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Thrombospondin-1 expression in breast cancer: prognostic significance and association with p53 alterations, tumour angiogenesis and extracellular matrix components

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Summary. Thrombospondin (TSP-1) is a 450-kd adhesive glycoprotein that was initially discovered in platelets and subsequently in a variety of cell types. Several reports suggest that TSP-1 possesses tumour suppressor function, through its ability to inhibit tumour neovascularization. In this study we investigated tissue sections from 124 breast carcinomas for the immunohistochemical expression of TSP-1 protein and its relationship to several clinicopathological parameters. The possible relationship to hormone receptors content, p53 protein, proliferation associated indices, angiogenesis, VEGF expression and extracellular matrix components (tenascin, fibronectin, laminin, collagen type IV and syndecan-1) was also estimated. TSP-1 was detected in the perivascular tissue, at the epithelial-stromal junction, in the stroma and in the tumour cells. High tumour cell TSP-1 expression was observed in 9.7%, moderate in 17.7%, mild in 10.5%, while 62.1% of the cases were negative for TSP-1 expression. The survival analysis showed an increased risk of recurrence associated with low TSP-1 tumour cell expression. High stromal TSP-1 expression was observed in 3.2% of the cases, moderate in 3.3%, mild in 27.4%, while 63.6% of the cases showed absence of TSP-1 expression. This expression was higher in invasive lobular type of breast cancer and inversely correlated with the lymph node involvement and the estrogen receptor content. Stromal TSP-1 expression was also positively correlated with extracellular matrix components expression, tenascin, fibronectin, collagen type IV, laminin, and syndecan-1. The relationship of TSP-1 expression with tumor angiogenesis, growth fraction and p53 protein expression was not significant. Our data suggest that TSP-1 expression seems to be associated with favorable biological behavior and may have clinical value in terms of predicting the risk of recurrence. In addition, TSP-1 might not be a direct anti-angiogenic factor, although it seems to be implicated in the remodeling of breast cancer tissue through interaction with other extracellular matrix components.

Key words: Thrombospondin-1, Breast cancer, Extracellular matrix, Angiogenesis, p53

Introduction

Thrombospondin-1 (TSP-1) is a 450a trimeric glycoprotein synthesized and incorporated into the extracellular matrix by a wide variety of epithelial and mesenchymal cells. TSP-1 is a multi-functional protein known involved in a variety of processes, including cell adhesion, cell migration, and angiogenesis (Qian and Tuszynski, 1996; Chen et al., 2000).

Angiogenesis is a multistep process, which involves changes in the extracellular matrix, and endothelial cell proliferation, migration, and differentiation into capillaries. Studies of tumour biology reveal a complex

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Abreviations: TSP-1, thrombospondin-1; MVD, microvessel density; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; TN, tenascin; FN, fibronectin; Coll, collagen type IV; LN, laminin; Synd-1, syndecan-1.

network of autocrine and paracrine interactions between tumour cells, stromal cells, and endothelial cells, which are in turn influenced by the composition of the extracellular matrix. The development of new blood vessels within a tumour depends on the local balance between angiogenic and anti-angiogenic factors. These factors may be produced by the tumour cells themselves or by the associated stromal and inflammatory cells. Thrombospondin-1 was the first naturally occurring inhibitor of angiogenesis identified. The pathways controlling the switch to an angiogenic phenotype in tumours are complex and poorly characterized. There is evidence that changes in oncogene and tumour suppressor gene expression influence new vessel growth during tumor progression (Rak and Klement 2000). There is a close relationship between the expression of TSP-1 and wild-type p53, loss of wild-type p53 being associated with loss of TSP-1 expression (Dameron et al., 1994). It has been shown that p53 status, especially p53 mutations determined by p53 gene sequence data, seems to correlate with VEGF expression (Linderholm et al., 2001, 2004) but not with TSP-1 expression (Linderholm et al., 2004). TSP-1 expression has been studied in benign and malignant breast tissue (Pratt et al., 1989; Wong et al., 1992; Clezardin et al., 1993; Tuszynski and Nicosia 1994; Serre et al., 1995; Bertin et al., 1997; Steward et al., 1998; Rice et al., 2002; Tokyol et al., 2009) with conflicting results, especially in the field of angiogenesis and tumour progression, due to the complexity of the TSP-1 molecule.

The aims of our study were: (a) to study the patterns of TSP-1 protein expression in tumour cells and tumour stroma in a series of 124 breast cancers, in correlation with clinicopathological features such as, tumour type, grade of differentiation, tumour size, lymph node involvement, (b) to evaluate the relationship between TSP-1 expression and other biological parameters including steroid receptor content (estrogen receptor-ER, progesterone receptor-PgR), proliferation associated indices (Ki-67, PCNA) and p53 protein, (c) to study the relationship between tumour cells and stromal TSP-1 expression and angiogenesis related factors, microvessel density (MVD) assessed by CD34 immunostaining and vascular endothelial growth factor (VEGF) expression and d) the correlation of its expression with the extracellular matrix components (ECM), tenascin (TN), fibronectin FN), collagen type IV (Coll), laminin (LN) and syndecan-1 (synd-1).

Materials and methods

Patients and study design

A cohort of 124 patients with primary invasive breast carcinoma treated by surgical resection were investigated. For 98 of patients we had complete follow up data and this number of patients was included in survival analysis. All patients had a mastectomy with axillary lymph node dissection performed as indicated and were followed up regularly at the Medical Oncology Department of University Hospital of Ioannina. Detailed clinical data were available for 98 patients: 24 had stage I disease, 53 patients stage II disease (pT1N1M0, pT2N0M0 and pT2N1M0) and 21 patients stage III (pT2N2M0, pT3N1M0 and pT3N2M0 or pT4N1M0). They were also clinically disease free and had baseline CA 15-3 serum levels below 30 U/ml at the initiation of adjuvant therapy. Adjuvant therapies were administrated according to standard guidelines and consisted in tamoxifen (38 patients), chemotherapy followed by tamoxifen (32 patients) and conventional chemotherapy (28 patients). After a median follow-up of 4 years (range 6-132 months), in 41.8% (41/98) of the patients the disease had progressed, and 34 of them had developed distant metastases (Table 1).

Archived material was used from formalin fixed and paraffin embedded breast carcinoma tissue, including adjacent non neoplastic tissue or fibrocystic disease. Each specimen was examined histologically on hematoxylin-eosin(H and E) stained slides. Tumour size varied from 1 to 17 cm (median=3.95cm) and graded as: ≤2), 2-5, >5. Tumour histotype, lymph node status and patient age were recorded for each patient. Tumour grade was assessed on H and E stained sections blinded to the results of immunohistochemistry. Tubule formation, nuclear morphology and mitotic rate were evaluated and scored in the neoplastic cells according to the Elston and Ellis modification of the Bloom and Richardson system: grade 1, grade 2 and grade 3 corresponding to well, moderately and poorly differentiated invasive carcinoma of the breast (Elston and Ellis, 1991).

Immunochistochemistry

Immunochistochemistry was performed on one or two selected paraffin blocks, from each case on 4Ìm tissue sections placed on poly-L-lysine-coated glass slides. In brief, tissue sections were deparaffinized in

Table 1. Clinical data, simple statistics.

	alive		recurrence		resistance	
	No	Yes	No	Yes	No	Yes
Type						
ductal	17	43	38	34	14	17
lobular	4	13	12	6	3	4
mixed	5	8	6	9	2	4
Size						
<2cm	3	9	8	6	1	5
2-5cm	13	39	30	29	14	12
>5cm	8	10	12	12	3	7
Grade						
1	2	8	9	3	1	5
2	8	35	26	23	10	9
3	15	18	18	22	8	11
Lymph node						
negative	1	24	24	6	3	7
positive	21	31	24	35	11	16

xylene and rehydrated. For the detection of syndecan-1 and p53, slides were immersed in citrate buffer (0.1m, pH 0.6) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. For the detection of TN, FN, Coll and LN, slides were pretreated with 1 μl/ml pronase (DAKO) for 10 min at room temperature. Subsequently, all sections were treated for 30min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. We used the method involving the avidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To access the specificity of the reaction, negative controls were included, where tumour sections were not incubated with the primary antibody. The antibody sources and dilutions are shown in Table 2.

Immunohistochemical evaluation

To evaluate the expression of Thrombospondin-1 and VEGF proteins we established a combined score, corresponding to the sum of both (a) staining intensity (0=negative, 1=weak, 2=intermediate, 3=strong, 4=very strong staining) and (b) extent of the staining, percentage of positive cells (0=0%, 1=1-25%, 2=26-50%, 3=50-75%, 4≥75%). The sum of both qualitative and quantitative immunostaining reached a maximum score of 8. The combined scores were then divided into 4 main groups: (-) = no immunostaining, score 0, (+) = weak immunostaining, scores 1-2, (++) = moderate immunostaining, scores 3-4, (+++) = strong immunostaining, scores 5-8.

In regard to the evaluation of extracellular matrix components, the tumours were classified as `positive' when there was unequivocal immunostaining of the matrix components in at least one representative area of the tumour. The positive tumours were semi-quantitatively scored as +, ++, and +++ corresponding to weak, moderate and extensive immunoreactivity respectively. The immunohistochemical evaluation of each protein studied was based both on the reports in literature and on our own experience (Poller et al., 1993; Ioachim et al., 2002; Tsanou et al., 2004).

The stromal positive cells (stromal fibroblasts and macrophages) were also estimated also semi-quantitatively and scored as +, ++, +++ corresponding to weak, moderate and severe.

All slides were reviewed and scored in a blind test by two Pathologists. Differences in interpretation were reconciled by re-review of slides separately or jointly at a double-headed microscope. The immunostaining was assessed from numerically coded slides without any knowledge of survival or other clinical data.

Microvessel count

The criteria that we used for microvessel recognition were the same as used in previous studies. Briefly, as microvessels we considered individual or clusters of cells with or without lumens, positively stained by anti-CD34. The lumen diameter had to be smaller than approximately eight red blood cells. Areas of fibrosis, necrosis and inflammation, and vessels with muscle wall were excluded from counting. In each case, the three areas with the highest vascularization ("hot spot") were selected. Individual microvessel counts were then made on a 250x field (25x objective and 10x ocular, corresponding to an area of 0.363 mm^2) by two independent observers. The average count from the two observers was used as the final score. A microvessel count <10 was considered as low MVD and >10 as high MVD.

Statistics

Superior Performance Software System (SPSS) software 10.0 for windows (SPSS Inc., 1989-1999) was used by the authors to compare morphological features and protein expression data. Significant differences between the expression of the target proteins with regard to clinicopathological parameters were computed by the t-test for paired or non-paired values or ANOVA test if the data were normally distributed. If the data did not show a normal distribution, differences were analysed by the Wilcoxon signed ranks test for paired values or the Mann-Whitney U test and the Kruskall-Wallis H test for independent values. Correlation between protein expression was computed using the Pearson's correlation coefficient for normally distributed data or the Kendall's Tau rank correlation coefficient where the data did not show a normal distribution. The prognostic significance of thrombospondin-1 expression, in determining the risk for recurrence, was studied with both univariate (log rank test) and multivariate (Cox proportional hazards) ways of analysis, separately for each group of patients. The same analysis was employed for the overall survival of patients. P-values ≤0.05 were considered statistically significant.

Table 2. Antibodies used.

Antibodies	Supplier	Dilution	Incubation time
TSP-1(A6.1)*	DBS	1:30	30 min
VEGF(JH121)*	Neomarker	1:50	1 hour
CD34(QBEnd/10)	Novo Castra	1:50	1 hour
Tenascin (TN2)#	Dako	1: 50	1 hour
Fibronectin (clone, 568)#	Novo Castra	1: 100	1 hour
Collagen IV (clone, CIV22)#	Dako	1: 50	1 hour
Laminin (An No 078P)#	Menarin	1: 1000	1 hour
Syndecan-1 (DL-101)*	Santa Cruz	1 :50	Overnight
P53 (DO-7)*	Dako	1: 50	1 hour
ER (M7047)	Dako	1:50	1 hour
PgR (M3569)	Dako	1:75	1 hour

*: with microwave oven antigen retrieval; #: incubation with pronase

Results

Thrombospondin-1 expression was detected in a band-like perivascular tissue, in epithelial-stromal junction (usually in the in situ component), in the stroma (Fig.1) and in tumour cells (Fig. 2).

In *tumour cells*, low TSP-1 expression was observed in 13/124 (10.5%), moderate in 22/124 (17.7%) and high in 12/124 (9.7%) of the cases, while 77/124 (62.1%) were negative for TSP-1. Low tumour cell thrombospondin-1 expression was correlated with survival after first documented relapse (p=0.015) (Fig. 3).

In *tumour stroma*, low TSP-1 expression was detected in 34/124 (27.4%) of the cases, moderate in 21/124 (16.9%), high in 4/124 (3.2%) while 65/124 (52.4%) showed absence of TSP-1 expression. A higher stromal TSP-1 expression was observed in lobular type compared to the ductal type (p<0.0001). TSP-1 expression was inversely correlated with lymph node involvement (p=0.02) and estrogen receptor status (p=0.04) (Table 3).

A positive relationship of stromal TSP-1 expression with the extracellular matrix components TN (p=0.03), FN (p=0.03), coll (p=0.004), LN (p=0.0001) and Synd (p=0.001) was also observed (Table 4).

The relationship of thrombospondin-1 expression (both tumour and stromal) with proliferation associated indices, MVD, VEGF expression and p53 protein status was not significant. A positive relationship of VEGF expression with TN (p=0.011), FN (p=0.042) and LN (p=0.006) was found. In addition, the p53 protein status analyzed by immunohistochemistry was positively correlated with VEGF protein expression (Kendall's tau-b and Spearman bivariable correlation p=0.014 and p=0.016 respectivelly). The relationship between MVD and extracellular matrix components was insignificant.

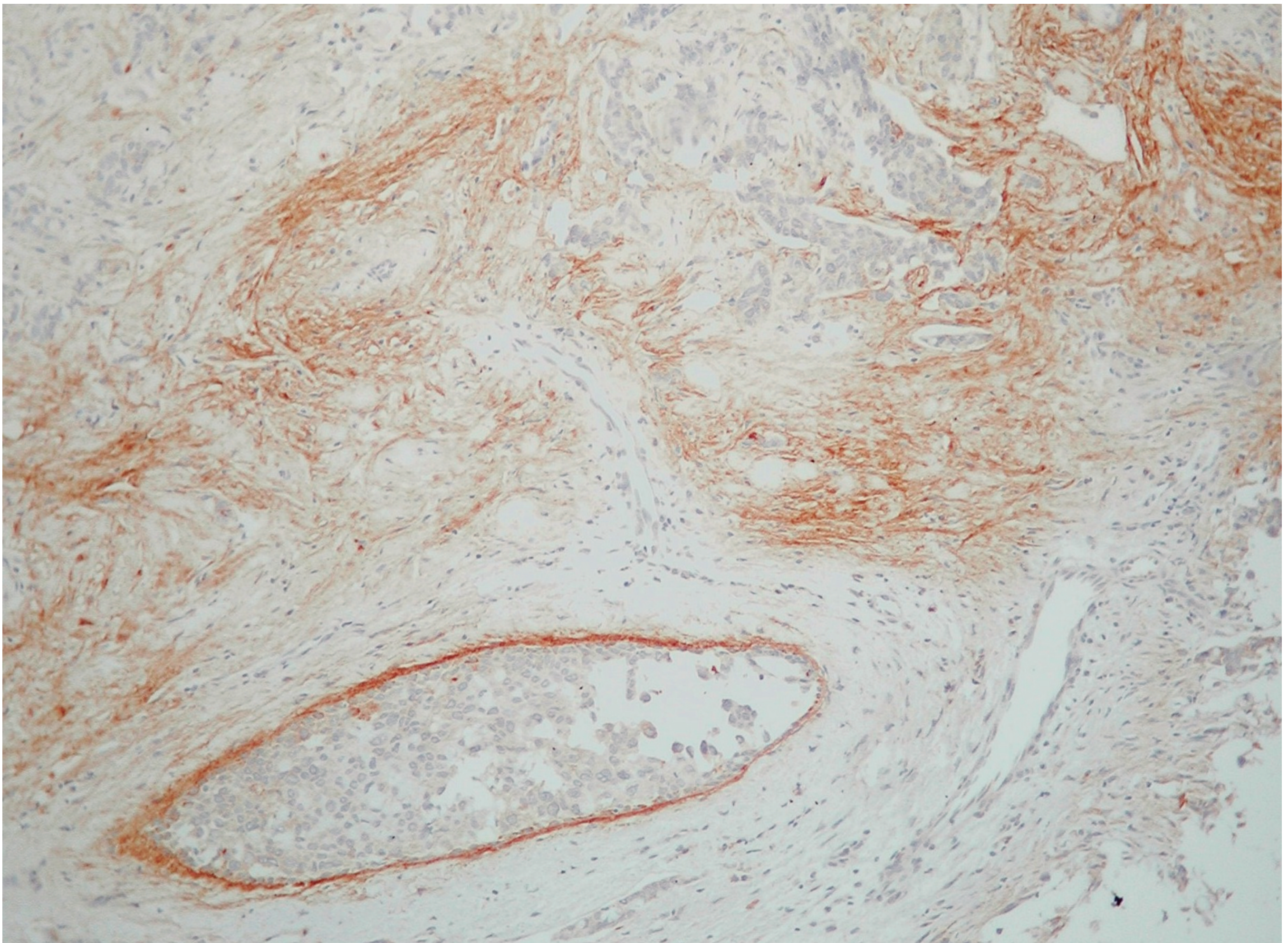

Fig. 1. Tumour cells TSP-1 expression in a case of infiltrating breast carcinoma. x 200

Discussion

TSP-1 protein expression and TSP-1mRNA are highly expressed in breast carcinomas, while benign lesions and normal breast show absent or low levels of TSP-1 (Pratt et al., 1989; Wong et al., 1992; Clezardin et al., 1993; Bertin et al., 1997) according to the results of the present study. We also found higher TSP-1 expression in lobular type of invasive breast carcinomas compared to the ductal type. In previous immunohistochemical studies it has been found that TSP-1 was distributed differently in invasive ductal and in lobular breast carcinoma, suggesting that these distribution patterns may reflect biological differences between the two types of breast carcinomas (Pratt et al., 1989; Serre et al., 1995). In a recent study it has been found that expression of stromal TSP-1 is lost in ductal carcinoma in situ with more aggressive histological feature (Rice et al., 2002). On the basis of anti-adhesive and haptotatic properties of TSP (Simantov et al., 2001; Taraboletti et al., 1987) a role has been proposed its role during tumor cell dissemination in breast cancer and in particular the diffuse invasive behaviour characteristic of lobular carcinoma cells (Serre et al., 1995).

The role of TSP-1 in tumour metastasis is controversial as TSP-1 has been proposed to have both pro-metastatic and anti-metastatic properties. TSP could be involved at several points in the metastatic process through potential interactions of malignant cells with extracellular matrix, endothelium or circulating platelets (Asch et al., 1987; Tokyol et al., 2009). It has been found that TSP-1 in the mammary tumor microenvironment inhibits angiogenesis and tumor growth, but promotes metastasis to the lung (Yee et al., 2009). In addition, an

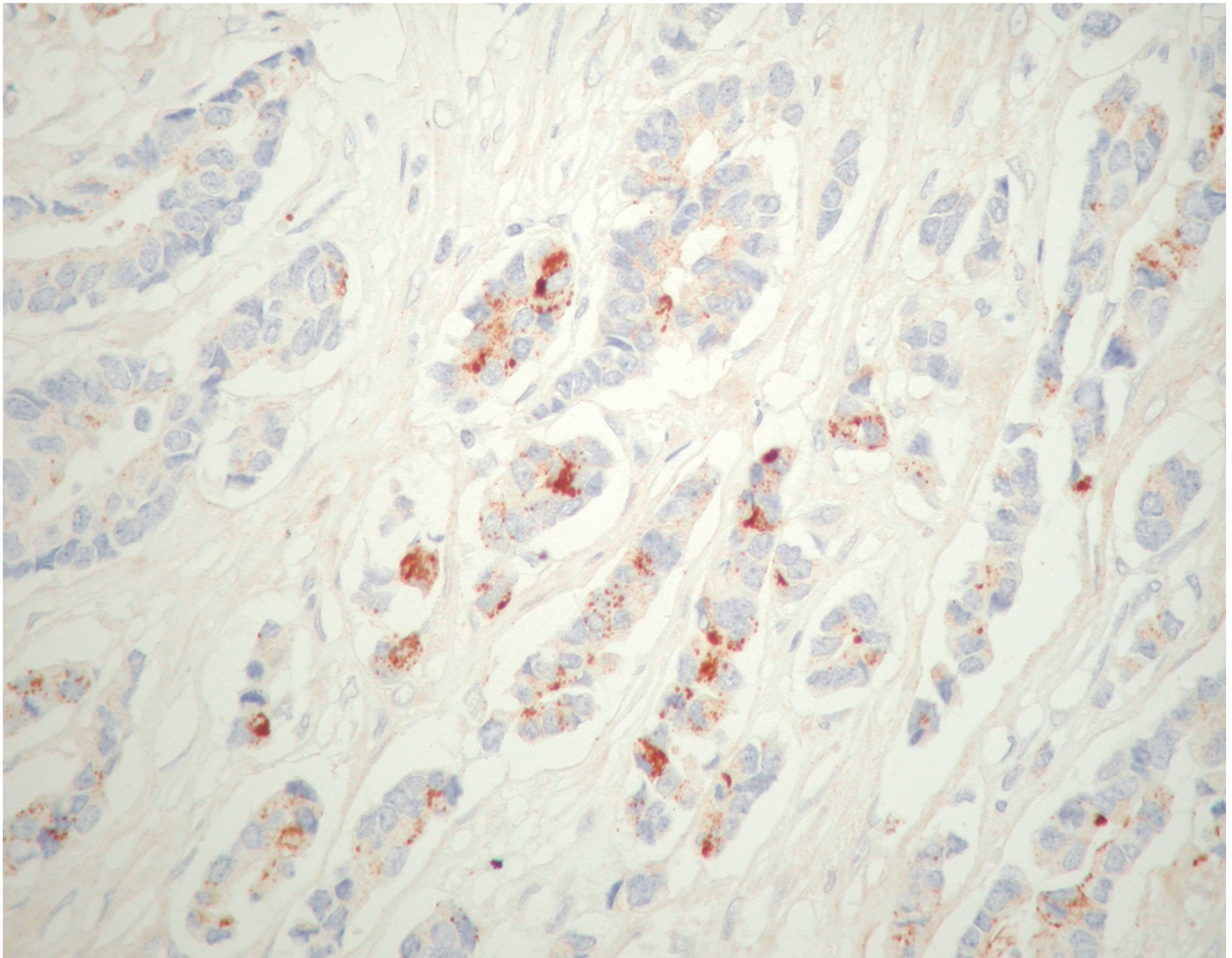

Fig. 2. Stromal and a band-like epithelial-stromal junction TSP-1 expression in a case of breast carcinoma. x 100

inverse correlation between TSP-1mRNA and protein expression, has also been reported and malignant progression in murine melanoma and human lung and breast cancer cell lines (Zabrenetzky et al., 1994). In the present study low TSP-1 stromal protein expression was correlated with lymph node involvement supporting its inhibitory role in cancer metastasis. However, other studies did not show any correlation of TSP-1 expression and lymph node status in breast cancer (Bertin et al., 1997; Wang-Rodriquez et al., 2003).

It has been shown that TSP-1 production is directly controlled by estrogens in ER-positive breast cancer cells lines (Hyder et al., 2009). In the current study we found an inverse correlation of stromal TSP-1 expression with estrogen receptor status, in contrast to the findings of other investigators (Bertin et al., 1997; Tokyol et al., 2009).

Although TSP-1 has been found to be implicated in the regulation of angiogenesis, its specific role in this process is not clear, as both stimulatory and inhibitory effects have been demonstrated in animal models (BenEzra et al., 1993; Simantov et al., 2001).

The TSP-1 molecule has multiple functional

Table 3. Stromal TSP-1 expression in correlation with clinico-pathological data in breast cancer.

	TSP-1 expression				
	0	+	++	+++	p value
Type					
ductal	30	22	20	4	p<0.0001
lobular	20	2	1		
mixed	10	17			
Size					
<2cm	7	6	2		
2-5cm	38	13	14	3	NS
>5cm	12	10	4	1	
Grade					
1	7	2	4		
2	32	11	10	2	NS
3	20	16	6	2	
Lymph node					
negative	13	9	10	2	p=0.02
positive	38	18	7	2	
ER					
negative	8	16	5		p=0.04
positive	39	10	11	3	
PgR					
negative	13	15	6	1	NS
positive	32	9	9	2	
In situ					
no	7	4	1		NS
yes	28	17	5	3	
Ki-67					
<10%	43	17	13	3	NS
>10%	15	14	7	1	
PCNA					
<50%	7	7	3	1	NS
>50%	51	24	15	2	
P53					
<5%	32	16	12		NS
>5%	28	15	9	4	
MVD					
low	36	22	13	3	NS
high	17	8	3	1	
VEGF					
<25%	7	7	3	1	NS
>25%	51	24	15	2	

Table 4. Correlation of stromal thrombospondin-1 expression with extracellular matrix components in breast carcinomas.

	Thrombospondin-1 expression		
	- , +	++, +++	p value
Tenascin			
-/+	45	8	p=0.03
++, +++	30	8	
Fibronectin			
-/+	53	7	p=0.03
++,+++	24	7	
Collagen			
-	45	6	p=0.004
+, ++	36	14	
Laminin			
-	68	12	p=0.0001
+, ++	17	7	
Syndecan-1			
+	28	5	p=0.001
++	19	7	
+++	13	10	

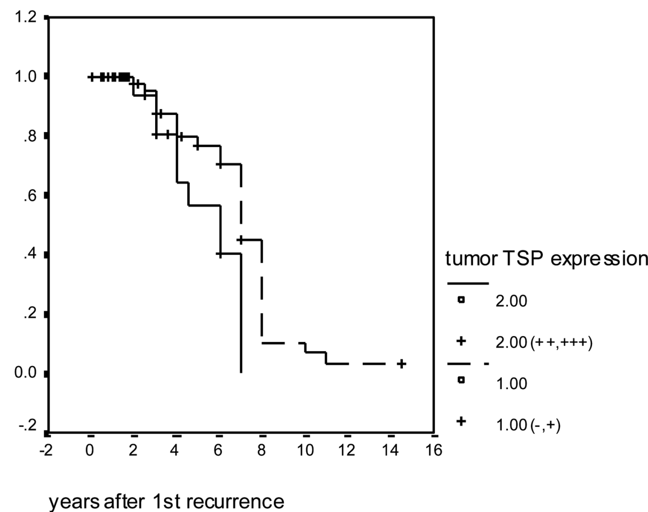

Fig. 3. Kaplan-Meier curves according to TSP-1 staining. Patients with low TSP-1 expression recurrence earlier compared with higher TSP-1 expression (p=0.015).

domains and appears to have a complex role in angiogenesis. Although TSP-1 is reported to have an anti-angiogenic effect in breast cancer cell lines (Volpert et al., 1995), studies of TSP-1 expression in tissue sections from invasive breast cancer show both positive and negative associations between TSP-1 and microvessel density (Tuszynski and Nicosia, 1994; Bertin et al., 1997; Steward et al., 1998).

TSP expression has also been linked to the p53 tumor suppressor gene. P53 is a transcriptional activator of the TSP gene, and it has been shown that increased expression of the wild type p53 protein results in increased expression of TSP (Dameron et al., 1994). A significant association between low levels of TSP expression and p53 accumulation has been demonstrated, as well as with microvessel density count, in bladder cancer tissue, supporting the hypothesis that the p53 gene exerts its influence on tumor angiogenesis through the regulation of TSP (Grossfeld et al., 1997). VEGF plays an important role in the biology of breast cancer activating two of the three receptors which are present on endothelial cells (VEGF-1 and VEGF-2). In the present study we found a positive relationship of p53 protein level with VEGF according to the results of other investigators concerning the p53 mutation status (Linderholm et al., 2001, 2004), but no correlation of TSP-1 with p53 protein status or VEGF. The last findings are in line with the results of other investigators (Rice et al., 2002; Gasparini et al., 2001; Linderholm et al., 2004; Toyol et al., 2009) suggesting that TSP-1 is not directly implicated in breast cancer angiogenesis.

Stromal components also play a critical role in the formation of vascular stroma through the regulation of functions such as cell adhesion and migration. In our previous studies (Ioachim et al., 2002; Ioachim, 2008), concerning the expression of ECM components in breast cancer tissue, we confirmed the implication of these proteins in the remodeling of breast cancer tissue and the possible role in tumor progression. In the present study we found a strong relationship of TSP-1 expression with the ECM components. In addition, a co localization of TSP and syndecan in epithelial cells during murine development has been found suggesting that TSP and syndecan may play an important role in cell-cell or cell-matrix interactions during development (Corless et al., 1992).

The ECM components, such as collagens, laminin, fibronectin, tenascin, osteonectin and thrombospondin-1 have been shown to modulate vascular endothelial cell adhesion, migration, proliferation, morphogenesis and responsiveness to angiogenic cytokines (Volpert et al., 1995; Wang-Rodriguez et al., 2003; Wong et al., 1992). The composition of the extracellular matrix may be modified in vivo in several ways. For example, angiogenic factors are known to modulate both the synthesis of matrix proteins as well as the synthesis of certain proteases and their inhibitors (Volpert et al., 1995; Wang-Rodriguez et al., 2003; Wong et al., 1992; Zabrenetzky et al., 1994). Our data provide more information of implication of other pathways in the regulation of TSP-1 and angiogenesis, through the interaction of ECM components. This information may offer other therapeutic anti-angiogenic targeting therapies.

Taking the above information into account, we can suggest that thrombospondin expression, although it does not seem to be a direct anti-angiogenic factor, could be implicated in the remodeling of breast cancer tissue through the interactions of other extracellular matrix components. In addition, its expression seems to be associated with favorable biological behavior and may have clinical value in terms of predicting the risk of recurrence.

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