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Histology and Histopathology

Histopathology and clinical assessment correlate with the cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) receptor of thrombospondin-1 in breast tumors

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Summary. Thrombospondin-1 (TSP-1) is a matrix protein implicated in mechanisms of tumor metastasis. TSP-1 has a characteristic Cysteine-Serine-Valine-Threonine-Cysteine-Glycine (CSVTCG) sequence that functions as a tumor cell adhesion domain. Our laboratory has isolated a novel CSVTCG specific tumor cell receptor. Immunohistochemical staining techniques and computerized image analysis were used to identify and quantitate the CSVTCG receptor of TSP-1 in a wide spectrum of human archival breast tumors. Histopathologic and quantitative examination was correlated with clinical findings two years post operation. Increasing amounts of CSVTCG receptor correlated positively with worsening histopathologic and clinical findings. These findings suggest a role for the TSP-1 CSVTCG receptor in breast tumor progression. This receptor may have utility for the diagnosis, staging, and treatment of this common and deadly disease.

Key words: Thrombospondin-1, CSVTCG receptor, Breast tumors, Cancer, Immunohistochemistry

Introduction

Thrombospondin-1 (TSP-1) is a 450,000 dalton glycoprotein. Platelets and a variety of other cells including endothelial (McPherson et al., 1981), and tumor cells (Mosher, 1990), synthesize and secrete TSP-1. Most of the cell synthesized TSP-1 becomes incorporated into the extracellular matrix (Jaffe et al., 1986). The extracellular matrix is an important mediator of tumor progression and metastasis. Such extracellular molecules as proteoglycan, fibronectin, laminin and TSP-1 provide vascular attachment sites for metastasizing tumor cells as well as a suitable environment for cell proliferation in the interstitial tissue (Aznavoorian et al., 1993). Detailed knowledge of the tumor cell binding domains of these matrix molecules has provided a unique opportunity to design peptide antagonists that prevent tumor cell implantation in animal models of tumor cell metastasis (Tuszynski, 1993). We believe tumor cell colonization is mediated by a novel tumor cell receptor which recognizes the adhesive domain on TSP-1 containing the CSVTCG amino acid sequence.

TSP-1 has been implicated in the malignant process. TSP-1 promotes the attachment and spreading of squamous carcinoma, glial, and melanoma cells (Varani et al., 1989). Numerous tumor cell lines including melanoma, fibrosarcoma, and primary neoplasms of unknown origin produce TSP-1 (Mosher, 1990). TSP-1 promotes motility of melanoma cells (Tarboletti et al., 1990). Malignant breast tumors have been found to contain large tissue concentrations of TSP-1, whereas benign tumors and breast cysts contain relatively low levels of TSP-1 (Pratt et al., 1989). TSP-1 is localized in the stroma of malignant breast tumors (Wong et al., 1992). Expression of TSP-1 promotes the malignant phenotype in small cell carcinoma (Castle et al., 1991), and highly metastatic small cell carcinomas are more chemotactic toward TSP-1 than those with a lower metastatic potential (Yabkowitz et al., 1993). Anti-peptide (CSVTCG) antibodies have been shown by our laboratory to inhibit tumor cell metastasis and TSP-1 mediated tumor cell adhesion (Tuszynski et al., 1992).

Our laboratory also found that TSP-1 promoted invasion of fibrin gels by a human lung cell carcinoma (Hosakowa et al., 1993). In the absence of TSP-1, the tumor cells were non-adherent and catalyzed the rapid degradation of the fibrin gel matrices. In contrast, addition of TSP-1 to the cells inhibited fibrinolysis in a dose-dependent manner, and promoted attachment and spreading of cells in the fibrin matrix. These results are consistent with the recently reported anti-plasmin activity of TSP-1, and suggest that TSP-1 may promote

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tumor cell metastasis not only by promoting cell attachment, but also by protecting tumor cell-fibrin emboli from degradation by the host fibrinolytic system.

Our laboratory has shown that TSP-1's CSVTCG sequence functions as a tumor cell adhesion domain, and CSVTCG peptides, as well as anti-CSVTCG antibodies, have anti-metastatic activity in a murine model of lung metastasis (Tuszynski et al., 1992). We have also isolated a novel CSVTCG specific tumor cell adhesion receptor (Tuszynski et al., 1993) which by immunohistochemistry localizes in malignant breast ductal epithelium (Tuszynski and Nicosia, 1994). In contrast, TSP-1 was demonstrated in the tumor stroma. Immunohistochemical analysis of benign breast tissue for TSP-1 and its CSVTCG receptor indicated that these molecules were not expressed by the epithelium or the stroma. We have further shown that this antibody to the CSVTCG receptor inhibits tumor progression of breast tumors in an athymic murine model (Wang et al., 1995).

These studies strongly indicate that the CSVTCG receptor and TSP-1 may function to promote the invasive behavior of breast epithelium and contribute to the development of malignancy. To further test this hypothesis, we immunohistochemically correlated the expression of the CSVTCG receptor of TSP-1 in a wide spectrum of human breast tumor samples stored in the Allegheny University of the Health Sciences, Medical College of Pennsylvania Hospital tumor archives, and retrospectively correlated them with two year follow up of metastasis and patient survival. We found a positive correlation between the expression of CSVTCG receptor, histologic type, and outcome. These studies provide information on the role of the CSVTCG specific TSP-1 receptor in breast tumor progression, and suggest the expression of the receptor in breast epithelium can be used in the diagnosis and management of breast neoplasia.

Material and methods

Samples of five micron thickness were taken from archival blocks, and placed on slides. Immunohistochemical staining of the breast tissue for the CSVTCG TSP-1 receptor was accomplished with the following protocol in accordance with the procedure provided by the Vectascan Avidin Biotin Complex (ABC) staining kit (Vector Laboratories, Burlingame, California). The samples were rinsed in methanol and then quenched on 3%H_2O_2 in methanol. Blocking solution (1% BSA/PBS, 1% Vectastain blocking serum, 4% horse serum), was applied for 10 minutes. Polyclonal primary rabbit antibody (1/200 dilution) vs. CSVTCG receptor was applied to the sample. Rabbit serum in the same concentrations was used as the control. The slides were incubated for 60 minutes. Secondary goat antibody (1/300 dilution of antibody, 1.5% blocking serum, 0.1% BSA/PBS) vs. rabbit was applied to the samples. The samples were treated with ABC, followed by DAB (5mg/ml), PBS, and H_2O_2 (added last). The slides were counterstained with hematoxylin, and then mounted wet in a glycerol based medium.

Quantitative analysis was accomplished using the CAS R 200 Image Analyzer system (Becton-Dickenson, Inc., San Jose, California). Briefly, ten stained tumor fields, were measured under a 40x objective lens by the CAS R 200 Image Analyzer system. This computerized video microscope emits a single wavelength (620 nm) and measures the optical density of the stained tumor fields. The objective antibody threshold for a specific staining was defined for each specimen by analyzing the negative control section (control rabbit serum) and subtracting this value from the receptor stained fields. This process eliminates background staining. The intensity of the immunohistochemical staining is directly proportional to the light absorbed by the section and the translated optical density on the analyzed sample. Software for the Quantitative Estrogen/Progesterone Analysis (Cell Analysis Systems, Inc.) provided by Becton-Dickenson, Inc., was used for the quantification of the samples. Positive controls using breast tumor cell line MDA-MB-231, and negative controls using normal breast tissue were performed. We have confirmed the specificity of our antibodies as previously reported (Tuszynski and Nicosia, 1994).

Results

The tissue samples were evaluated with the methods as described previously. A wide spectrum of breast tumors was evaluated. The samples were selected in a blinded fashion, evaluated by immunohistochemistry and computerized image analysis, and then correlated with the assigned pathological diagnosis and reviewed by a blinded senior pathologist. These findings were then correlated with the medical records available. The CSVTCG receptor quantity is expressed in units of absorbance.

Quantitative analysis (Table 1)

The measured absorbance of the CSVTCG receptor positively correlates with poor histologic type. The measured absorbance of CSVTCG receptor also correlates positively with poor clinical presentation. The patients with higher expression of the receptor tend to do worse, as determined by follow up evaluation.

Statistical analysis (Table 2)

There is a statistically significant difference between each of the above groups. Each of these groups, the carcinomas, the non-carcinoma masses, and the normal controls, expresses a unique amount of TSP-1's CSVTCG receptor. The higher CSVTCG receptor level positively correlates with the poorer histologic type. This is seen both quantitatively (with statistical correlation), and histologically as shown in the next section.

Histopathological assessment

Histopathologic assessment found minimal or no receptor expression in the normal breast tissue (Fig. 1A). The fibrocystic disease samples demonstrated minimal expression of the receptor in the ductules and lobules (Fig. 1B). The lobular carcinoma samples demonstrated stronger expression (Fig. 1C). The ductal carcinoma samples demonstrated even more significant expression of the CSVTCG receptor (Fig. 1D). Of note was increased capillary expression of CSVTCG receptor in the lobular and ductal carcinoma samples (Fig. 1E). These histopathological findings correlate with the quantitative results as described above.

Another interesting histopathological finding is that significantly more receptor expression was seen in invasive and anaplastic cells as compared with adjacent tumor cells with better morphologic and noninvasive features (Fig. 1E,F). This correlates with the histologic and quantitative findings summarized in Table 1.

The samples were also evaluated for TSP-1. As previously reported, increased amounts of TSP-1 were localized in the stroma of the tumor tissue (data not shown).

Table 1. Expression of CSVTCG receptor in benign and malignant breast tumors.

CLINICAL DESCRIPTION	ABSORBANCE
Poorly differentiated infiltrating ductal carcinoma. Aprocrine metaplasia, sclerosing adenosis, multiple fibroepithelial polyps. Some bone pain, normal bone scan.	34.98
Ductal carcinoma. Excision of chest wall for mass. Recurrent breast carcinoma found, on tamoxifin.	24.58
Infiltrating ductal carcinoma with medullary features, moderately differentiated. Hot spot on bone scan 1994	23.28
Infiltrating ductal carcinoma. Tamoxifin discontinued secondary to vaginal bleeding. Bone, occipital brain, liver metastasis. Nodule removed from left deltoid muscle. Metastatic ductal carcinoma, consistent with primary.	22.99
Infiltrating lobular carcinoma with bone metastasis. Given radiation, treatment, and tamoxifen. Bone metastasis, liver metastasis/sp chemotherapy/ radiation. Excellent palliative response to lumbar spine radiation.	17.76
Fibrocystic changes with focal stromal fibrosis and fibroadenomatoid changes.	14.91
Fibrocystic changes, apocrine metaplasia and stromal fibrosis.	13.38
Fibroadenoma with fibrocystic changes, apocrine metaplasia, sclerosing adenosis.	10.08
Atypical intraductal hyperplasia with fibrocystic changes, apocrine metaplasia, cysts and ductal hyperplasia.	9.12
Ductal carcinoma in situ with thickening of right breast. FNA of right breast negative for malignancy.	7.03
Negative control. Normal breast tissue.	4.51

Discussion

Breast cancer is a major health problem in developed countries. It is the leading cause of death among American women aged 40-55, and at least 12% of the female population of the United States will be diagnosed with breast cancer (Harris et al., 1992). Early detection is important so that the tumor may be removed before metastasis occurs. A critical question in the management of breast cancer is the determination of whether non-invasive cancers such as in-situ carcinoma or hyperplasia will progress to breast cancer requiring mastectomy and possible adjunctive therapy to effect a cure. Forty percent of patients with in-situ ductal carcinoma treated with excision alone have recurrence of invasive carcinoma after five years (Harris et al., 1992). Determination of prognosis of breast carcinoma based on morphological appearance is inadequate. An accurate predictor of tumor progression and outcome is needed. Some tumor markers have been identified (e.g. S ploidy, cathsepin D, p53, Her-B oncogenes, etc). The TSP-1 CSVTCG specific receptor was evaluated as a marker of tumor progression and patient outcome. Our long term goal is to develop antibodies or probes specific against the CSVTCG receptor that can be used for both diagnosis and treatment.

A previous study done in our laboratory investigated human invasive breast carcinoma samples as well as benign and normal tissue. These samples were examined immunohistochemically for the expression of TSP-1 and its CSVTCG receptor using polyclonal antibodies. TSP-1 expression was present in all breast ductal carcinomas. In contrast, all benign lesions stained negatively for TSP-1 and the CSVTCG receptor except for two fibrocystic samples with hyperplasia, which showed either weak TSP-1 expression of receptor in the ductal epithelium. TSP-1 staining in ductal carcinoma was localized in the dense stromal collagen adjacent to the tumor whereas the CSVTCG receptor localized to the tumor cells. Our previous results suggested that increased expression of stromal TSP-1 and the CSVTCG receptor in ductal epithelium correlates with neoplastic transformation. Further studies showed that both malignant and benign breast tissue can stimulate surrounding capillaries to express the TSP-1 receptor. By

Table 2. Statistical analysis between carcinoma, non-carcinoma, and normal groups.

	MEAN±SEM	p
Carcinoma (n=5)	24.7±2.82	vs. normal, p=0.002 vs. Non-CA, p=0.002
Non-carcinoma masses (n=5)	10.9±1.43	vs. Normal, p=0.02 vs. CA, p=0.002
Normal (control) n=3	4.51±0.41	vs. CA, p=0.002 vs. Non-CA, p=0.02

Statistical analysis by ANOVA and Student's t-test, using the Sigma Stat Program, Jandel Scientific, San Rafael, California, USA.

contrast, only carcinoma has the capacity to stimulate surrounding nonendothelial stromal cells such as myofibroblasts, to secrete a TSP-1 rich matrix which may contribute to the desmoplastic stromal reaction characteristic of breast cancer. This TSP-1 rich matrix may promote tumor cell attachment, migration, and angiogenesis (Tuszynski et al., 1993). The receptor rich capillary endothelium may promote the cell adhesive interactions important in tumor invasion. This provides a rational basis

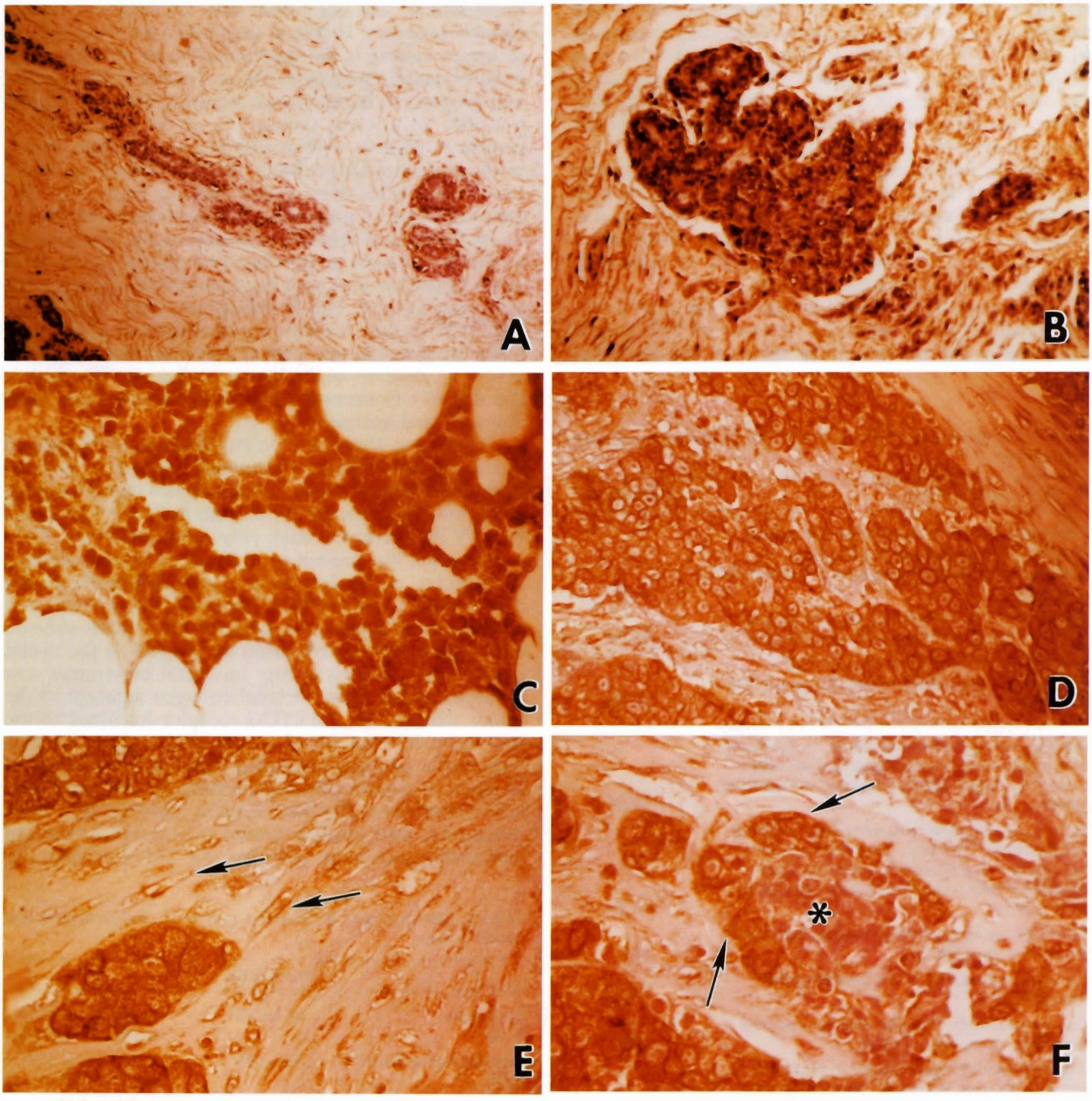

Fig. 1. Benign, hyperplastic and neoplastic breast tissue immunostained by the ABC immunohistochemical method for the CSVTCG receptor. **A.** Normal breast tissue. x 10. **B.** Hyperplastic lobule in fibrocystic disease. x 10. **C.** Lobular carcinoma. x 40. **D.** Infiltrating ductal carcinoma. x 40. **E.** Newly formed microvessels (arrows) in the stroma of ductal carcinoma. x 100. **F.** Infiltrating ductal carcinoma. Note the invading tumor cells (arrows) express the CSVTCG receptor while non-invading cell at the center of the tumor nest (asterisk) are negative. x 100

for a role of TSP-1 in tumor angiogenesis and metastasis.

The findings of this study, investigating a spectrum of breast tumors, continues to support the conclusion that the CSVTCG receptor of TSP-1 is expressed by tumor tissue and is associated with poor clinical prognosis. The negative control absorbance of 4.51 represents a "normal" or background level of receptor expression, and was found in the matrix of breast tissue. In contrast, the fibroadenoma was found to have an absorbance of almost twice that of control. This suggests that the CSVTCG receptor may upregulate growth of tissue, perhaps through mediating cell-cell interactions, since the receptor has been shown to mediate cell adhesion (Tuszynski et al., 1993) and invasion (Wang et al., 1995). The fact that receptor expression was observed in the microvasculature adjacent to invasive tumor both in this study and in our previous study suggests that receptor expression may promote tumor angiogenesis. Our data in the present study shows that there is an increasing level of receptor in progressively more neoplastic breast lesions. Even fibroadenoma, a benign lesion, with rare transformation to carcinoma, shows low levels of receptor expression. Remarkably, our study also shows that the levels of CSVTCG receptor correlate with the clinical presentation. Those patients whose tumors had higher levels of the CSVTCG receptor tended to have worse clinical presentations. This becomes apparent after comparing the clinical outcomes of a patient with intraductal carcinoma, (Absorbance 34.98), not invasive at the time of diagnosis, now presenting with a recurrence after chemotherapy, with that of a patient with fibrocystic disease (Absorbance 14.91), who is doing well clinically, and without recurrence.

These results are consistent with our previously published data in head and neck tumors which showed that the CSVTCG receptor expression correlated with poor patient outcomes better than histologic grading and TNM status (Arnoletti et al., 1994).

Another interesting finding in this study, is that the CSVTCG receptor localized to the tumor tissue. More significantly, predominant staining was noted in the more invasive cells (Fig. 1E, F). This further demonstrates the CSVTCG receptor correlation with subsequent poor outcome.

The proposed role of TSP-1 and its CSVTCG receptor in tumor adhesion, matrix breakdown, and angiogenesis have been described (Nicosia and Tuszynski, 1994; Tuszynski and Nicosia, 1996). This study shows that the amount of CSVTCG receptor positively correlates with the more malignant clinical presentations and less favorable outcomes in a wide variety of breast tumors. This suggests a role for the TSP-1 CSVTCG receptor both as a significant diagnostic indicator and possible target for therapeutic intervention.

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