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Advances in Brief

Thrombospondin-1 and -2 Messenger RNA Expression in Normal, Benign, and Neoplastic Human Breast Tissues: Correlation with Prognostic Factors, Tumor Angiogenesis, and Fibroblastic Desmoplasia[1]

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Abstract

Thrombospondin-1 (TSP1) is a M_r 450,000 extracellular matrix glycoprotein that modulates tumor growth, angiogenesis, and metastasis. Of the five structurally different TSPs described to date, only TSP2 is similar to TSP1 in terms of its molecular architecture, and TSP2 also modulates angiogenesis. Angiogenesis plays a relevant role in the biological aggressiveness of breast cancer, and TSP1 is present in the tumor stroma (termed desmoplasia) of invasive human breast ductal carcinoma not otherwise specified (NOS). The present study was designed to identify and quantify TSP1 and TSP2 mRNAs in normal, benign, and neoplastic human breast tissues using the reverse transcriptase PCR technique. We found that TSP2, like TSP1, was expressed in human breast tissues, and that TSP1 and TSP2 mRNA expression in invasive breast carcinoma NOS was significantly increased compared to that observed in normal and benign tissues. The expression of TSP1 and TSP2 in invasive breast ductal carcinoma NOS did not significantly correlate with any of the prognostic factors studied (tumor size, lymph node status, morphology, and hormone receptor status). However, when our study population was divided according to the quantity of tumor stroma, TSP1 (and possibly TSP2) mRNA expression and microvessel counts in desmoplastic-rich stroma of breast carcinoma NOS were significantly increased compared to those observed in desmoplastic-poor stromata.

Introduction

TSP1[3] is a M_r 450,000 extracellular matrix glycoprotein present in desmoplasia of invasive human breast ductal carcinoma NOS (1–3). Fibroblasts are responsible for TSP1 synthesis in desmoplastic areas of breast carcinomas (2). Although the exact role of TSP1 in breast carcinoma is unknown, fibroblasts are believed to be critically important in malignant growth (4), and it is now well established that TSP1 functions by modulating tumor growth and metastasis (5). Furthermore, TSP1 modulates angiogenesis (5, 6), possibly through an indirect mechanism involving fibroblasts (7, 8). Of the five structurally different TSPs (TSP1, TSP2, TSP3, TSP4, and cartilage oligomeric matrix protein) described to date, only TSP2 is similar to TSP1 in terms of its molecular architecture (9), and TSP2 also modulates angiogenesis (10). Because angiogenesis plays a relevant role in the biological aggressiveness of breast cancer (11), an important question is whether TSP2 is expressed in the tumor stroma together with TSP1 to modulate tumoral neovascularization. TSP1 and TSP2 mRNAs are expressed by cultured fibroblasts (7), suggesting that TSP2, like TSP1 (1–3), could be present in desmoplastic areas of invasive human breast carcinomas. However, antibodies against human TSP2 are not yet available. To overcome this problem, we have used in the present study the reverse transcriptase PCR technique to identify and quantify TSP1 and TSP2 mRNAs in normal, benign, and neoplastic human breast tissues. Our findings indicate that TSP2, like TSP1, is expressed in human breast tissues, and that TSP1 and TSP2 mRNA expression in invasive breast carcinoma NOS is significantly increased compared to that observed in normal and benign tissues. The expression of TSP1 and TSP2 in invasive breast carcinoma NOS does not significantly correlate with any of the prognostic factors studied (tumor size, lymph node status, morphology, and hormone receptor status). However, when our study population is divided according to the quantity of tumor stroma, TSP1 (and possibly TSP2) mRNA expression and microvessel counts in desmoplastic-rich stroma of breast carcinoma NOS are significantly increased compared to those observed in desmoplastic-poor stromata.

Materials and Methods

Breast Tissue. Breast samples were obtained from patients undergoing tumorectomies and systematized mammary exereses. Benign lesions included fibroadenomas (n = 3), cystic disease (n = 1), hamartoma (n = 1), and intraductal papilloma (n = 2). Neoplastic lesions comprised invasive ductal carcinoma NOS (n = 31) and lobular carcinomas (n = 2). Normal breast tissue was obtained from patients (n = 6) undergoing mammoplastic surgery. Tissues were snap frozen in liquid nitrogen-cooled isopentane and subsequently stored in liquid nitrogen until used.

Immunohistochemistry. Formalin-fixed, paraffin-embedded breast tissue sections (5 μm thick) were cut using a microtome (Leitz). Tissue sections were microwave heated (6 min, 750 W) then incubated with anti-CD31 monoclonal antibody 5.6E (Immunotech, Luminy, France) previously diluted at a concentration of 0.1 μg/ml in PBS (pH 7.2) containing 1% (mass/vol) BSA. Negative control experiments were performed by omitting the primary antibody. The remaining part of the procedure was as described previously (2).

Microvessel Scoring. Areas representative of the invasive tumor were selected, and highly vascularized regions ("hot spots") of these invasive tumor areas were identified according to the recommendations for assessing microvessel density using CD31 as a marker of microvessels (11). Microvessel counts were performed in 10 consecutive fields at a high magnification (×400). Each evaluation was performed independently by two examiners. In case of disagreement between examiners, sections were reviewed, and a consensus opinion was obtained.

Construction of RNA Internal Standards for TSP1 and TSP2. Human TSP1 and TSP2 cDNA probes were kindly provided by Dr J. Lawler (Brigham and Women's Hospital, Boston, MA). TSP1 and TSP2 cDNA probes were inserted into the *Eco*RI site of pGEM-2 and pBluescript KS (Promega

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[3] The abbreviations used are: TSP1, TSP2, TSP3, and TSP4, thrombospondin-1, -2, -3, and -4, respectively; NOS, not otherwise specified.

Biotech), respectively. TSP1 and TSP2 constructs were linearized by overnight digestion with *Hin*dIII and *Sma*I, respectively. A TSP1 cDNA standard was performed by deletion with *Pst*I of a 246-bp cDNA fragment. A deletion with *Nla*III of a 70-bp cDNA fragment was performed to obtain the TSP2 cDNA standard. TSP1 and TSP2 cDNA standards were then transcribed with T3 RNA polymerase (Boehringer Mannheim, Meylan, France) to obtain standard RNA sense probes.

Reverse Transcription and PCR. Frozen breast tissue samples were homogenized in liquid nitrogen, and total RNA was extracted by using the RNazol B method (Bioprobe Systems). Reactions were performed with a constant amount of total RNA (1 μg) from breast tissue samples in 20 μl of a buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 5 mM $MgCl_2$] containing 0.1% Triton X-100, decreasing amounts of internal RNA standards, 1 mM of each of the deoxyribonucleoside triphosphates, 0.5 unit of rRNasin RNase inhibitor, and 100 ng of each of the primers. TSP1 primers used were 5′-CGT CCT GTT CCT GAT GCA TG-3′ (position 99–118) and 5′-GGC AGG ACA CCT TTT TGC AGA-3′ (position 1115–1135) corresponding to the 5′ and 3′ primers, respectively. TSP2 primers were 5′-CAA GGT GCC TCG CTG TGT CA-3′ (position 2054–2073) and 5′-GGC GTC ACC CTC TCC ATT GT-3′ (position 2614–2633) for 5′ and 3′ primers, respectively. After initial denaturation for 2 min at 92°C, 15 units of AMV reverse transcriptase (Promega) were added to the reaction mixture and incubated for 20 min at 42°C. Reverse transcriptase was then heat inactivated (3 min at 94°C), and after cooling to 0°C, PCR amplification of the cDNA products was carried out by adding 0.5 unit of *Taq*I DNA polymerase (ATGC). PCR amplification was done using 30 cycles in a DNA thermocycler (Thermojet; Eurogentec) in the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and extension for 1.5 min at 72°C. To ascertain that TSP1 and TSP2 transcripts were specifically amplified from breast samples, sequence analysis of PCR products was performed. Amplified cDNA products fully matched TSP1 or TSP2 nucleotide sequences (results not shown).

Amplified PCR products were run on a 1.3% agarose gel containing 0.1 μg/ml ethidium bromide. Gels were photographed under UV light using noncoated instant film (Polaroid 667; Eastman Kodak Co.). Photographs were scanned (Canon CLC 10), and the intensity of the two bands corresponding to cellular and competitor amplification products was measured using Image analyzer software (Wayne Rasband, NIH). The areas of the peaks were plotted on the *Y* axis of a chart against the corresponding copy numbers of the serial dilution of RNA standards. A regression line was then used as standard curve of the assay. The absolute copy numbers of specific RNA in breast tissue samples (*i.e.*, the number of amplified copies/μg of total RNA $\times$ 10^6) were then calculated by interpolating the areas from the amplified band of each sample to the standard curve. An index of TSP mRNA expression was then determined as follows:

$$\text{TSP mRNA expression index} = \frac{\text{Number of amplified copies}/\mu\text{g of total RNA} \times 10^6 \text{ in breast tissues}}{\text{Mean of amplified copies}/\mu\text{g of total RNA} \times 10^6 \text{ in normal breast tissue}}$$

Statistical Analysis. Data were analyzed using the one-way ANOVA test followed by a Fischer's PLSD test and a Scheffé's F-test. Differences or correlations were considered significant at $P < 0.05$.

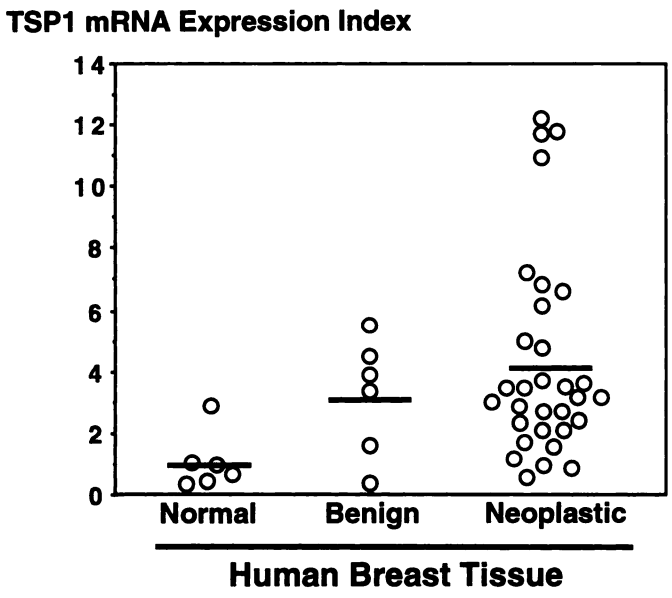

Fig. 1. TSP1 mRNA expression in normal, benign, and neoplastic human breast tissues. ○, TSP1 mRNA values. *Horizontal lines*, median values. The difference between neoplastic and normal breast tissues was significant ($P < 0.05$).

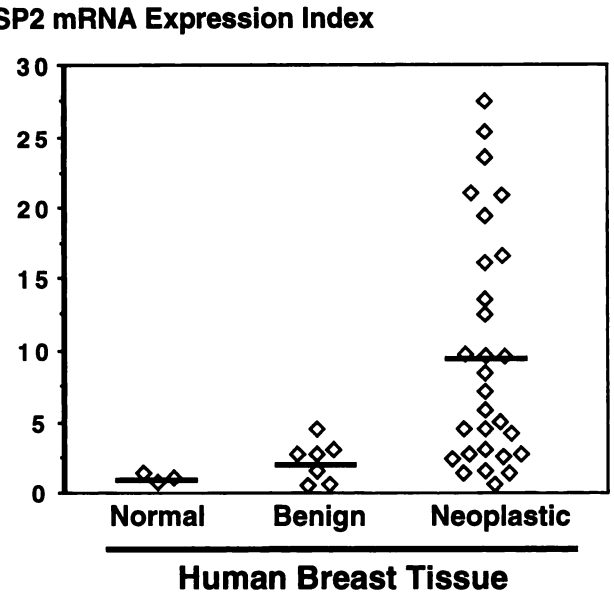

Fig. 2. TSP2 mRNA expression in normal, benign, and neoplastic human breast tissues. ◇, TSP2 mRNA values. *Horizontal lines*, median values. The difference between neoplastic and normal and benign breast tissues was significant ($P < 0.05$).

Results

TSP1 and TSP2 mRNA Expression in Normal, Benign, and Neoplastic Human Breast Tissues. TSP1 mRNA expression in benign and neoplastic human breast tissues was 3–4-fold higher than that observed in normal breast tissues (3.14 ± 1.92, 4.08 ± 3.34, and 1 ± 0.94, respectively; Fig. 1). Significant differences in TSP1 mRNA expression between neoplastic and normal breast tissues were observed ($P < 0.05$). A significant 9-fold increase in TSP2 mRNA expression was also observed in neoplastic breast tissues (9.67 ± 8.18; $P < 0.05$) compared to that obtained in normal breast tissues (1 ± 0.35; Fig. 2). TSP2 mRNA expression in benign breast tissues was slightly increased (2.07 ± 1.53; Fig. 2).

Association of TSP1 and TSP2 mRNA Expression with Prognostic Factors and Microvessel Score. Clinical and biological characteristics and TSP mRNA values in patients with invasive breast carcinoma NOS were shown in Table 1. The tumor grade positively correlated with the tumor size (F-test, $P = 0.03$) and the estrogen receptor status (F-test, $P = 0.02$; *i.e.*, tumors with a high grade had usually a large size and were estrogen receptor negative). There was no meaningful association between TSP1 and TSP2 mRNA expression and any other tumor characteristics including microvessel density.

Association of TSP1 and TSP2 mRNA Expression with Fibroblastic Desmoplasia of Ductal Breast Carcinoma NOS and Microvessel Density. The desmoplastic reaction was graded according to the extent of the stroma area. When the stroma area exceeded 75% of the whole tumor, it was graded 3+, 2+ between 50 and 75%, + be-

Table 1 *Clinical and biological characteristics and TSP1 and TSP2 mRNA expression in 31 patients with invasive breast ductal carcinoma NOS*

	Patients			
	No.	%	TSP1[a]	TSP2[a]
Age				
≤50 yr	9	29	4.9 ± 4.3	9.6 ± 9.2
>51 yr	22	71	3.6 ± 2.7	9.7 ± 7.9
Tumor Size (cm)				
≤1	5	16	4 ± 2.3	14 ± 9.7
1–3	21	68	4 ± 3.7	9.6 ± 8
>3	5	16	4.3 ± 3.5	5.5 ± 6.1
Histological grade of Scarff, Bloom, and Richardson				
I	3	10	3.9 ± 2.3	12.5 ± 9.5
II	23	74	4.3 ± 3.4	10.8 ± 8.3
III	5	16	6 ± 8	7.2 ± 8.8
Estrogen receptor status				
<10 fmol/mg protein	6	19	2.6 ± 2.3	6.9 ± 8.4
≥10 fmol/mg protein	23	75	4.4 ± 3.5	9.4 ± 7.9
ND[b]	2	6	4.6 ± 0.2	18.9 ± 9
Progesterone receptor status				
<10 fmol/mg protein	6	20	2.9 ± 2.5	3.8 ± 3.4
≥10 fmol/mg protein	23	74	4.4 ± 3.7	10.2 ± 8.2
ND[b]	2	6	4.6 ± 0.2	18.9 ± 9
Nodal status				
0	19	61	3.8 ± 3.5	8.9 ± 7
1–3	8	26	4.9 ± 3.9	9.3 ± 8.9
>3	3	10	2.8 ± 1.6	14.7 ± 15
ND[b]	1	3	6.6	23.4
Microvessel density (microvessel counts/mm^2)				
≤100	11	37	3.8 ± 1.8	11.8 ± 7.4
>100	15	48	4.5 ± 3.9	7.4 ± 7.7
ND[b]	5	15	3.6 ± 4.6	10.9 ± 10.9

[a] TSP-1 and -2 mRNA levels were expressed as the ratio of the number of amplified copies/μg of total RNA × 10^6 in neoplastic tissues over that obtained in normal breast tissues. This ratio defines a TSP mRNA expression index shown in the table. Results were expressed as the mean ± SD.

[b] ND, not determined.

tween 25 and 50%, and +/− when the area was less than 25% of the whole tumor (Fig. 3). TSP1 mRNA expression progressively and significantly increased as desmoplasia became more and more prominent (Table 2). For example, a 5-fold increase of TSP1 mRNA expression was observed between ductal carcinomas with strong (graded 3+) and poor (graded ±) desmoplasia. Although it was not statistically significant, TSP2 mRNA levels were also increased in ductal carcinomas with moderate-to-strong desmoplasia (graded as 2+ and 3+). The microvessel density was significantly higher in desmoplastic-rich stroma of ductal carcinomas (recorded as 3+) compared to that observed in invasive carcinomas with a lower desmoplastic reaction (Table 2). The increase of TSP1 and TSP2 mRNA expression in desmoplastic-rich stroma of ductal carcinomas did not significantly correlate with microvessel counts (P = 0.08 and 0.7, respectively) or with any other prognostic factors. Although invasive lobular carcinomas have a poor desmoplastic reaction, TSP1 and TSP2 mRNA expression in these tumors was significantly higher than that observed in normal and benign breast tissues (Table 2).

Discussion

The present study was designed to identify and quantify TSP1 and TSP2 mRNAs in normal, benign, and neoplastic human breast tissues using the reverse transcriptase PCR technique. We found that TSP2, like TSP1, was expressed in human breast tissues, and that TSP1 and TSP2 mRNA expression in invasive breast carcinoma NOS was significantly increased compared to that observed in normal and benign tissues. The observation that TSP1 mRNA expression was increased in breast carcinoma is in close agreement with the findings that cytosols of neoplastic breast tissue contain significantly higher levels of TSP1 protein than normal and benign breast tissues (12), and that strong localization of TSP1 protein is observed in desmoplastic areas of invasive breast carcinomas, whereas there is little or no immunostaining of normal breast tissue with an anti-TSP1 antibody (1–3). Although antibodies against human TSP2 are not yet available, the concomitant increase of TSP1 and TSP2 mRNA levels in desmoplasia of neoplastic breast tissues strongly suggested that TSP2 protein, like TSP1, was present in the stroma of invasive breast carcinoma NOS. The increased expression of TSP1 and TSP2 in invasive breast carcinoma NOS did not significantly correlate with any of the prognostic factors studied (tumor size, lymph node status, morphology, and hormone receptor status). Similarly, it has been shown previously that high TSP1 levels in cytosols of neoplastic breast tissue do not correlate with estrogen and progesterone receptors (12). It is now well established (although poorly understood) that the quantity of stroma termed desmoplasia often varies strikingly from one tumor to

Table 2 *Association of TSP1 and TSP2 mRNA expression with fibroblastic desmoplasia of invasive ductal carcinoma NOS or lobular carcinoma and microvessel density*

Fibroblastic desmoplasia	Ductal carcinoma				Lobular carcinoma
	3+	2+	+	+/−	
TSP1 mRNA[a,b]	11.4 ± 0.5 (n = 4)	4.2 ± 1.8 (n = 11)	2.3 ± 0.8 (n = 10)	2.1 ± 2.2 (n = 6)	4.86 ± 2.53 (n = 2)
TSP2 mRNA[a]	13.3 ± 13 (n = 4)	11.4 ± 8 (n = 11)	4.9 ± 2.6 (n = 8)	10.6 ± 9.3 (n = 6)	7.8 ± 5.7 (n = 2)
Microvessel[c] density	269 ± 83 (n = 3)	110 ± 59 (n = 10)	124 ± 52 (n = 8)	157 ± 133 (n = 4)	

[a] TSP-1 and -2 mRNA levels were expressed as the ratio of the number of amplified copies/μg of total RNA × 10^6 in neoplastic tissues over that obtained in normal breast tissues. This ratio defines a TSP mRNA expression index shown in the table. Results were expressed as the mean ± SD.

[b] TSP1 mRNA values obtained for each group were statistically different ($P < 0.05$) when comparing 3+, *versus* 2+, 3+ *versus* +, 2+ *versus* +/−, 2+ *versus* +, and 2+ *versus* +/−.

[c] Microvessel density was expressed as the mean ± SD of microvessel counts/mm^2. Microvessel counts obtained for each group were statistically different ($P < 0.05$) when comparing 3+ *versus* 2+ and 3+ *versus* +.

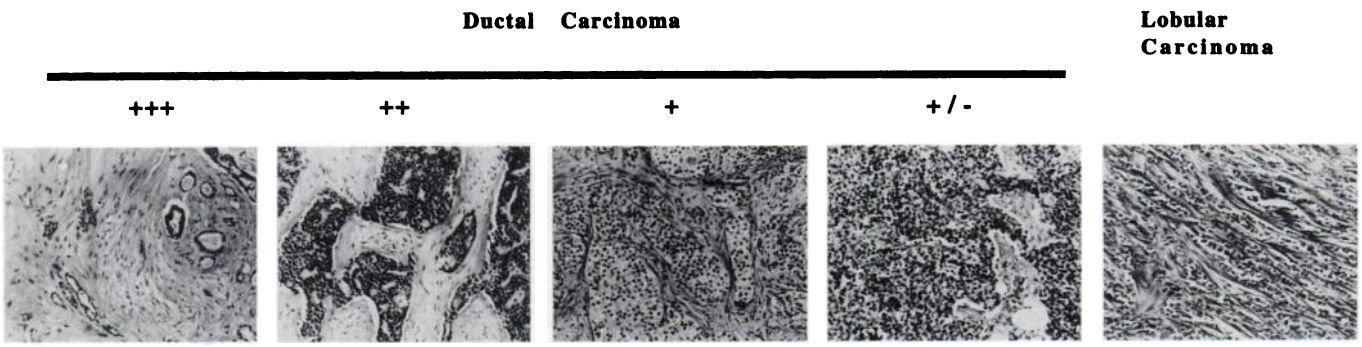

Fig. 3. Fibroblastic desmoplasia of invasive ductal and lobular breast carcinomas. The desmoplastic reaction of ductal carcinoma NOS was graded ±, +, 2+, or 3+ according to the extent of the stroma area. Lobular carcinomas have a poor desmoplastic reaction.

another (4). When our population was divided according to the quantity of stroma, TSP1 (and possibly TSP2) mRNA expression drastically increased as the extent of desmoplasia became more and more pronounced. Tumor stroma is composed of diverse elements including new blood vessels, inflammatory cells, and fibroblasts and myofibroblasts, which are responsible for matrix synthesis (4). We observed in this study a significant increase of microvessel density in desmoplastic-rich stroma of ductal carcinomas, confirming the presence of blood vessels in the tumor stroma. In addition, increased TSP1 mRNA levels found in desmoplastic-rich tumors correlated with our previous findings showing that fibroblasts present in the tumor stroma are responsible for TSP1 synthesis (2). Because cultured fibroblasts express TSP1 and TSP2 mRNAs (7), it is conceivable that TSP2, like TSP1, is also produced by fibroblasts present in desmoplasia. By contrast, invasive lobular carcinomas, which have usually a poor desmoplastic stroma, produced significant amounts of TSP1 and TSP2 mRNAs. These findings are in accordance with our previous study showing that invasive breast lobular carcinoma cells express TSP1, whereas no expression is observed in invasive breast ductal carcinoma cells (2). The role of TSP1 and TSP2 in breast carcinoma is unknown. *In vitro,* TSP1 promotes attachment, migration, and invasion of human MDA-MB-231 breast carcinoma cells (6), suggesting that the TSP1-rich matrix in invasive ductal carcinoma is a favorable factor in the prognosis for tumor progression. However, TSP1 and TSP2 exhibit antiangiogenic activities in different experimental models of angiogenesis (5, 7, 10). In addition, overexpression of TSP1 in MDA-435 breast carcinoma cells inhibits tumor growth *in vivo* through a reduction of angiogenesis (13). By contrast, when it is matrix-bound, TSP1 stimulates myofibroblasts which, in turn, produce angiogenic factors (6, 8). In this study, we followed closely the microvessel scoring protocol used in different breast cancer studies (11). Although it has been suggested recently that the heterogeneity of tumor vascularity may be a potentially important source of variance (14), we observed that high TSP1 and TSP2 mRNA expression in desmoplastic-rich invasive ductal carcinoma NOS coincided with a high microvessel density. Our present study did not indicate, however, whether TSP1 and TSP2 exert antiangiogenic or proangiogenic activity.

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