Expression of Extracellular Matrix Components Versican, Chondroitin Sulfate, Tenascin, and Hyaluronan, and Their Association with Disease Outcome in Node-Negative Breast Cancer

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ABSTRACT

Purpose: **The purpose is to determine whether the levels of expression of extracellular matrix components in peritumoral stroma are predictive of disease outcome for women with node-negative breast cancer.**

Experimental Design: **Tumor tissue from 86 patients with node-negative breast cancer was examined by immunohistochemical staining for the expression of versican, chondroitin sulfate (CS), tenascin, and hyaluronan (HA). With the exception of HA, the expression of the extracellular matrix components was measured by video image analysis. Statistical correlation of the immunohistochemical data with clinicopathological characteristics and disease outcome was performed.**

Results: **All of the extracellular matrix components were present in the peritumoral stroma of the entire study cohort. In contrast, immunoreactivity within the cancer cell was observed in 82% of tumors for HA, 12% for CS, and 4% for tenascin; no immunostaining of cancer cells for versican was observed for any of the tumors. Cox regression and Kaplan-Meier analyses indicated that elevated expression of stromal versican predicted increased risk and rate of relapse in this cohort. Elevated expression of tenascin was predictive of increased risk and rate of death only. Although neither CS nor HA were predictive of disease outcome in this cohort, tumor size was predictive of increased risk and rate of both relapse and survival.**

Conclusions: **Elevated expression within peritumoral stromal matrix of versican and tenascin was predictive of relapse-free and overall survival, respectively, in women with node-negative breast cancer.**

INTRODUCTION

The extracellular matrix (ECM) provides a physical framework for cellular attachment and facilitates the normal physiological regulation of cell proliferation, migration, and differentiation. Recently, we reported that disease relapse for patients with node-negative breast cancer is associated with elevated expression of the chondroitin sulfate (CS) proteoglycan, versican, in the peritumoral stromal tissue (1). Individual matrix components do not exist in isolation, however, but function as a complex due to the numerous binding associations and biological interactions of these molecules. Versican binds to the linear unsulfated glycosaminoglycan hyaluronan (HA) via the NH_2-terminal tandem repeat peptide sequence of versican, which is homologous to link protein and has inherent affinity for HA (2). Versican also binds to the hexameric glycoprotein tenascin-R, possibly via interaction between the lectin domain of versican with either a carbohydrate moiety on tenascin (3) or with the fibronectin type III repeats within tenascin (4). Individual reports have cited increased levels of versican, HA, or tenascin-C within neoplastic stroma of breast cancer tissues, especially at the leading edges of invasive foci (5–7). In addition to our observed association of versican with relapse, both HA and tenascin-C have been associated with a poor disease outcome for breast cancer (8, 9). Thus, these three molecules appear to be coexpressed and associated with clinical outcome from breast cancer, and all are putative modulators of cellular attachment and motility *in situ* (10–12). However, it is not clear which of these matrix factors might have the greatest importance with respect to determination of patient outcome from breast cancer.

The focus of this study, therefore, was to examine the level of versican, CS, tenascin, and HA in breast cancer tissues and to determine which of these components within the matrix complex is the strongest predictor of outcome for women with node-negative disease.

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Table 1 Clinical and pathological characteristics of 86 patients with node-negative, infiltrating ductal carcinoma

Median age (range)	63 (28–82) years
Median follow-up (range)	65.5 (11–116) months
Menopausal status	
Premenopausal	15
Postmenopausal	68
Unknown	3 (45, 46, 47 years)
Tumor grade	
Grade I	13
Grade II	34
Grade III	39
Tumor size	
<20 mm	47
≥20 mm	39
Stage	
1	46
2a	39
2b	1
Hormone receptor status	
Estrogen receptor <10 fmol/1 mg protein	32
Estrogen receptor ≥10 fmol/1 mg protein	54
Progesterone receptor <10 fmol/1 mg protein	42
Progesterone receptor ≥10 fmol/1 mg protein	44
Recurrence	
Nonrelapse	58
Relapse	28
Death during follow-up	
Alive	59
Breast cancer death	18
Other causes	9

MATERIALS AND METHODS

Patients and Tissues. A retrospective cohort of 86 women who underwent surgery for primary breast cancer between 1987 and 1991 was assembled with approval from the Research Ethics Committee of Flinders Medical Centre, Adelaide, South Australia. All patients were node negative, and all tumor tissue samples were diagnosed as infiltrating ductal carcinoma (carcinoma of no special type). Thirty-five patients were in common with our previously reported cohort (1). No patients in either cohort had previously received chemotherapy or hormonal therapy individually or in combination. The breast tumor tissue samples were graded according to the Elston and Ellis modification of the Bloom and Richardson grading system (13). Steroid receptor status was routinely assessed using the radioligand binding assay (14). Clinical and pathological data for the cohort is summarized in Table 1. Clinical follow-up was derived from patient case notes. Follow-up was 3-monthly. Recurrences were determined by Union International Contre Cancer method and reported by the Flinders Medical Centre Cancer Registry. Deaths were reported by the Flinders Medical Centre and South Australian Cancer Registries.

Immunoprobes. The following antibodies were used in this study: monoclonal anti-TN2 (Dako, Carpinteria, CA) against human tenascin hexabrachion structure; monoclonal anti-CS56 (Sigma, St. Louis, MO) reactive for native CS epitopes; and rabbit antihuman versican (15). In addition, a biotinylated complex of bovine cartilage aggrecan HA-binding region and link protein (bHABC) (16) was used to detect HA.

Immunohistochemical Staining. Sections (4 μm) from archival formalin-fixed, paraffin-embedded blocks of breast cancer tissue were mounted on Histogrip-coated slides (Zymed Labs, San Francisco, CA) and baked for either 60 min or overnight at 50°C–60°C. They were then deparaffinized in xylene, rehydrated in ethanol, and rinsed in PBS (pH 7.4). Endogenous peroxidase was blocked using 0.3% H_2O_2 in PBS for 5 min. To detect antigenicity of versican, the sections were predigested with chondroitinase ABC [Sigma; 0.5 units/ml in 0.1 M Tris acetate buffer (pH 7.8), 0.1% BSA] 90 min at room temperature. After blocking nonspecific binding sites with 10% goat serum, the sections were incubated with rabbit antihuman versican (1:1000) overnight at 4°C, followed by addition of biotinylated goat antirabbit IgG secondary antibody (Vector Labs, Irvine, CA) and peroxidase-conjugated streptavidin (Dako). After each step, the sections were washed with PBS twice for 5 min. To detect CS, tissue sections were blocked with goat serum, followed by incubation with monoclonal mouse antibody CS56 (1:500) 60 min at room temperature. Subsequently, biotinylated goat antimouse IgG secondary antibody (Vector Labs) and peroxidase-conjugated streptavidin were applied to the sections. To detect tenascin, tissue sections were digested with 0.1% pepsin in 0.01 M HCl 60 min at 37°C before blocking with 10% normal horse serum. Sections were then incubated with monoclonal mouse antibody TN2 (1:400) 120 min at room temperature, followed by biotinylated horse antimouse IgG secondary antibody (Vector Labs), and then avidin-biotin-peroxidase complex (Vector Labs). To detect HA, tissue sections were blocked for nonspecific binding with 1% BSA in PBS, incubated with bHABC probe (2.5 μg/ml, diluted in 1% BSA) overnight at 4°C, and then treated with avidin-biotin-peroxidase complex (1:200; Vector Labs) as reported previously (16). The specificity of staining for HA was examined using tissue sections predigested with *Streptomyces* hyaluronidase (Seikagaku, Tokyo, Japan) in the presence of protease inhibitors.

Visualization of all of the various immunoreactions was achieved using diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide [0.03% in 50 mM Tris-HCl (pH 7.6)] for 6 min. Sections were rinsed in tap water, counterstained with hematoxylin (excepting sections stained for HA), dehydrated through graded alcohols, cleared in xylene, and mounted using DPX (BDH Labs, Poole, United Kingdom).

Evaluation of Staining. Immunostaining for the individual ECM components was performed in 86 patients. However, after staining, some cases were excluded because of insufficient residual tumor area for adequate analysis, resulting in 85 cases being available for versican staining, 85 cases for CS staining, 81 cases for tenascin staining, and 79 cases for HA staining.

The level of immunostaining for versican, CS, and tenascin was measured using an automated image analysis system (VideoPro 32; Leading Edge P/L, Marion, South Australia) as described previously (1). Twenty images from random areas of each breast cancer tissue section were captured at ×66 magnification and edited using VideoPro 32 software. The images included both epithelial and stromal areas. Epithelial areas were evaluated visually for immunostaining and recorded for each of the above matrix components. Epithelial areas were then edited from the captured images, retaining only the stromal areas for quantitative image measurement. The following image measurements were recorded for the stromal tissue: total area and immunostained area (in pixels) and absorbance (*i.e.*, reciprocal of optical density, in arbitrary density units), which represents the intensity of staining for each matrix component. The mean

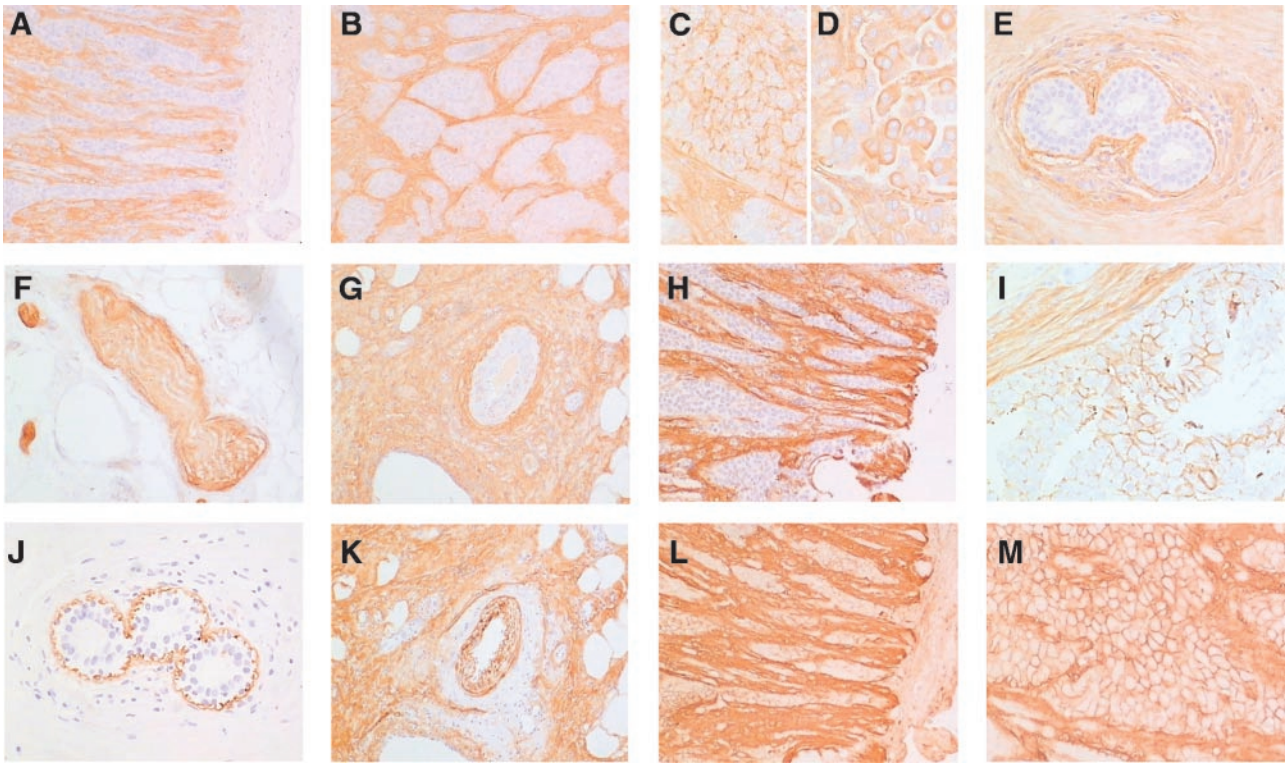

Fig. 1 Staining for extracellular components in serial sections from a representative, paraffin embedded, primary, node-negative, infiltrating ductal carcinoma. *A*, versican present in peritumoral stroma. *B*, chondroitin sulfate (CS) present in peritumoral stroma. *C*, CS present in plasma membrane of breast cancer cells. *D*, CS present in the cytoplasm of breast cancer cells. *E*, CS present in periglandular basement membrane of normal breast tissue. *F*, CS present in myelin sheath of nerve bundles. *G*, CS present in collagenous adventitia of muscular artery. *H*, tenascin present in peritumoral stromal tissue. *I*, tenascin present in cancer cell plasma membranes. *J*, tenascin present in periglandular basement membrane in normal breast tissue. *K*, tenascin present in smooth muscle cells of blood vessels within normal breast tissue. *L*, hyaluronan present in peritumoral stroma. *M*, hyaluronan present in cancer cell plasma membranes. *A*, *B*, *F*, *G*, *H*, *K*, and *L*, magnification, ×100; and *C*, *D*, *E*, *I*, *J*, and *M*, magnification, ×200.

integrated absorbance (MIOD) was determined from the formula: stained area × integrated absorbance/divided by total area, for each section, and then the mean was calculated for the 20 images from each patient. Hence, MIOD is equivalent to the average stromal tissue concentration (expressed in arbitrary density units/pixel) of the individual matrix components.

The intensity of HA in peritumoral stromal tissue was graded visually as weak (+), moderate (++), and strong (+++). The expression of HA in breast cancer cells was classified as negative when <5% of cancer cells were stained, weak when 5–40% cells stained, moderate when 41–70% cells stained, and strong when 71–100% cells stained.

Statistical Analysis. Statistical analysis was performed using SSPS 10 for Windows software (SPSS, Inc., Chicago, IL). The relationship between clinicopathological variables and the concentration (MIOD) of ECM components was determined using χ^2 test. Spearman's rho analysis was used to test for correlation between expression levels of the individual ECM components. Relapse-free survival was defined as the time from the date of diagnosis of disease to the date of disease recurrence (local or systemic). Overall survival was defined as the time from diagnosis of disease to death from breast cancer. Cox regression was used to correlate risks of relapse and death with the level of expression of individual matrix components. Kaplan-Meier product limit plots and log-rank tests were used to compare the rates of relapse or death for groups of patients dichotomized for high or low expression of matrix component. Statistical significance was set at $P < 0.05$.

RESULTS

Immunolocalization and Expression of Versican, CS, Tenascin, and HA. Versican immunoreactivity was observed in the peritumoral stromal matrix of all breast cancer tissues examined (Fig. 1*A*). The intensity of staining varied between patient samples, the median (range) concentration of versican in peritumoral stromal tissue being 8.68 (1.82–24.48) density units/pixel. In nonmalignant breast tissue, versican was either absent or presented as a thin discontinuous band of weak staining of the periductal stroma. Versican immunoreactivity was not observed in either cancer cells or the nonmalignant epithelial cells in any of the specimens.

CS immunoreactivity (epitope CS56) was observed in the peritumoral stromal tissue of all breast cancer tissues examined (Fig. 1*B*), the median (range) concentration of CS being 16.97 (7.14–25.60) density units/pixel. In 10 of the 85 (12%) specimens, membranous and cytoplasmic staining of the malignant epithelial cells was evident (Fig. 1, *C* and *D*). Weak immuno-

Table 2 Correlation between versican, tenascin, chondroitin sulfate (CS), and hyaluronan (HA) (Spearman's rho correlation)

Versican and CS	$r = 0.334$	$P = 0.002$[a]
Versican and tenascin	$r = 0.046$	$P = 0.684$
CS and tenascin	$r = 0.204$	$P = 0.068$
Versican and HA in stroma	$r = -0.068$	$P = 0.552$
CS and HA in stroma	$r = 0.207$	$P = 0.069$
Tenascin and HA in stroma	$r = 0.322$	$P = 0.005$[a]

[a] Statistically significant at $P < 0.05$.

staining for CS was observed within the basement membrane of nonmalignant glands and in the surrounding collagenous stromal matrix (Fig. 1*E*). CS was also present in nerve bundles and the adventitia of arteries (Fig. 1, *F* and *G*). CS was not detected in normal ductal epithelial cells.

Tenascin immunoreactivity was observed in the peritumoral stroma of all breast cancer tissues examined (Fig. 1*H*), the median (range) concentration of tenascin being 19.44 (3.48–34.69) density units/pixel. Tenascin immunoreactivity was observed in the cytoplasm and cell membrane of the malignant epithelial cells in only 3 of 81 (4%) of patients (Fig. 1*I*). Tenascin was also detected as a thin band of weak immunoreactivity surrounding normal ducts and in the endothelial and smooth muscle cells of blood vessels within normal breast tissue (Fig. 1, *J* and *K*). No tenascin expression was detected in normal ductal epithelial cells.

Staining for HA was present in the peritumoral stromal tissue of all specimens of breast carcinoma, with variable intensity between tumors (Fig. 1*L*). Weak HA staining of the stromal tissue was observed in 10 of 79 (13%) of tumors, moderate staining in 17 of 79 (21%), and strong staining in 52 of 79 (66%). HA was also detected in the malignant epithelial cells of 65 of 79 (82%) specimens (Fig. 1*M*). In 42 of these specimens, HA was detected in >40% of the carcinoma cells. HA staining was associated with the plasma membrane in 40 of 65 specimens, with the plasma membrane and within the cytoplasm in 16 of 65 specimens and with the plasma membrane and within the cytoplasm and the nucleus in 9 of 65 patients.

Relationship between Expression of the Individual ECM Components and Their Relationship with Clinicopathological Features of Node-Negative Primary Breast Cancer. The relationships between the concentrations of the individual matrix components in peritumoral stromal tissue are shown in Table 2. Significant associations were detected between the levels of versican and CS (Fig. 2; Spearman's rho, $r = 0.334$, $P = 0.002$) and between the levels of tenascin and HA in stroma ($r = 0.322$, $P = 0.005$). The relationships between the concentrations of versican, CS, tenascin, and HA and patient clinicopathological factors (tumor size, grade, stage, and steroid receptor status) were determined by χ^2 analysis and are shown in Table 3. The level of versican staining of peritumoral stromal tissue was not related to any clinicopathological factor examined other than progesterone receptor status ($P = 0.015$). Increased expression of tenascin in peritumoral stromal tissue was significantly associated with increased tumor size ($P = 0.014$), tumor grade ($P = 0.004$), and stage ($P = 0.008$). Increased expression of HA in peritumoral stromal tissue and expression of HA by cancer cells were significantly associated with tumor grade ($P = 0.0001$ and $P = 0.025$, respectively).

Prediction of Disease Outcome Based on Immunoreactivity of the ECM Components. An increased risk of relapse was predicted by increasing tumor size ($P = 0.036$) and by high levels of versican expression when tested as either a continuous ($P = 0.047$) or a dichotomized variable (cut point MIOD $<$ 13 *versus* $\geq$ 13 density units/pixel, $P = 0.007$; Table 4*A*). The cut point for versican was previously determined to be in the range of MIOD 13–14 density units/pixel (1). Tenascin levels did not predict relapse-free survival. An increased risk of death was predicted by increasing tumor size ($P = 0.05$) and high levels of tenascin expression when tested as either a continuous ($P = 0.024$) or a dichotomized variable (cut point MIOD $<$ 19.4 *versus* $\geq$19.4 density units/pixel, $P = 0.019$). Because there was no established MIOD cut point for tenascin, the median MIOD for the cohort was used. Versican levels in peritumoral stromal tissue did not predict overall survival. Neither the level of CS nor HA predicted relapse-free survival and overall survival in this cohort of node-negative breast cancer patients. Multivariate analysis comparing risks of relapse for tumor size and versican concentration indicated that these variables independently predicted relapse (Table 4*B*). Multivariate analysis comparing risks of death for tumor size and tenascin concentration indicated that tenascin was the stronger predictor of overall survival (Table 4*B*).

Versican and tenascin levels were investigated as dichotomized variables by Kaplan-Meier product limit and log-rank tests for rates of relapse and death (Fig. 3). The rate of relapse was greater for the group of patients with versican level MIOD $\geq$ 13 (Fig. 3*A*, relapse-free survival at 5 years, 74 *versus* 35% for patients with versican levels MIOD $<$ 13 and $\geq$ 13, respectively). Versican levels were not predictive of rate of death (Fig. 3*B*), and tenascin levels were not predictive of rate of relapse (Fig. 3*C*). The rate of death was greater for the group of patients with tenascin level MIOD $\geq$ 19.5 (Fig. 3*D*, overall survival at 5 years, 92 *versus* 66% for patients with tenascin levels MIOD $<$ 19.5 and $\geq$ 19.5, respectively).

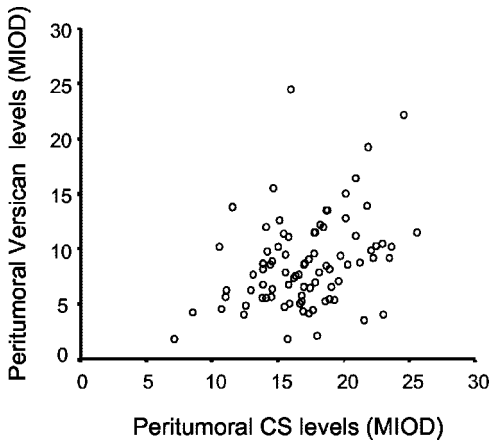

Fig. 2 Correlation of immunostaining for versican and chondroitin sulfate (CS) in node-negative, primary breast cancer. Concentration mean integrated absorbance (MIOD) expressed in density units/pixel. Spearman rho analysis; $r = 0.334$, $P = 0.002$.

Table 3 Relationship of versican, tenascin, and hyaluronan (HA) expression with clinicopathological parameters in node negative primary breast cancer (χ^2 test)

	Versican		Tenascin		HA in stroma			HA in cancer cells	
Variable	<13	≥13	<19.4	≥19.4	Weak	Moderate	Strong	Negative	Positive
Tumor size									
<20	41	5	27	16	8	10	25	8	35
≥20	34	5	13	25	2	7	27	6	30
	$P = 1.00$		$P = 0.014$[a]			$P = 0.16$		$P = 1.00$	
Grade									
I	12	1	11	2	6	2	4	5	7
II	28	5	17	14	3	8	18	5	24
III	35	4	12	25	1	6	30	3	34
	$P = 0.72$		$P = 0.004$[a]			$P = 0.0001$[a]		$P = 0.025$[a]	
Stage									
1	40	5	27	15	8	10	24	8	34
2a	34	5	12	26	2	7	27	5	31
2b	1	0	1	0	0	0	1	1	0
	$P = 0.91$		$P = 0.008$[a]			$P = 0.352$		$P = 0.080$	
Estrogen receptor status									
Negative	31	1	13	18	3	7	10	4	26
Positive	44	9	27	23	7	10	32	10	39
	$P = 0.082$		$P = 0.36$			$P = 0.84$		$P = 0.549$	
Progesterone receptor status									
Negative	41	1	17	23	3	7	29	5	34
Positive	34	9	23	18	7	10	23	9	31
	$P = 0.015$[a]		$P = 0.27$			$P = 0.245$		$P = 0.378$	

[a] Statistically significant at $P < 0.05$.

Because tumor size and versican concentration were independent variables in Cox multivariate analysis, combined scores for these variables were compared between patient subgroups in Kaplan-Meier plots to determine whether a more robust prediction of the rate of relapse for patients with node-negative, primary breast cancer could be achieved. Fig. 4 illustrates that patients with small tumors and low versican concentration experienced fewer relapses than patients with large tumors and low versican level. In contrast, patients with high versican concentration, irrespective of tumor size, experienced a high rate of relapse (relapse-free survival at 5 years, 82 *versus* 64 *versus* 35% for the groups of patients with low versican and small tumors, low versican and large tumors, and high versican irrespective of tumor size, respectively).

DISCUSSION

A diagnosis of node-negative primary breast cancer segregates patients into a category of low risk of relapse and death but uncertainty of individual outcome remains. Although surgery is curative for the majority of women in this category, ~30% of patients will relapse with progressive disease. Currently, there is no reliable means to predict those patients who are likely to relapse and would potentially benefit from more aggressive treatment at diagnosis. We recently reported that high levels of versican in peritumoral stromal tissue predicted relapse in a cohort of women with node-negative, primary breast cancer (1). Versican is a recognized modulator of cellular adhesion and motility for mesenchymal cells such as fibroblasts, smooth muscle, and neural cells (17), and increased expression of versican in their malignant derivatives, *e.g.*, melanoma and astrocytoma, appears to contribute to a more aggressive phenotype (18, 19). There have been few studies, however, regarding the presence of versican and the level of tumor aggression for adenocarcinomas of tissues such as breast, prostate, and colon (1, 20).

Versican is a CS-proteoglycan and can bind specifically to other matrix molecules, principally HA and tenascin, leading to structural aggregations of matrix. CS proteoglycans, HA and tenascin, have been individually implicated in modulating cellular adhesion, and several reports suggest that these matrix components colocalize within the neoplastic stroma of tumors and the mesenchyme in embryonic tissues (5, 21–23). This suggests that these molecules may act in concert to achieve modulation of cellular attachment and motility. The results of our study indicate that within the putative matrix complex, the concentration of versican is related to the concentration of CS but not to the concentrations of tenascin and HA. The correlation between versican and CS suggests that versican is the predominant CS-proteoglycan present in the peritumoral stromal tissue of breast cancer. The variability in the ratio of versican to tenascin and HA concentrations between individual patients suggests that the putative matrix complex in peritumoral stroma is not assembled in a fixed ratio of composition. Reinforcing this structural variability, no uniform association is observed between the matrix components and the clinicopathological features of the individual carcinomas. Increased tenascin levels are related to increased tumor size, higher grade, and stage, all accepted features of more aggressive carcinomas. Increased versican is independent of these features. The association of tenascin expression with a number of features of tumor aggression is consistent with earlier studies (24–26). In agreement with other studies, HA (stromal and cancer cell associated) is related only to grade (8).

An examination of the predictive power of the individual matrix components by Cox regression analysis indicated that

Table 4 Cox regression analyses for disease-free survival and overall survival

A. Univariate analysis

Variable	Disease-free survival			Overall survival		
	Relative risk	95% confidence interval	*P*	Relative risk	95% confidence interval	*P*
Tumor size ($n = 86$)[a]	2.27	1.06–4.87	0.036[b]	2.67	1.00–7.10	0.050[b]
Grade						
Grade I ($n = 13$)	1.00					
Grade II ($n = 34$)	0.44	0.10–1.97	0.282			
Grade III ($n = 39$)	1.49	0.69–3.23	0.314			
Estrogen receptor status ($n = 86$)[c]	1.34	0.60–2.99	0.472	0.744	0.29–1.92	0.541
Progesterone receptor status ($n = 86$)[d]	1.88	0.87–4.09	0.111	1.14	0.45–2.89	0.786
Versican ($n = 85$)[e]	1.08	1.00–1.16	0.047[b]	1.06	0.96–1.17	0.255
Versican ($n = 85$)[f]	3.12	1.37–7.11	0.007[b]			
Chondroitin sulfate ($n = 85$)[g]	1.07	0.97–1.19	0.168	1.09	0.95–1.24	0.206
Tenascin ($n = 81$)[h]	1.04	0.99–1.09	0.128	1.07	1.00–1.14	0.024[b]
Tenascin ($n = 81$)[i]				3.80	1.25–11.55	0.019[b]
Hyaluronan in stroma						
Weak ($n = 10$)	1.00			1.00		
Moderate ($n = 17$)	1.21	0.30–4.88	0.788	0.895	0.15–5.36	0.904
Strong ($n = 52$)	1.01	0.29–3.50	0.986	0.945	0.20–4.37	0.942
HA in cancer cells ($n = 79$)	0.948	0.36–2.53	0.916	0.936	0.26–3.32	0.919

B. Multivariate analysis of predictors significant by univariate analysis

Variable	Disease-free survival ($n = 85$)			Overall survival ($n = 81$)		
	Relative risk	95% confidence interval	*P*	Relative risk	95% confidence interval	*P*
Tumor size ($n = 85$)[a]	2.34	1.09–5.08	0.030[b]	1.96	0.72–5.33	0.187
Versican[c]	3.36	1.46–7.73	0.004[b]			
Tenascin[d]				3.28	1.06–10.18	0.040[b]

[a] Tumor size (mm) as a dichotomous variable cut point <20 *versus* ≥ 20.
[b] Statistically significant at $P < 0.05$.
[c] ER status (fmol/mg protein) as a dichotomous variable cut point <10 *versus* ≥ 10.
[d] PR status (fmol/mg protein) as a dichotomous variable cut point <10 *versus* ≥ 10.
[e] Versican immunostaining [mean integrated absorbance (MIOD) units] as continuous variable.
[f] Versican immunostaining (MIOD units) as dichotomous variable, cut point <13 *versus* ≥ 13.
[g] Chondroitin sulfate immunostaining (MIOD units) as continuous variable.
[h] Tenascin immunostaining (MIOD units) as continuous variable.
[i] Tenascin immunostaining (MIOD units) as continuous variable, cut point <19.4 *versus* ≥ 19.4.

tumor size predicts both risk of relapse and risk of death, whereas versican and tenascin content of the peritumoral stromal tissue predicts only risk of relapse and risk of death, respectively. Neither CS nor HA are predictive of outcome in this cohort of node-negative, primary breast cancer. In agreement with this study, HA expression was not predictive of outcome for the subgroup of node-negative women[7] within a previously published cohort of women with breast cancer (8). An important finding of this study is that not only is versican concentration independent of tumor size (χ^2), but versican concentration and tumor size are independent predictors of outcome (Cox multivariate analysis) for patients with node-negative breast cancer. Because of this independence, a robust prediction of outcome can be achieved by combination of tumor size and versican concentration in Kaplan-Meier analysis. Patients with high peritumoral versican concentration experience the highest rate of relapse irrespective of the tumor size (>75% at 5 years). Patients with low versican concentration and small tumors experience the lowest relapse rate (<20% at 5 years).

The scatter of points in the Spearman plot for women with node-negative breast cancer (Fig. 2, $r = 0.334$) denoting the ratio of concentration of versican core protein to CS side chains, when compared with the same ratio in men with early stage prostate cancer ($r = 0.742$; Ref. 20), suggests a greater variability in CS proteoglycan type and/or glycosylation level in breast cancer. In support of this observation, the level of CS in peritumoral stroma in breast cancer is unrelated to risk and rate of relapse, whereas there is a highly significant association in prostate cancer (27, 28). This suggests that there may be a fundamental difference in the expression of CS proteoglycan types between breast and prostate cancers. One prime example of this difference in expression is that unlike prostate cancer cells, 12% of breast cancer specimens contain CS-immunoreactive cancer cells.

This study suggests that within the putative matrix complex of versican, tenascin, and HA in peritumoral stromal tissue, versican alone predicts risk of relapse and may be the principal component responsible for cancer cell metastasis in node-negative, primary

[7] R. Tammi, unpublished data.

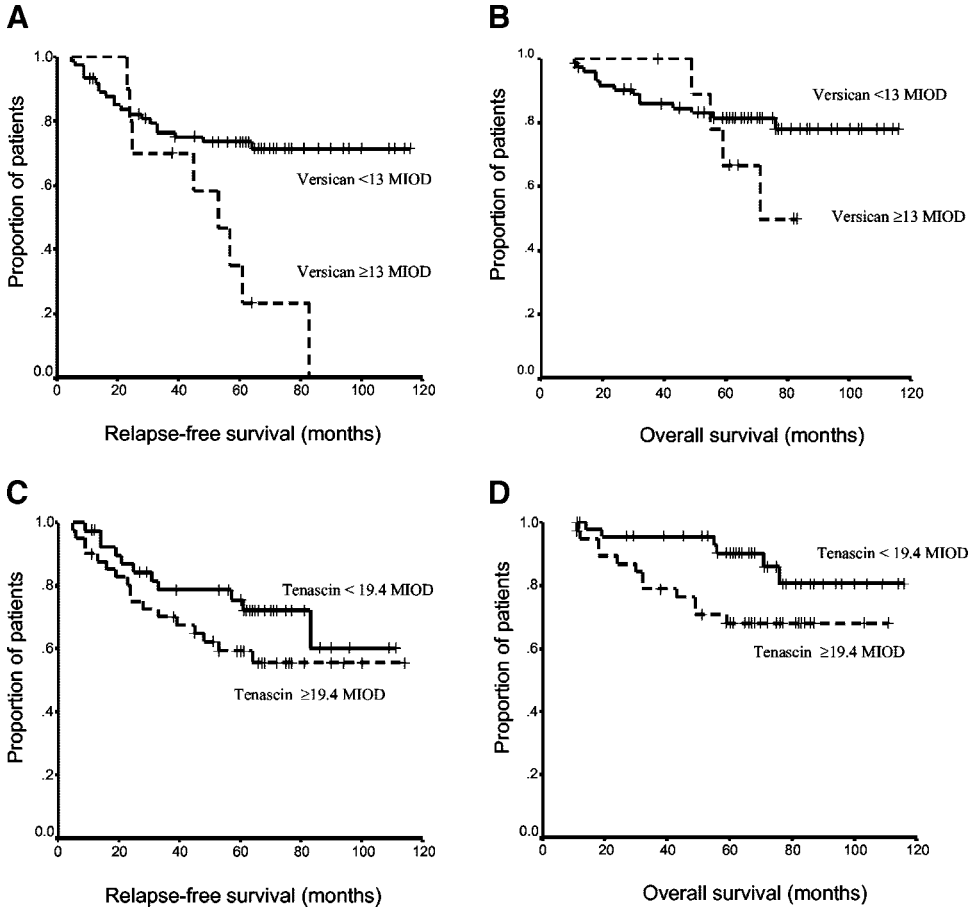

Fig. 3 Kaplan-Meier plots for relapse-free survival and overall survival in node-negative breast cancer patients. *A*, relapse-free survival, variable stromal versican concentration, $n = 85$, cut point mean integrated absorbance (MIOD) $<$ 13 *versus* $\geq$ 13 density units/pixel, number of relapses 20 of 75 (26.7%) *versus* 8 of 10 (80%), log-rank statistic, 8.22; $P = 0.0041$. *B*, overall survival, variable stromal versican concentration, $n = 85$, cut point $<$ 13 *versus* $\geq$ 13 density units/pixel; number of relapses, 14 of 75 (18.7%) *versus* 4 of 10 (40%), log-rank statistic 1.43; $P = 0.233$. *C*, relapse-free survival, variable stromal tenascin concentration, $n = 81$, cut point $<$ 19.4 *versus* $\geq$ 19.4 density units/pixel; number of relapses, 11 of 48 (27.5%) *versus* 17 of 41 (41.5), log-rank statistic 1.75; $P = 0.186$. *D*, overall survival, variable stromal tenascin concentration, $n = 81$, cut point MIOD $<$ 19.4 *versus* $\geq$ 19.4 density units/pixel; number of relapses, 4 of 40 (10%) *versus* 14 of 41 (34.1%), log-rank statistic 6.44; $P = 0.0112$.

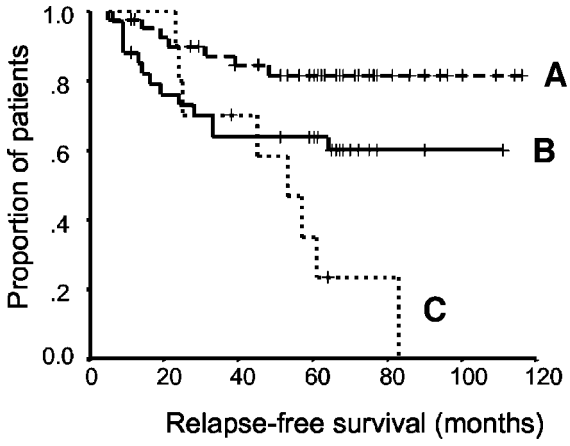

Fig. 4 Kaplan-Meier plots for relapse-free survival in node-negative breast cancer patients, according to tumor size and versican concentration. $n = 85$, cut points tumor size $<$ 20 *versus* $\geq$ 20 mm, stromal versican concentration, mean integrated absorbance (MIOD) $<$ 13 *versus* $\geq$ 13 density units/pixel. Patient groups were as follows: *A*, tumor size $<$ 20 mm and versican concentration $<$ 13 density units/pixel, number of relapses 7 of 41 (17%). *B*, tumor size $\geq$ 20 mm and versican concentration $<$ 13 density units/pixel, number of relapses 13 of 34 (38%). *C*, versican concentration $\geq$ 13 density units/pixel and tumor size $<$ 20 or $\geq$ 20 mm, number of relapses 8 of 10 (80%). Log-rank statistic 11.76; $P = 0.0028$.

breast cancer. The biological role of HA in breast cancer appears less clear. Although HA appears not to be associated with disease outcome in node-negative breast cancer, this molecule is associated with outcome for unselected primary breast cancer patients (8). Moreover, HA is clearly involved in cellular motility by virtue of its ability to form a pericellular sheath in combination with aggregating proteoglycans such as versican and aggrecan (12). Although the sheath is anchored by HA-receptors such as CD44, which have themselves been associated with outcome in breast cancer (29), the existence of sheath relies on its aggregating proteoglycan content (30).[8] To clarify the independent roles of HA and versican, *in vitro* studies need to be performed to determine whether versican promotes motility and invasion of breast cancer cells in the absence of a pericellular sheath of HA.

The biological role of tenascin in breast cancer also remains unclear despite its reported antiadhesive activity, which would favor cell motility and growth promotion (10). Tenascin expression has been associated with cell proliferation and may represent a strong marker for local and distant recurrence (31). In addition, a series of reports suggest that tenascin expression may be a suitable

[8] C. Ricciardelli unpublished data.

marker for predicting the invasive potential of premalignant breast lesions, including ductal carcinoma *in situ* (9, 32, 33).

In conclusion, this study has shown that although the ECM components versican, tenascin, and HA appear to be colocated within a putative complex with anticellular adhesive properties, the ratio of versican content to the other matrix components varies between individual tumors. Within this complex, versican appears to be the sole predictor for risk and rate of relapse, independent of tumor size, and this would suggest that this molecule has a primary role in breast cancer spread for patients with node negative disease. Tenascin, on the other hand, is related to a number of features associated with tumor aggression and is associated only with risk and rate of death. Its role in progression of breast cancer remains unclear. HA appears from this study to be unrelated to progression of node-negative breast cancer, despite the reported necessity of the versican-cross-linked pericellular sheath of HA for cell motility. Further study is required to determine how these molecules cooperate to control cancer cell metastasis.

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