% level, only TIMP-4 was statistically significant, with none of the other markers being statistically significant even at the 10% level (data not shown). In an initial analysis, ER status had the widest interval and an estimate of 1.175. With several specimens missing information on ER status, it was excluded from the final analysis to add confidence to the results for the other factors.

Stratification of the TIMP-4 level in subgroups of low (score 1) or medium/high (scores 2 and 3) staining intensities demonstrated not just a lower proportion of patients with 5-year disease-free survival within the medium/high subset, but also a greater likelihood of early onset of disease progression ($P = 0.0052$ by log-rank analysis) (Figure 2B). The age at time of death ranged from 34 to 84 years (median of 68 years) with 57% of all deaths occurring among patients younger than 70 years. However, the follow-up data provided with the TMA specimens did not discriminate between death due to breast cancer and death due to other causes. Thus, the Kaplan-Meier analysis was performed on patients for whom censoring information was available during the first 5 years of follow-up. When the results were considered together, they strongly suggested an unfavorable survival prognosis associated with TIMP-4 elevation in primary breast carcinoma.

TIMP-4 Expression Is Associated with a Particularly Unfavorable Survival Prognosis in ER-Negative Early-Stage Ductal Carcinoma

Estrogen receptor status has been used as a prognostic marker with receptor presence indicating favorable long-term disease-free survival. Patients with ER-negative tumors are generally regarded as more likely to have a relapse or progression of cancer within a shorter time. Still, some patients within this group will experience long-term survival without further complications. However, there is no known marker that can predict survival prognosis among patients with early stage (T1N0) ER-negative breast tumors. Our analysis clearly indicated that the presence of TIMP-4 protein was associated with a shorter disease-free survival time. Patients in the TIMP-4-positive group had early progression to more aggressive disease

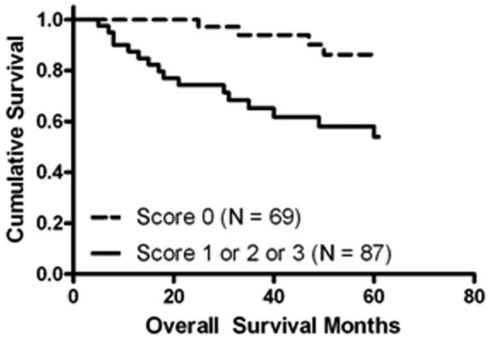

Figure 3. Kaplan-Meier survival graph for estrogen receptor-negative early-stage IDC. Overall survival according to the presence (scores 1, 2, and 3) or absence (score 0) of TIMP-4 in tumors on the TMA for patients with estrogen receptor-negative early-stage IDC ($N = 156$) (log-rank $P = 0.0011$).

and were less likely to survive 5 years after diagnosis (Figure 3). Comparison of the overall survival for early-stage ER-negative tumors showed a strong correlation between the presence of TIMP-4 (score = 1, 2, or 3) and poor survival ($P = 0.0011$ by log-rank analysis), relative to the survival period for those without detectable levels of TIMP-4. Clinically, these findings suggest that ER-negative tumors are more sensitive to growth-promoting pathways involving TIMP-4 that are as yet undefined.

Discussion

This study demonstrates that breast cancer patients with early-stage infiltrating ductal carcinoma whose tumors express TIMP-4 have shorter disease-free periods compared with patients whose tumors have undetectable TIMP-4. Further, high levels of TIMP-4 were found predominantly in patients with a survival period of less than 3 years, indicating that TIMP-4 is a marker for aggressive disease and shorter survival expectancy.

A biological mechanism to explain the link between TIMP-4 level and poor survival prognosis in breast cancer patients remains to be determined. Even though the TIMPs are usually only regarded as regulators of matrix metalloproteinase activities, there is a growing body of evidence demonstrating that the TIMPs are multifunctional proteins that can affect cell growth, apoptosis, and angiogenesis.[1–3] Expression of TIMP-4 might be an early sign of malignancy as it has been shown that TIMP-4 expression is associated with the progression of ductal carcinoma *in situ* to infiltrating ductal carcinoma.[15] In addition, others have demonstrated that TIMP-4 expression is associated with poor survival prognosis among colon cancer patients.[25] Still others have shown that TIMP-1 inhibits intrinsic apoptosis by inducing survival pathways involving focal adhesion kinase.[26] For example, high levels of TIMP-1 are associated with poor survival outcome for breast cancer patients,[27] and they are also predictive of response to chemotherapy.[28] Based on our current observation, TIMP-4 in breast cancer tissue would be predicted to mediate a tumor progression event(s) contributing to reduced life expectancy.

It is becoming increasingly important to identify markers that can stratify patients with small but aggressive tumors so that appropriate treatment can commence at the earliest possible time point. In the past, patients with early-stage breast cancer were generally given a good prognosis for disease-free survival. However, it now clear that even tumors with low TNM scores can recur or progress within a few years of diagnosis. This occurrence is mostly observed among the ER-negative tumors, which frequently also lack the progesterone receptor (progesterone receptor-negative), making them unresponsive to estrogen and therapies that interfere with estrogen signaling. Among the most aggressive and difficult types of breast cancers to treat are "triple-negative" tumors, ie, those lacking ER, progesterone receptor, and amplified Her-2. For these patients, standard chemotherapy is the only modality of systemic therapy. However, triple-negative tumors are frequently associated with a high rate of local and systemic relapse even after chemotherapy.[29] With no access to the Her-2 status of the TMA tumors, it is a possibility that at least some tumors identified as ER-negative are also triple-negative. With a substantial overlap between the triple-negative and the basal-like breast carcinomas,[30] the results could be affected by these subcharacteristics. On-going prospective studies of TIMP-4 in early-stage breast cancer will provide clarification as to whether TIMP-4 is associated with ER status alone or whether it is associated with triple-negative/basal-like tumor types.

Another factor that could influence the observed association between TIMP-4 and survival is the therapies given after surgery. This question will also need to be addressed. However, because it is known that some but not all patients with ER-negative tumors will have a relapse within a few years after diagnosis,[16,17] it is tempting, based on the survival outcome among those with TIMP-4-positive/ER-negative tumors, to suggest that TIMP-4 can identify these patients. On the basis of the results from this retrospective study, TIMP-4 might help satisfy the need for markers to identify the tumors that pose the most risk in this regard.

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