Tenascin Expression in Cancer Cells and Stroma of Human Breast Cancer and Its Prognostic Significance

Akinori Ishihara, Toshimichi Yoshida, Hisao Tamaki, and Teruyo Sakakura

ABSTRACT

Sections of formalin-fixed, paraffin-embedded tissues from 210 human breast cancers were immunohistochemically examined using the mAb against human tenascin (TN) RCBI. Immunoreactive TN was detected in the breast cancer stroma in 77 (36.7%) cases, whereas the remaining 133 (63.3%) were negative. Of the 77, 12 (5.7%) cases also showed positive staining in the carcinoma cell cytoplasm. The positive cells were often observed in the margin of the cancer nests at the site adjacent to the stroma. According to the staining pattern of TN, the breast cancer cases were classified into the three groups of cancer cell TN(+)/stromal TN(+), cancer cell(-)/stromal TN(+), and cancer cell(-)/stromal TN(-). Analysis of the relationship of these TN patterns with various clinicopathological characteristics of the tumors and the patient outcome revealed that, in comparison to the cancer cell(-)/stromal TN(-) group, the cancer cell TN(+)/stromal TN(+) group exhibited increased frequency of lymph node metastasis and exceptionally poor outcome, and the cancer cell(-)/stromal TN(+) group also showed more frequent metastasis and poorer outcome. Most of the cancer cell TN(+)/stromal TN(+) cases were c-erbB-2 positive and estrogen receptor negative. Furthermore, in situ hybridization of freshly obtained breast cancer tissues demonstrated that both cancer cells and stromal cells express TN mRNA. These results indicate that the TN in breast cancer is produced by cancer epithelial cells as well as by stromal mesenchymal cells, and that cancer cell TN might be involved in cancer spreading, resulting in unfavorable patient prognosis.

INTRODUCTION

TN is an extracellular matrix glycoprotein with a unique six-armed macromolecular structure, which is known to be an essential factor for modulation of reciprocal interactions between the epithelium and mesenchyme during embryogenesis (1–3). A number of studies have demonstrated prominent TN expression in human cancers including those of brain (4), colon (5, 6), liver (7, 8), lung (9, 10), uterus (11), skin (12), prostate (13), and mammary gland (14–17). Because it was initially proposed as a stromal marker for epithelial malignancy (14), TN expression in cancers has usually been observed in the stroma. Examining human breast cancers by immunohistochemistry, investigators have found that TN is consistently present in the stroma of malignant tumors (14–16). Our previous examination also indicated intense staining in the connective tissue of invasive ductal carcinomas (17). Accordingly, it has been generally accepted that TN is produced by the mesenchyme and has an active function in cancer development, probably by promoting cancer cell proliferation and invasion.

Shoji et al. (18) proposed TN to be a marker useful in predicting the survival of breast cancer patients. They investigated immunoreactive TN in 82 patients with primary invasive breast carcinomas, and found a significantly superior outcome during the 5-year period after surgery in the TN-positive patients compared with the negative patients. Immunohistochemistry of primary colon carcinomas demonstrated no lymphogenous metastasis in patients in whom the cancer stroma showed strongly positive staining for TN (19). Taking these results into consideration, it is conceivable that TN may prevent rather than enhance the cancer cell outgrowth, probably by creating a barricade surrounding the cancer nests and inhibiting the movement of these cells. In fact, TN has been proposed as a boundary molecule in the somatosensory cortical barrel field during the development of mouse cerebral cortex (20). Thus, there are two different lines of speculation as to the role of TN in cancer development.

Lightner et al. (21) have recently demonstrated that normal mammary epithelial cells express TN in culture and incorporate the protein into the underlying matrix. They have also examined human breast tissues by in situ hybridization and found that both normal and malignant mammary epithelial cells can express TN mRNA (21). Our previous findings have shown that A431 epidermal cancer cells which express no TN in culture can produce TN, with accumulation in the surrounding mesenchyme, when injected into the nude mouse subcutis (22). Thus, the TN in the breast cancer stroma is considered to be originated from both the epithelium and mesenchyme. We have proposed that TN in the tissue is heterogeneous in its structure and function (23). In breast cancer, the structure and function of epithelial TN are possibly different from those of the mesenchymal TN. Therefore, it is important to determine by which cells the TN in the cancer stroma was produced.

In this study, we used archival formalin-fixed and paraffin-embedded surgical materials of human breast cancers. In immunohistochemical studies of cancers using anti-TN antibody, we have found positive staining in the cytoplasm of cancer cells. Therefore, this study was designed to investigate the presence of both the cancer cell TN and stromal TN in breast cancer, and the implications regarding the relationship between the TN staining
pattern and clinicopathological characteristics. TN staining was detected in cancer cells in the patients whose outcome was poor. The results were also compared with the c-erbB-2 and ER status. Furthermore, the cellular source of TN was identified in freshly obtained cancer tissues by in situ hybridization with human TN mRNA.

MATERIALS AND METHODS

Tissues. Primary breast cancers and axillary lymph nodes from 210 female patients who had undergone surgical resection at Matsusaka Chuo General Hospital between 1979 and 1987 were used in this study. The average age at the time of diagnosis was 54.4 (range, 28–89) years. The tumor diameter varied from 0.6 to 12 cm (mean diameter, 2.8 cm). Five years after the initial operation, 167 patients were still alive, 32 had died from relapsed breast cancer, and 11 had died of other causes.

The tumors were classified according to the WHO histological typing for breast cancer (24). The histological malignancy was categorized into three grades (I–III), based on the grades of architectural atypia and nuclear atypia, along with the number of mitotic figures as described initially by Bloom and Richardson (25). All cases were classified into the clinical TNM stages I, II, IIIa and b, and IV according to the size of the tumor, degree of lymph node metastasis, and distant metastasis status (26). The ER levels were determined at a commercial laboratory (Otsuka Assay Company, Tokushima, Japan).

Survival times were measured from the date of diagnosis using the multiple regression model developed by Cox (27) for censored survival data. The curves of the probability of survival were obtained using the product limit method of Kaplan and Meier (28). Differences among the groups for other prognostic parameters were analyzed using Student’s t test with the Cochran–Cox method and χ² test.

Immunohistochemistry. The surgical specimens were fixed routinely in 10% phosphate-buffered formalin, embedded in paraffin, and cut at 4-µm thickness. Sections were examined by immunostaining for TN, c-erbB-2, and vimentin. After deparaffinization with xylene and hydration with downgraded ethanol, the sections were incubated in 0.3% H₂O₂ in methanol for 15 min to block endogenous peroxidase activity. For the staining of TN, the sections were treated in 10 mM TBS solution parallel fashion but incubated with normal sera instead of the primary antibodies. The TN-positive staining in the cancer stroma was evaluated, but the staining in the basement membrane and subbasement membrane zone was excluded, since it was observed around the normal ducts as well. The cytoplasmic staining of the cancer cells was also determined. The staining intensity was scored into three grades: strongly positive (+ + +), positive (+), or negative (–).

In Situ Hybridization. Freshly dissected breast cancer tissues were trimmed and fixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C. After being rinsed in the same buffer, they were dehydrated by a graded ethanol series and xylene, embedded in paraffin, cut at 3-µm thickness, and placed on glass slides coated with Biobond (British BioCell International, Cardiff, United Kingdom). TN mRNA expression was detected by in situ hybridization using a previously described method (30) with slight modifications. Briefly, antisense and sense cRNA probes were prepared by in vitro transcription of human TN cDNA (kindly given by Dr. L. Zardi, Genoa), using a DIG RNA labeling kit (SP6/T7; Boehringer Mannheim, Mannheim, Germany). Sections were deparaffinized with xylene, digested with 0.3 units/ml proteinase K (Boehringer Mannheim), postfixed with 4% paraformaldehyde, washed in PBS three times, then treated in 0.2 M HCl for 10 min. After being washed again in PBS, they were treated in 0.1 M triethanolamine (pH 8.0), washed in PBS, treated with 0.1 M triethanolamine/0.25% acetic anhydride, washed in PBS, dehydrated in upgraded ethanol, and air dried. The specimens were prehybridized with hybridization buffer composed of 50% formamide, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 600 mM NaCl, 10 mM DTT, 1 × Denhardt’s solution (Sigma), 0.25% SDS, 10% dextran sulfate, and 200 µg/ml Escherichia coli tRNA (Boehringer Mannheim) for 2 h at 50°C, and incubated with 40 µl hybridization buffer containing alkaline-hydrolyzed DIG-labeled antisense or sense probes for 16 h at 50°C. After hybridization, sections were rinsed in 4× SSC followed by 50% formamide/2× SSC at 50°C for 15 min, and incubated in 1× STE (10 mM Tris-HCl, pH 7.5/500 mM NaCl/1 mM EDTA) at

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Expression of immunoreactive TN in human breast cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological classification</td>
<td>No. of cases</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>183</td>
</tr>
<tr>
<td>Noninvasive ductal carcinoma</td>
<td>9</td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>6</td>
</tr>
<tr>
<td>Other types</td>
<td>12</td>
</tr>
</tbody>
</table>
| *Numbers in parentheses, numbers of cases with TN-positive cancer cells.
Fig. 1  TN immunohistochemistry in human breast cancer using the mAb RCB-1. Positive TN staining is widely distributed in the stroma of infiltrating ductal carcinoma (a). In another case, TN staining is not shown in the tissue (b). The cytoplasm of some cancer cells shows the intense positive reaction (c). TN-positive cancer cells are often located in the margin of cancer nests (d). Bar in d, 200 μm for a, b, and d, and 100 μm for c.

Table 2  Clinicopathological data in the 12 breast cancer cases with TN-positive cancer cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Tumor size (cm)</th>
<th>Histology (grade)</th>
<th>Lymph node metastasis</th>
<th>Clinical stage</th>
<th>Outcome (mo)</th>
<th>Immunohistochemistry*</th>
<th>Stromal TN</th>
<th>c-erbB-2</th>
<th>Vimentin</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>7.0</td>
<td>Inv.D. (3)</td>
<td>16/25</td>
<td>IIIb</td>
<td>Died (27)</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>2.7</td>
<td>Inv.D. (3)</td>
<td>12/18</td>
<td>IIIa</td>
<td>Died (47)</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>3.0</td>
<td>Inv.D. (2)</td>
<td>0/18</td>
<td>II</td>
<td>Died (80)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>10.0</td>
<td>Inv.D. (3)</td>
<td>3/10</td>
<td>IIIa</td>
<td>Died (5)</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>2.1</td>
<td>Inv.D. (3)</td>
<td>1/18</td>
<td>II</td>
<td>Died (55)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>1.5</td>
<td>Inv.D. (3)</td>
<td>8/13</td>
<td>IIIa</td>
<td>Died (36)</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>31</td>
<td>4.0</td>
<td>Inv.D. (2)</td>
<td>0/11</td>
<td>II</td>
<td>Alive</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>1.5</td>
<td>NonI.D. (1)</td>
<td>0/11</td>
<td>I</td>
<td>Alive</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>2.5</td>
<td>Inv.D. (3)</td>
<td>0/19</td>
<td>II</td>
<td>Died (52)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>7.0</td>
<td>Inv.D. (3)</td>
<td>4/19</td>
<td>IIIa</td>
<td>Died (10)</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>85</td>
<td>2.2</td>
<td>Ad-sq (3)</td>
<td>4/13</td>
<td>IIIa</td>
<td>Alive</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>34</td>
<td>4.0</td>
<td>Inv.D. (3)</td>
<td>9/19</td>
<td>IIIa</td>
<td>Died (58)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Intensity of immunostaining: -, negative; +, positive; ++, strongly positive.

* Inv.D., invasive ductal carcinoma; NonI.D., noninvasive ductal carcinoma; Ad-sq, adenosquamous carcinoma.
100
50
20.
50 100 150
Months
Table 3
TN immunohistochemistry in relation to the clinicopathological characteristics of 210 breast cancer patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CaC(+)</th>
<th>CaC(−)</th>
<th>CaC(−)</th>
<th>Total</th>
<th>P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>12</td>
<td>65</td>
<td>133</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Mean age at diagnosis (yr)</td>
<td>54.4</td>
<td>53.9</td>
<td>55.1</td>
<td></td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Mean tumor diameter (cm ± SD)</td>
<td>4.0 ± 2.7</td>
<td>2.9 ± 1.8</td>
<td>2.7 ± 1.8</td>
<td></td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>+</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>8 (67%)</td>
<td>34 (52%)</td>
<td>49 (37%)</td>
<td>91</td>
<td>0.043</td>
</tr>
<tr>
<td>−</td>
<td>4 (33%)</td>
<td>31 (48%)</td>
<td>84 (63%)</td>
<td>119</td>
<td>0.038</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1 (8%)</td>
<td>12 (18%)</td>
<td>42 (32%)</td>
<td>55</td>
<td>0.067</td>
</tr>
<tr>
<td>II</td>
<td>5 (42%)</td>
<td>33 (51%)</td>
<td>61 (46%)</td>
<td>99</td>
<td>0.111</td>
</tr>
<tr>
<td>III</td>
<td>6 (50%)</td>
<td>20 (31%)</td>
<td>30 (22%)</td>
<td>56</td>
<td>0.460</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>8 (67%)</td>
<td>19 (29%)</td>
<td>29 (22%)</td>
<td>56</td>
<td>0.001</td>
</tr>
<tr>
<td>−</td>
<td>4 (33%)</td>
<td>46 (71%)</td>
<td>104 (78%)</td>
<td>154</td>
<td>0.252</td>
</tr>
<tr>
<td>ER statusc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>2 (17%)</td>
<td>30 (46%)</td>
<td>72 (54%)</td>
<td>104</td>
<td>0.007</td>
</tr>
<tr>
<td>−</td>
<td>10 (83%)</td>
<td>31 (48%)</td>
<td>53 (40%)</td>
<td>94</td>
<td>0.279</td>
</tr>
</tbody>
</table>

a The patients were divided into the following three groups according to the results of TN immunohistochemistry: CaC(cancer cell TN)(+)/St(stroma TN)(+), CaC(−)/St(+), and CaC(−)/St(−).
b The P value in the upper row for each parameter is that for the comparison between CaC(+)St(+) and CaC(−)/St(−), and that in the lower row is that for the comparison between Ca(−)/St(+) and CaC(−)/St(−).
c ER levels were not available for 12 patients.

37°C for 15 min. To remove unhybridized RNA, the sections were incubated with 20 µg/ml RNase A in 1X STE at 37°C for 30 min, followed by one washing in 1X STE at 37°C for 10 min, twice in 2X SSC at 50°C for 10 min, and twice in 0.2X SSC for 10 min at 50°C. In situ hybridization signals were detected immunohistochemically with alkaline phosphatase-conjugated anti-DIG antibody used according to the manufacturer’s instructions (DIG Nucleic Acid Detection kit; Boehringer Mannheim). When satisfactory signals were obtained, the sections were washed in 10 mM Tris-HCl/1 mM EDTA, soaked in distilled water, and counterstained with 0.1% nuclear fast red. The slides were air dried and mounted.

RESULTS

Histological Diagnosis. Of the 210 breast cancers, 183 cases were invasive ductal cancer, 9 were noninvasive intraductal cancer, 6 were lobular cancer, and 12 were other types of cancer. Microscopic metastasis in axillary lymph nodes had been found in 84 patients at surgery.

Immunohistochemistry. The results of immunostaining for TN are summarized in Table 1. Positive staining for TN in cancer stroma (Fig. 1a) was found in 77 of the 210 cases, while 143 cases were negative (Fig. 1b). Further examination of the 77 TN-positive breast cancers revealed that 12 showed intense staining for TN in the cytoplasm of the cancer cells (Fig. 1c). Histologically, TN-positive cancer cells were often located in the margin of cancer nests in contact with the underlying mesenchyme (Fig. 1d). Positive reactivity was also demonstrated in single cells or small groups of cells in a fibrous stroma. These cancers showed a high malignancy in terms of histological grading, and included 11 ductal carcinomas and 1 adenosquamous carcinoma (Table 2). Regional lymph node metastasis was found in eight of the 12 patients. Positive TN staining of cancer cells at a site of metastasis was found in only one case, and the other seven were negative. Among these 12 cases, positive staining for vimentin in cancer cells was found in 1, and the other 11 were negative. The epithelial cells in normal, hyperplastic, and benign neoplastic mammary lesions in the present samples showed no significant staining for TN.

Relationship of TN Immunohistochemistry with Clinicopathological Characteristics. The results of analysis of the relationship between TN immunohistochemistry and the clinicopathological characteristics of the patients are presented in Table 3. Compared with the patients with TN-negative breast cancers, those with positive staining of TN in the cancer cell cytoplasm showed greater frequency of lymph node involve-
Fig. 3 In situ hybridization of human breast cancers using a DIG-labeled cRNA probe for TN mRNA. In an infiltrating ductal carcinoma, a large proportion of the stromal cells show positive signals for TN mRNA, but the cancer cells are not labeled (a). Immunoreactive TN is densely deposited in the stroma (b). Another carcinoma shows the cancer cells with positive signals for in situ hybridization (c), and the cells are also labeled by TN immunohistochemistry (d, arrows). Bar in d, 100 μm.

DISCUSSION

In breast cancers, an elevation of c-erbB-2 protein levels and this gene amplification have been found to be associated with increased tumor grade (31–34). The ER status of the breast cancer cells is correlated well with the response to hormonal treatment and to some extent with the risk of recurrence, and thus overall survival (35, 36). Each of these two factors is accepted as a possible prognostic marker in human breast cancer, but it is generally agreed that they do not correlate well with each other (31). Comparison of the results for these two parameters suggest that c-erbB-2 is much more effective than ER and a rather independent factor (37). In this study, the possible utility of TN as a prognostic marker of breast cancers was examined. Seventy-seven of 210 breast cancers showed stromal staining for TN; the survival of the patients was poorer than that in the other 133 stromal TN(−) patients. Among these 77 cases, we found 12 also showing TN(+) cancer cells; the 5-year survival of these patients was extremely poor. Thus, we identified TN to be a prognostic marker, especially when it is expressed in cancer cells, which permits the discrimination of a subgroup at a high risk of early relapse among breast cancer patients. However, we found that many of these cases were ER
negative and c-erbB-2 positive. There was also a significant relationship of cancer cell TN positivity to lymph node metastasis. Therefore, TN expression of cancer cells may not be an independent parameter.

It is generally accepted that the mesenchymal cells are the cellular source of TN (1, 14, 38, 39). Also, a few investigators have reported TN expression in the epithelium. In chick developing lung, in situ hybridization demonstrates TN mRNA in the most distal epithelial cells of the bronchi as well as the adjacent fibroblasts (40, 41). During chick feather morphogenesis, TN mRNA is detected initially in the overlying epithelia and then in the underlying mesenchyme (42). In these developing tissues, however, immunoreactive TN is positive in the basement membrane and/or the surrounding mesenchyme, but not in the epithelia. The results suggest that TN synthesized by the epithelium can be released promptly into the extracellular space and deposited there. Immunohistochemistry of cancer tissues, including comedo carcinomas of the breast (16), metastasized prostatic carcinoma in a lymph node (13), and squamous cell carcinomas and adenocarcinomas of the lung (9), has demonstrated cytoplasmic TN staining, suggesting that cancer cells also produce TN. The present results acquired by in situ hybridization clearly demonstrate TN synthesis by cancer cells as well as by the stromal cells. Four of the nine cases were positive for TN mRNA, but immunoreactive TN was found in cancer cells in only two of them. TN produced by cancer cells may be also released promptly into the extracellular space, but a small amount, probably caused by extreme overexpression or retardation of its release, is retained in the cancer cell cytoplasm.

Of the various proposed functions of TN, antiadhesion would seem to be the most probable (43, 44). Fibroblast attachment to fibronectin-coated plates is inhibited by TN addition to the culture medium in a concentration-dependent manner (45). TN released from cancer cells may loosen adhesions between the cells and adhesive extracellular matrix proteins, such as fibronectin, and may increase the mobility of cancer cells, with resulting increased invasive capacity. The TN function, at least partially, could account for the relationship between the TN positivity of the cancer cells and the clinicopathological characteristics, such as the tendency to larger tumor size and the significantly elevated incidence of lymph node metastasis. We also found that the outcome associated with TN(+) stroma was poor compared with TN(−) stroma, and that lymph node metastasis occurred more frequently in association with TN(+) stroma than with TN(−) stroma. According to this result, stromal TN in the cancer tissues may promote cancer spreading. Our study also indicates that the stromal TN detected by immunohistochemistry could have been secreted from both the cancer cells and the stromal cells, a state which may make it more complicated to determine TN functions in cancer stroma. Therefore, it is necessary to further clarify the TN-secreting cells and the timing of deposition during tumor progression.

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