ORIGINAL

Thrombospondin-1 is highly expressed in desmoplastic components of invasive ductal carcinoma of the breast and associated with lymph node metastasis

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Abstract : Desmoplastic (scirrhous) invasion and lymph node metastasis are critical for the treatment and prognosis of invasive ductal carcinoma of the breast. Despite being an anti-angiogenic therapeutic candidate, Thrombospondin-1 (TSP-1) promotes invasion and metastasis of some carcinomas. To clarify the effect of TSP-1 on invasion and metastasis, we obtained 101 invasive ductal carcinomas of the breast with axillary lymph node resection. All tumors were histologically divided into two categories, carcinomas with, and those with non- /minimal desmoplastic component. Immunohistochemistry for TSP-1 was performed on all primary tumors and axillary lymph nodes with tumor metastasis. Fifty-four (53.5%) of 101 tumors were recognized as positive for TSP-1 in the cytoplasm of tumor cells. Histological study showed that significantly more cancers with desmoplastic components (46/69, 66.7%) manifested TSP-1 expression than did cancers with no- or minimal (less than 20%) desmoplasia (8/32, 25.0% ; p<0.001). Axillary lymph node metastasis was significantly higher in TSP-1-positive- (28/54, 51.9%) than TSP-1-negative cancers (11/47, 23.4% ; p<0.005). The present study indicates that tumor cells in the desmoplastic component strongly expressed TSP-1 in invasive ductal carcinoma of the breast and TSP-1 participates in invasion of these tumors. Our findings also suggest that TSP-1 promotes lymph node metastasis and TSP-1 potentially could be a predictive marker for metastasis. J. Med. Invest. 60 : 91-96, February, 2013

Keywords **:** *axillary lymph node, breast carcinoma, desmoplasia, metastasis, thrombospondin-1*

INTRODUCTION

Stromal invasion and metastasis are critical prognostic factors in breast cancer. Invasive ductal carcinoma of the breast often demonstrates invasive feature with marked desmoplasia, a so-called "scirrhous" type. The molecular mechanism of the desmoplastic reaction has not yet been fully clarified. On the other hand, axillary lymph node metastasis is also common in invasive ductal carcinoma of the

Abbreviations : HER2, human epidermal growth factor receptor 2 ; MMP, matrix metalloproteinase ; TGF, transforming growth factor ; TSP-1, thrombospondin-1

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breast. The decision to perform extended dissection of residual lymph nodes is based on microscopic findings on sentinel lymph nodes, but the microenvironmental mechanism(s) involved in axillary lymph node metastasis remains obscure. At present regarding lymph node metastasis, there seem to be few useful predictive biomarkers using histology and immunohistochemistry of primary tumors.

Thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein (1), was initially recognized as an anti-angiogenic factor (2, 3). TSP-1 inhibits tumor growth by inhibiting endothelial migration and proliferation via the induction of apoptosis (4) and results from clinical trials suggested TSP-1 as a potential therapeutic candidate in several cancers (5-7). However, TSP-1 induced pro-angiogenic activity in breast cancer cell cultures (8) and several studies demonstrated that TSP-1 promotes invasion and metastasis in other cancers (9-11). These findings indicate that TSP-1 has diverse and complex functions with respect to cancer invasion and metastasis.

It is not yet known if in invasive ductal carcinomas with much desmoplasia TSP-1 affects the stromal microenvironment with respect to invasion. To our knowledge, no studies on the relationship between TSP-1 expression and the histological structural pattern of invasive ductal carcinoma of the breast have been reported. We studied the expression of TSP-1 by, and the microscopic structural pattern of, invasive ductal carcinoma to assess whether TSP-1 participates in the stromal reaction and invasion of these tumors. We also investigated the role of TSP-1 by examing metastatic axillary lymph nodes using surgical specimens from patients with invasive ductal carcinoma of the breast.

MATERIALS AND METHODS

Case selection and histological categorization

We obtained 101 surgical tissue samples from 101 women (age range 39-92 years) who underwent resection for invasive ductal carcinoma of the breast and axillary lymph nodes at Hyogo Prefectural Awaji Hospital, Sumoto City, Japan between 2005 and 2011. Permission for scientific research using resected tissue was given under a comprehensive agreement with ethical regulation in Hyogo Prefectural Awaji Hospital. None had received radio- or chemotherapy prior to surgery and all underwent mastectomy or tumor excision and axillary lymph node dissection. None had undergone preoperative sentinel lymph node biopsy. Resected tissues were fixed in 10% neutral formalin. Samples from tumors and lymph nodes were cut at their maximum diameter, fixed in 10% neutral formalin, embedded in paraffin, and mounted on glass slides.

Slides were stained with hematoxylin and eosin for histological diagnosis. We distinguished two categories of microscopic structural pattern ; tumors with a desmoplastic (tumor cell nest surrounded by thick collagenous fibers) component and tumors with a non- or minimally (less than 20%) desmoplastic component. To avoid accounting for equivocal immunoreactivity or false-positivity, a cut-off value of 20% was applied by modifying the HER2 testing guideline (12) for TSP-1 positivity.

Immunohistochemistry

For immunohistochemical studies we used 3 micrometer-thick unstained tissue samples mounted on silane-coated slides (PLC-15, Matsunami, Tokyo, Japan). Antigen retrieval for TSP-1 was in neutral citrate buffer using an autoclave (115°C, 2 min).

Immunoreaction was performed in an Autostainer system (UNIVERSAL STAINING SYSTEM, Dako, CA, USA) apparatus. Internal peroxidase was blocked with Dako REAL Peroxidase (Dako) for 5 min at room temperature. An antibody to TSP-1 (Novocastra, Newcastle, UK ; diluted 1 : 100) was applied for 30 min at room temperature ; the reaction was completed using the DakoChemMate ENVISION kit/HRP (DAB) (Dako). Diaminobenzidine was the chromogen. Cases were considered to be TSP-1-positive when at least 20% of the tumor cells expressed TSP-1 in their cytoplasm.

Statistical analysis

Statistical analyses were carried out with the Chi square test using Statcel 3 software (The Publisher OMS Ltd., Tokorozawa, Japan). Differences of $p < 0.05$ were considered to be statistically significant.

RESULTS

Microscopic structural pattern and thrombospondin-1 expression

All 101 tumors manifested the typical histology of invasive ductal carcinoma with a definite stromal invasion. As shown in Table 1, 69 (68.3%) were carcinomas with a desmoplastic component and 32 (31.7%) were non- or minimal desmoplastic.

Table 1. Microscopic structural pattern and thrombospondin-1 (TSP-1) expression in invasive ductal carcinoma of the breast (n=101)

TSP-1 expression	Tumor with a non- or minimally desmoplastic component n (%)	Tumor with a desmoplastic component n (%)
Negative (n=47)	24 (75.0)	23 (33.3)
Positive (n=54)	8 (25.0)	46 (66.7)*
Total (n=101)	32	69

*p< 0.001 vs. Tumor with a non- or a minimally desmoplastic component by Chi square test

Immunoreactivity for TSP-1 was detected in the cytoplasm of cells from primary tumors and metastatic foci in the lymph nodes (Figures 1 and 2). In primary tumors, the percentage of TSP-1 immunoreactive cells varied from case to case among the 101 cases ; in 54 cases, at least 20% of the tumor cells were TSP-1-positive. Histologically, 46 of the 54 (85.2%) TSP-1-positive tumors had a desmoplastic component (Table 1), and of the 47 TSP-1-negative tumors, 23(48.9%) manifested a desmoplastic component.

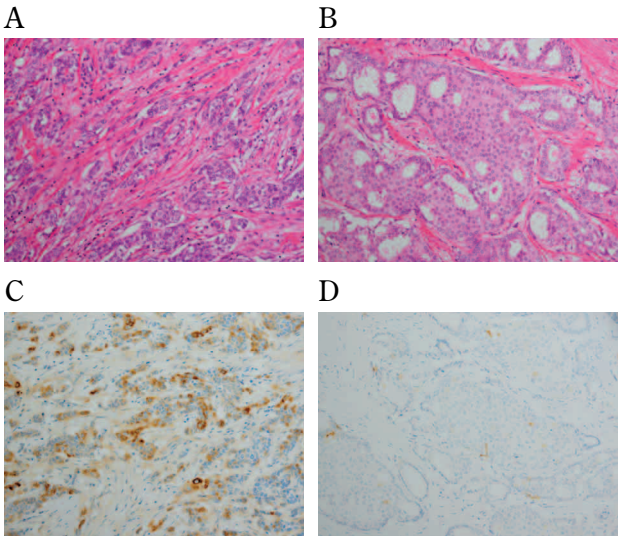

Figure 1. Invasive ductal carcinoma of the breast with a desmoplastic reaction (A) and with a non- or minimally desmoplastic component (B). Carcinomas with a desmoplastic component (C) showed TSP-1 immunoreactivity significantly higher than carcinomas with a non- or minimally desmoplastic component (D).

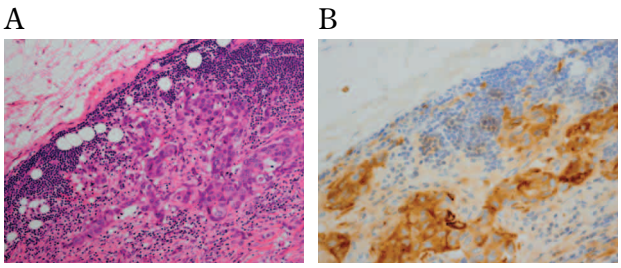

Figure 2. (A) Metastatic invasive ductal carcinoma in axillary lymph nodes. (B) Tumor cells in lymph nodes also showed TSP-1 expression at a high rate.

Among the 69 tumors with desmoplastic components, 46 (66.7%) were TSP-1-positive while of 32 tumors without- or minimally desmoplastic components, 8 (25.0%) were positive for TSP-1. These findings indicate that the incidence of TSP-1 expression was significantly higher in tumors with a desmoplastic component (p< 0.001, Table 1).

Axillary lymph node metastasis and thrombospondin-1 expression

Axillary lymph node metastasis from primary breast carcinoma was found in 39 of the 101 patients (38.6%) (Figure 2). Among the 54 patients with TSP-1-positive primary tumors, 28 (51.9%) manifested axillary lymph node metastasis (Table 2). On the other hand, 11 of 47 (23.4%) patients with TSP-1-negative primary tumors had axillary lymph node

Table 2. Axillary lymph node metastasis and thrombospondin-1 (TSP-1) expression in invasive ductal carcinoma of the breast (n=101)

TSP-1 expression	Tumor without axillary lymph node metastasis n (%)	Tumor with axillary lymph node metastasis n (%)
Negative in primary tumor (n=47)	36 (58.7)	11 (26.3)
Negative in axillary lymph nodes		5
Positive in axillary lymph nodes		6
Positive in primary tumor (n=54)	26 (41.3)	28 (73.7)*
Negative in axillary lymph nodes		8 (28.6)
Positive in axillary lymph nodes		20 (71.4)
Total (n=101)	62	39

*p< 0.005 vs. Tumor without axillary lymph node metastasis by Chi square test

metastasis. These findings indicate that in patients with TSP-1-positive primary breast tumors, the incidence of lymph node metastasis was significantly higher ($p<0.005$, Table 2) than in patients with TSP-1-negative primary tumors. TSP-1-positive cells were also present in the lymph nodes with a high rate of 20 of the 28 cases (71.4%) with TSP-1 positivity in primary tumors (Figure 2 and Table 2).

Tumor size and thrombopondin-1 expression

According to the TNM classification (13), primary tumors ranged 43 of T1 (≦2 cm), 53 of T2 (>2 to 5 cm) and 5 of T3 (>5 cm) in size. TSP-1 expression was observed in 19/43 of T1, 32/53 of T2 and 3/5 of T3 tumors. There was no significant difference in TSP-1 expression between any categories (T1 vs. T2, $p=0.169$; T1 vs. T3, $p=0.843$; T2 vs. T3, $p=0.644$)

DISCUSSION

Our immunohistochemical investigation of invasive ductal carcinomas showed that approximately half of these tumors expressed TSP-1 in the cytoplasm. The expression of TSP-1, a modulator of cell-matrix interactions whose molecular mechanisms play a role in the disease microenvironment, is usually detected in endothelial cells, platelets, fibroblasts, and macrophages in non-neoplastic tissue (14-18). TSP-1 induces cellular adhesion and motility, matrix metalloproteinase -2 activation, and endothelial cell apoptosis in inflammation and wound healing (15, 19-21). In early reports, invasive ductal carcinomas expressed TSP-1 protein in various locations such as basement membrane of ducts, stromal tissue and cytoplasm of tumor cells (22-24). Our findings in the present study indicate that TSP-1 is primarily produced and regulated by tumor cells rather than stroma. This suggests that tumor cells produce TSP-1 to remodel tissue as a better environment for invasion.

There has been no consensus with respect to the role of TSP-1 in tumor invasion and metastasis. Cell culture experiments suggested that the presence of TSP-1 accelerates invasion and metastasis in many types of cancer (24-27). On the other hand, Iddings *et al.* (27, 28), who studied human colorectal cancers immunohistochemically, found that significantly more tumors without than with lymph node metastasis expressed TSP-1, suggesting that TSP-1 inhibits tumor metastasis. In surgical samples from 31 patients with invasive ductal carcinoma of the breast, Wang-Rodriguez *et al.* (29) found no significant correlation between TSP-1 and lymph node metastasis. In their study, cases were relatively limited (twenty-two primary tumors with metastasis and nine without metastasis), and histological categorization including desmoplasia was not described. However, in the present study we had quite a large number of cases, 101 tumors. We focused on the desmoplastic reaction and TSP-1 expression and found that the incidence of lymph node metastasis was significantly higher in invasive ductal carcinomas expressing TSP-1 and that axillary lymph nodes involving metastasis tended to express TSP-1. Based on these observations we suggest that TSP-1 expression by primary breast tumors may be suggestive of their metastatic potential.

Histology of invasive ductal carcinoma of the breast varies. Desmoplastic carcinomas tend to be highly invasive (30). In our desmoplastic carcinomas, TSP-1 expression was primarily detected in the tumor cells immunohistochemically, and the desmoplastic components expressed TSP-1 strongly. In fact, 2/3 (66.7%) of the tumors with desmoplastic components expressed TSP-1 and the difference between desmoplastic- and non- or minimally desmoplastic tumors was significant ($p<0.001$). Our results suggest that TSP-1 released from carcinoma cells plays a role in the manifestation of the characteristic desmoplastic (scirrhous) features and stromal invasion of breast carcinoma.

Desmoplasia is also recognized in other carcinomas. Diffuse-type gastric carcinoma shows similar histological features to desmoplastic breast carcinoma. Several reports suggested that expression of transforming growth factor (TGF)-β and TSP-1 is closely associated with formation of fibrosis in diffuse-type gastric carcinoma (31-33). TGF-β causes desmoplasia by induction of myofibroblasts (34, 35). It is not clear whether gastric and breast carcinomas share the same mechanism of desmoplastic formation. Interaction of TGF-β with TSP-1 should be investigated in vitro and in vivo in future research.

Our study showed that the incidence of TSP-1 expression was significantly higher in invasive ductal carcinomas of the breast with axillary lymph node metastasis and in carcinomas with strong desmoplastic components. Our results suggest that TSP-1 plays a role in the invasion and metastasis of these tumors. Although TSP-1 has been documented to be anti-angiogenic and suggested as a candidate

anti-cancer drug, in patients with invasive ductal carcinoma of the breast it may promote tumor invasion and is associated with lymph node metastasis.

CONFLICT OF INTEREST

The authors of this manuscript have no conflict of interest.

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