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Significance of Membrane Type 1 Matrix Metalloproteinase Expression in Breast Cancer

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Expression of matrix metalloproteinases (MMPs) plays an essential role in tumor metastasis and invasion through the degradation of extracellular matrix (ECM). MT1-MMP (membrane type 1 matrix metalloproteinase), a membrane-type MMP, is responsible for the activation of MMP2. In this study the significance of MT1-MMP expression in human breast tumors was investigated by immunocytochemical assay, and its correlation with clinicobiological features was analyzed. MT1-MMP expression was detected in tumor cells and/or stromal cells, and there was a strong correlation between the expressions of MT1-MMP in the two cell types. Out of 183 primary tumors, 103 (56.2%) showed positive staining of MT1-MMP in tumor cells. MT1-MMP expression showed no significant correlation with any of the clinicobiological parameters examined, including hormone receptor status and angiogenesis. In postoperative survival analysis, MT1-MMP expression itself was not a significant prognostic factor. However, in the particular subgroup with the accumulation of thymidine phosphorylase (TP)-positive stromal cells, which have been activated by various stimuli, such as cytokines and hypoxia, MT1-MMP expression had a significant prognostic value. These data suggested that MT1-MMP might function cooperatively with tumor-associated stromal cells for the progression of breast cancer.

Key words: Breast cancer — Matrix metalloproteinase (MMP) — Membrane type 1 matrix metalloproteinase (MT1-MMP) — Thymidine phosphorylase (TP)

Proteolytic degeneration of the extracellular matrix (ECM) is a major step in tumor invasion. Matrix metalloproteinases (MMPs) are zinc proteinases that degrade various ECM components, including collagen types I–V, laminin, fibronectin, gelatin, elastin and proteoglycan.[1–5] In various cancers, MMP-2 (gelatinase A/72 kDa type-IV collagenase) and MMP-9 (gelatinase B/92 kDa type-IV collagenase) play important roles in tumor-cell invasion and metastasis.[6–8] In addition, MMP-2 and integrin $\alpha v\beta 3$ are known to be functionally associated with each other on the endothelial cell surface during angiogenesis.[9] A recent report indicated that the C-terminal hemopexin-like domain of MMP-2, termed PEX, can interact with integrin $\alpha v\beta 3$ and thereby block MMP-2 binding to this integrin.[10]

Membrane type 1 (MT1) MMP is a membranous type of MMP, which is activated by furin in the cells through processing of the RRKR sequences.[11, 12] MT1-MMP specifically activates pro-gelatinase, MMP-2.[13–22] The 68 kDa form (in gelatin zymography) of pro-MMP-2 is converted to the 62 kDa activated form by MT1-MMP through a 64 kDa intermediate.[15] MT1-MMP, which is predominantly immunolocalized in tumor cells and stromal cells,[6, 23–25] also has collagenolytic activity, sharing substrate specificity with interstitial collagenases. This implies a pivotal role of MT1-MMP in digestion of ECM by direct cleavage of the substrate and activation of pro-MMP-2.[7] Ueno *et al.* found a significant relationship between the level of MT1-MMP expression and the clinical stage, including the presence of lymphatic and distant metastasis, clinical stage and tumor size.[25] The relationship suggests the importance of MT1-MMP expression in breast cancer progression.

With their proteolytic capacities, it is possible for MMPs to process the precursor of tumor necrosis factor (TNF)-α into mature TNF.[26, 27] The cleaved soluble factors can elicit subsequent stromal reactions, such as promotion of angiogenesis and activation of tumor-associated stromal cells. Furthermore, in primary breast cancer, most of the MMPs are produced by activated stromal cells rather than tumor cells, which indicates a close linkage between tumor cells and stromal cells through soluble mediators and MMPs.[28]

In this study, we examined MT1-MMP expression by immunocytochemical assay and compared it with various clinico-biological features, particularly parameters of angiogenesis, such as microvessel density (MVD) and the

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expression of thymidine phosphorylase (TP). TP can stimulate endothelial chemotaxis and its expression is regulated by various microenvironmental factors.[29–34] The prognostic value of MT1-MMP expression will also be discussed.

PATIENTS, MATERIALS AND METHODS

Tumor samples Primary breast tumors from 183 unselected primary breast cancer patients who had undergone resection of the tumor, including mastectomy with dissection of axillary lymph nodes were examined in this study. Tumor samples were immediately frozen after removal and were stored at −80°C until use. The frozen tissue sample (0.2 g) was homogenized and extracted with 50 m*M* Tris-HCl buffer (pH 7.4), containing 0.25% Triton X-100 (2 ml). The tumor extracts were diluted according to their protein concentration and then assayed.

Immunocytochemistry For assessing MT1-MMP, TP, and MVD accumulation, 3–5 μm sections of paraffin-embedded primary tumor tissues were subjected to indirect anti-peroxidase immunocytochemical assays (Dako, Carpinteria, CA) using anti-MT1-MMP monoclonal antibody, anti-TP monoclonal antibody (Japan Roche Inst., Kamakura), and anti-factor-VIII related antigen antibody (Wako, Carpinteria, CA). Both MT1-MMP expression in tumor cells and that in stromal cells were assessed in terms of the staining intensity, which was categorized as "negative," "weak," "positive," "strong" and "very strong" by observation under an optical microscope. "Positive" gave a clearly higher staining intensity than normal mammary epithelium, and "positive," "strong," and "very strong" tumors were considered to be MT1-MMP-positive (+). Stromal cells include monocytic cells and fibroblastic cells. Stromal TP-positive cell density was counted in the five densest areas, "hot spots," identified visually in the microscopic field (per mm^2). The average of the three highest counts was taken as the stromal TP-positive cell count. Similarly, MVD was evaluated by counting the endothelial deposits in the most vascularized areas, as described previously.

Cytoplasmic staining of TP in tumor cells was graded as "negative," "weak," "positive" and "strong" according to the staining intensity, and "positive" and "strong" tumors were regarded as tumor cell TP-positive.

The immunocytochemical and pathological assessments were carried out by two pathologists who were blinded as to clinical information.

Hormone receptor assay Estrogen receptor (ER) and progesterone receptor (PgR) in the cytoplasmic fractions of the tumor extracts were measured by enzymatic immunoassay (EIA). For both ER and PgR, tumors containing more than 5 fmol/mg protein were designated as positive.

Adjuvant treatments and patient follow-up The indication and protocol of adjuvant treatment were chosen based on the patient status, i.e., nodal involvement (n), tumor size (T), age and ER. Polychemotherapy was given to patients who were node-positive at the age of 55 or younger. Hormonal therapy (tamoxifen, 2 years or more)

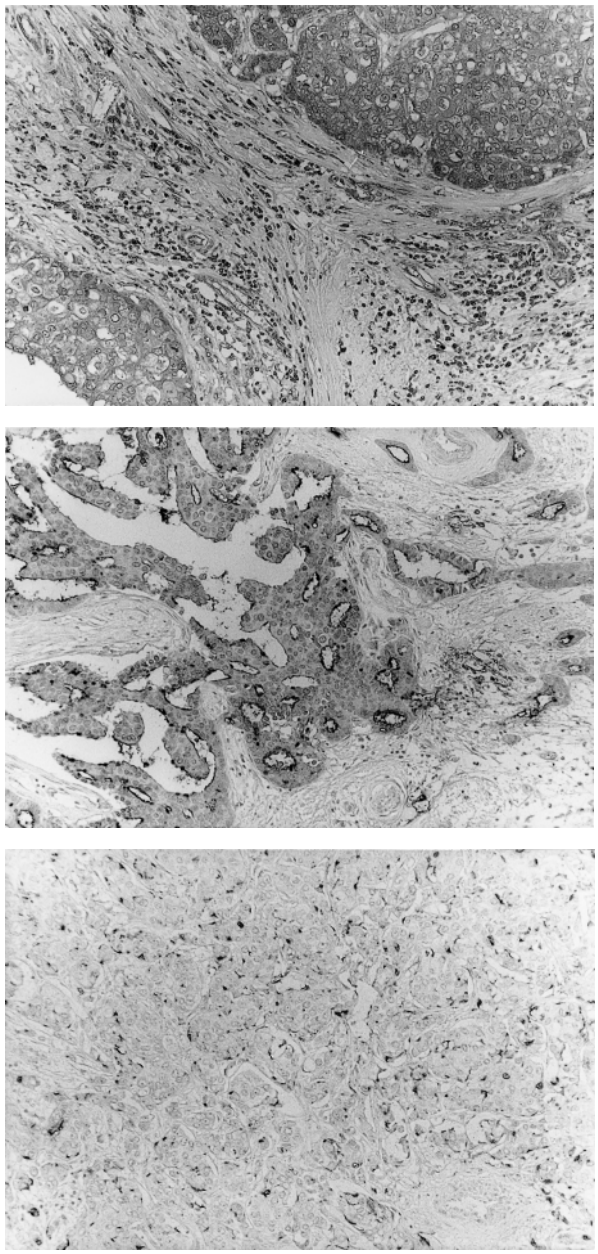

Fig. 1. Immunolocalization of MT1-MMP in breast cancer tissues. A, MT1-MMP is expressed in both tumor cells and stromal cells (stained); B, MT1-MMP is expressed predominantly in tumor cells; C, MT1-MMP is expressed predominantly in stromal cells.

was given to ER-positive patients. No information about MT1-MMP, TP, or MVD was available for any patient before the treatment. The condition of the patients was monitored at least every 3 months. Recurrence was diagnosed on the basis of histological examinations or radiographic, scintigraphic and CT scan images.

Statistics The χ^2 test and unpaired Student's *t* test were used for analyzing the relationship between patients' background and biological parameters. Survival curves were drawn by the Kaplan and Meier method. The difference in relapse-free survival was evaluated by means of log rank tests. The Beccel Mark-II analyzing system (Bec-

Table I. MT1-MMP Expression in Tumor Cells and Stromal Cells

Category	MT1-MMP in tumor cells			MT1-MMP in stromal cells		
	– (%)	+ (%)	*P* value	– (%)	+ (%)	*P* value
All patients	80	103		106	77	
Menopause						
pre-	28 (35.0)	51 (49.5)	NS	44 (41.5)	35 (45.5)	NS
post-	52 (65.0)	52 (50.5)		62 (58.5)	42 (54.5)	
Tumor size						
–2 cm	5 (6.2)	5 (4.8)		5 (4.7)	5 (6.5)	
2.1–5 cm	52 (65.0)	69 (70.0)	NS	73 (68.9)	48 (62.3)	NS
5.1 cm–	23 (28.8)	29 (28.2)		28 (26.4)	24 (31.2)	
No. of nodes						
0	31 (38.8)	43 (41.7)		39 (36.8)	35 (45.5)	
1–3	18 (22.5)	24 (23.3)	NS	27 (25.5)	15 (19.5)	NS
4–	31 (38.8)	36 (35.0)		40 (37.7)	27 (35.1)	
ER –	30 (37.5)	55 (53.4)		43 (40.6)	29 (37.7)	
+	42 (52.5)	42 (40.8)	NS	56 (52.8)	41 (53.2)	NS
unknown	8 (10.0)	6 (5.8)		7 (6.6)	7 (9.1)	
PgR –	35 (43.8)	41 (39.8)		40 (37.7)	36 (46.8)	
+	33 (41.3)	51 (49.5)	NS	55 (51.9)	29 (37.7)	NS
unknown	12 (15.0)	11 (10.7)		11 (10.4)	12 (15.6)	
Adjuvant therapy						
none	6 (7.5)	2 (1.9)		3 (2.8)	5 (6.5)	
endocrine	11 (13.8)	18 (17.5)	NS	16 (15.1)	13 (16.9)	NS
chemo	8 (10.0)	15 (14.6)		13 (12.3)	10 (13.0)	
chemo-endocrine	55 (68.8)	68 (66.0)		74 (69.8)	49 (63.6)	
MT1-MMP tumor						
–				60 (56.6)	20 (26.0)	$P<0.001$
+				46 (43.4)	57 (74.0)	$\chi^2=17.0$

Statistical analysis, χ^2 test; NS, not significant.

Table II. MT1-MMP and Angiogenesis

Category	MT1-MMP in tumor cells			MT1-MMP in stromal cells		
	– (%) *n*=80	+ (%) *n*=103	*P* value	– (%) *n*=106	+ (%) *n*=77	*P* value
MVD						
–100 counts/mm^2	46 (57.5)	60 (58.3)	NS	60 (56.6)	46 (59.7)	NS
101–	34 (42.5)	43 (41.7)		46 (43.4)	31 (40.3)	
TP in stromal cells –	44 (55.0)	63 (61.2)	NS	58 (54.7)	49 (63.6)	NS
+	36 (45.0)	40 (38.8)		48 (45.3)	28 (36.4)	

Statistical analysis, χ^2 test; NS, not significant.

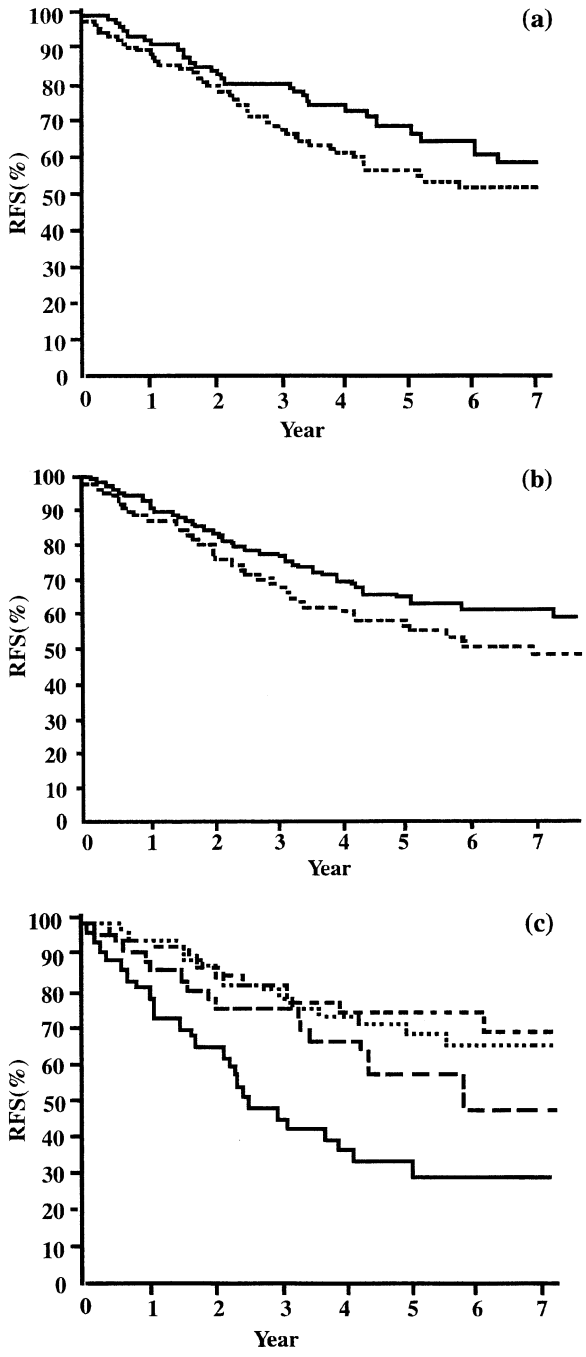

Fig. 2. (a, b) Disease-free survival plotted for MT1-MMP expression in tumor cells and in stromal cells. No significant difference was obtained. (c) Disease-free survival stratified by tumor cell MT1-MMP status and stromal cell TP status. There is a significant difference between the MT1(+), stromal TP(+) and MT1(–), stromal TP(+) groups ($P<0.05$). (a) —— MT1 –(80), ... MT1 +(103), (b) —— MT1 stroma –(106), ... MT1 stroma +(77), (c) —— MT1 +, TP stroma +(40), ---- MT1 –, TP stroma +(36), ····· MT1 –, TP stroma –(44), ····· MT1 +, TP stroma –(63).

cel, Tokyo) using the Cox proportional hazards model was used in multivariate analysis.

RESULTS

MT1-MMP expression was found in breast tumors in various patterns. In some cases, MT1-MMP was localized

Table III. Statistical Analysis

	Univariate analysis		Multivariate analysis	
	χ^2	*P* value	χ^2	*P* value
Tumor size (–3.0 cm vs 3.1 cm–)	3.397	0.045	3.844	0.0499
Nodal status (n– vs n+)	25.312	<0.001	17.929	<0.0001
ER (– vs +)	1.494	NS	—	—
MVD (–100 vs 101–)	17.978	<0.01	5.640	0.0175
TP in stromal cells (– vs +)	13.631	<0.01	18.524	<0.0001
MT1-MMP in tumor cells (– vs +)	0.572	NS	—	—
MT1-MMP in stromal cells (– vs +)	1.285	NS	—	—

NS, not significant.

Table IV. Subgroup Analysis

	MT1-MMP in tumor cells (– vs +)		MT1-MMP in stromal cells (– vs +)	
	χ^2	*P* value	χ^2	*P* value
Tumor size				
–3.0 cm	1.733	NS	0.217	NS
3.1 cm–	1.575	NS	0.028	NS
Nodal status				
n–	0.247	NS	0.663	NS
n+	0.351	NS	0.073	NS
ER				
–	0.998	NS	0.021	NS
+	1.330	NS	2.276	NS
MVD				
–100	2.693	NS	3.543	<0.1
101–	0.345	NS	0.022	NS
TP in stromal cells				
–	0.144	NS	0.920	NS
+	4.012	<0.05	0.439	NS

NS, not significant.

only in tumor cells or stromal cells, but in others, it was found in both tumor cells and stromal cells. One hundred and three cases (56.2%) showed MT1-MMP(+) expression in tumor cells, and 77 cases (42.1%) in the stroma. In 57 cases (31.1%) MT1-MMP was strongly immunolocalized in both tumor cells and stromal cells (Fig.1 and Table I).

There was no significant relationship between MT1-MMP expression, either in tumor cells or in stromal cells, and clinicopathological factors: T, n and hormone receptor status (Table I). With respect to the relationship with angiogenesis, no significant correlation between MT1-MMP and MVD, tumor cell TP and stromal TP status was obtained (Table II).

Neither tumor cell status, nor stromal MT1-MMP status showed a significant prognostic value for relapse-free survival (Fig. 2, a and b). In the multivariate analysis, nodal status, stromal TP expression and MVD had independent prognostic value (Table III). According to 2×2 subgroup analysis between MT1-MMP expression in either tumor cells or stromal cells and other parameters, stromal TP-positive and tumor cell MT1-MMP-positive phenotype showed a significantly poorer prognosis compared to the other three combination categories (Fig. 2c, $P<0.05$; log rank test). There was no combination effect between MT1-MMP expression and other factors (Table IV). In the subgroup with low MVD, stromal MT1-MMP(+) tumors tended to show poor prognosis compared to stromal MT1-MMP(−) tumors; however, the statistical significance was marginal.

DISCUSSION

MT1-MMP not only plays an important role in the specific activation of pro-MMP-2, but also shows a distinctive collagenolytic activity.[21] Four types of MT-MMPs have been identified, and MT1-MMP and MT2-MMP expressions were demonstrated to be enhanced in several types of human cancer tissues; for instance, breast tumors.[6, 16–19, 24] Ueno *et al*. detected MT1-MMP protein expression in both tumor cells and stromal cells by immunocytochemical analysis.[25] In this study using paraffin-embedded formalin-fixed sections, we confirmed that MT1-MMP protein expression is localized in tumor cells and stromal cells, including fibroblasts, endothelial cells and tissue-infiltrating monocytic cells. Although much remains unclear about the mechanism of MT1-MMP expression on tumor cells, there was a strong correlation between tumor cell MT1-MMP expression and stromal cell MT1-MMP expression. This clearly indicates the importance of the intratumoral microenvironment for the induction of MT1-MMP in breast cancer.

As to the regulation of MT1-MMP expression, concanavalin A is known to elevate MT1-MMP expression level in breast cancer cells.[35] TNF-α is also reported to be an inducer of MT1-MMP expression in synovial fibroblasts.[36] Because TNF-α can also stimulate the production of MMPs in monocytes and leucocytes,[29, 37, 38] TNF-α may be the key cytokine to regulate MMP expression level and activation. Indeed, TNF-α is expressed in the infiltrating monocytes in breast tumors.

In this study using 183 primary breast tumors, we found no significant correlation between MT1-MMP expression and various clinicopathological factors, including T, n and hormone receptor status. In the previous report, Ueno *et al*. showed that the expression of MT1-MMP mRNA was positively correlated with lymph node metastasis.[25] The reason for this discrepancy is not clear, but the difference of methodology is one possibility.

MT1-MMP status also showed no correlation with angiogenesis. It is evident that activated MMPs, such as MMP-2, are deeply involved in neovascularization, because new vessel formation obviously requires tissue degradation and remodeling. Further, intrinsic MMP inhibitors, which include TIMP-1, TIMP-2 and PEX are known to inhibit angiogenesis in experimental models. Therefore, the relationship between MT1-MMP and other parameters including other endothelial regulators and TIMPs needs to be investigated more thoroughly. In the previous analysis, we found that coexpression of plural factors, such as vascular endothelial growth factor (VEGF) and TP or VEGF and MMP-9, was important for increase of the grade of angiogenesis.[39]

MT1-MMP status showed no significant prognostic value in this study using immunocytochemical analysis. However, the subgroup analysis provided the intriguing result that the tumors with tumor cell MT1-MMP(+) and stromal cell TP(+) phenotype had a significantly worse prognosis as compared to those with stromal cell TP(+) but MT1-MMP(−) phenotype. According to recent studies, stromal TP expression is regarded as an indicator that the stromal cells are activated.[40] TP is well known to be upregulated by several cytokines, such as TNF-α interleukin (IL)-1, interferon-γ and hypoxia.[29, 41] Stromal cell TP expression was an independent prognostic indicator, although tumor cell TP status was not (data not shown), which suggests that stromal cell TP status may reflect a particular microenvironmental condition. Thus, TP-positive stromal cells may be activated and may be producing various protumor mediators, including MMP-2, which is mainly derived from the stromal cells in primary breast carcinoma tissues. Thus, the interaction between MT1-MMP(+) tumor cells and TP(+) activated stromal cells may be an important determinant for tumor cells to acquire aggressive malignant nature. The results are consistent with the idea that invasive tumor cells utilize pro-MMP-2 produced by stromal cells through MT1-MMP on the cell surface. In addition, TP itself can stimulate angio-

genesis through the induction of endothelial chemotaxis by 2-deoxy-D-ribose, which is an active metabolite of thymidine.[31] MT1-MMP and TP/2-deoxy-D-ribose might function cooperatively *in situ*. Several MMP inhibitors are under clinical trial. It will be of great interest to see how the effect of MMP inhibitors is associated with MT1-MMP expression and angiogenesis in the clinical setting.

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