MIAME guidelines issued by the Microarray Gene Expression Data group. Further analyses were done using GeneSpring version 7.3 (Silicon Genetics, San Carlos, CA). Microarray data were available from our previous study (http://cibex.nig.ac.jp/cibex2/index.jsp).[13]

Serum Collection

At 5 min prior to surgery, venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 min. They were then centrifuged at 1,500 rpm for 15 min. Serum was immediately aliquoted and kept at −80°C until assayed.

Serum Samples and MMP-1 Analysis

MMP-1 was determined using a quantitative solid-phase sandwich enzyme linked immunosorbent assay (ELISA) and tested in duplicate.[14,15] We also examined serum MT1-MMP by ELISA.[16]

Statistical Methods

Recorded variables included age, gender, date of surgery, date of death or last follow-up, histological records such as site of primary cancer, tumor size, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion and Dukes' stage, and serum level of MMP-1, as described previously. Missing values (<2%) were omitted from multivariate analyses.

A disease-free survival curve and overall survival curve were plotted according to the Kaplan–Meier method. The difference between the survival curves was analyzed by the Wilcoxon and log-rank test. The effects of various clinicopathological factors on disease-free and overall survival (including the serum level of MMP-1) were assessed by the Cox proportional hazards model. All analyses were performed using Stat View 5.0 for Windows (SAS Institute, Cary, NC). The study protocol was approved by the Institutional Review Board, and informed consent was obtained from all patients.

RESULTS

Gene Expression Within the MMP Family in CRC and Selection of Candidate Markers for Serum Analysis

Figure 1 shows the expression of all *MMP* family genes in stromal cells and cancer cells excised by laser microdissection. These data show the mean values from cancer cells and normal cells in colon epithelial tissues from 73 CRC patients. In addition, normal cells from non-CRC patients were analyzed at the same time, and the normal level of gene expression was set at 1.00 for each *MMP*. In cancer cells, expression values for *MMP-1*, *MMP-3*, *MMP-7*, *MMP-11*, and *MMP-12* were 3 times higher than those observed in normal cells. In fact, expression of those *MMPs* was elevated in interstitial cells as well as in cancer cells in CRC. In addition, expression levels of *MMP-2*, *MMP-9*, and *MMP-10* in stromal cells were more than 10 times higher than in normal controls. Other *MMPs* showed

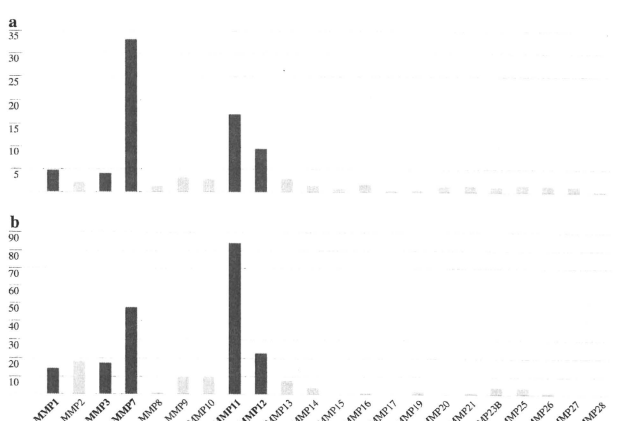

FIG. 1 Relative expression of MMPs in interstitial cells adjacent to cancer tissues (**a**) and in cancer cells (**b**). **a** The vertical axis showed the relative expression ratio of MMPs in interstitial cells in comparison to that in normal control cells. MMP-1, MMP-3, MMP-7, MMP-11, and MMP-12 were three times higher than normal controls. **b** In addition to the above 5 MMPs, expression levels of MMP-2, MMP-9, and MMP-10 were more than 10 times higher than normal controls. Therefore, expression of MMP-1, MMP-3, MMP-7, MMP-11, and MMP-12 (colored *red*) was elevated in interstitial cells as well as in cancer cells in CRC. Other MMPs (colored *blue*) showed little expression in this microarray analysis

little expression in this microarray analysis. These data identified MMP-1, MMP-3, MMP-11, and MMP-12 as candidates for further serum analysis. Although MMP-11 seemed to be an attractive candidate because of its high expression values, we focused on MMP-1 because this enzyme seems to play a more important role than the other 3 MMPs both in the transition from adenoma to carcinoma, and CRC progression as well as positive interactions of MMP-2, MMP-9, and MMP-7.[8] MMP-11 seems to contribute only to the transition from adenoma to carcinoma.[8]

Patient Characteristics and Serum Levels of MMP-1 in CRC Patients Receiving Curative Resection

During the study period, 100 patients with CRC were diagnosed for the first time and underwent colectomy at Teikyo University. Within this group, 25 patients were found to be surgically noncurative, and 75 patients were potentially surgically curative and were approved for the current study. Median age of the patients in our sample was 68.6 years, and 57.3% were males. The mean and median serum levels of MMP-1 were 49.2 and 48 ng/ml, respectively (range 8–161 ng/ml).

The correlations between serum MMP-1 levels and clinicopathological factors, including recurrence, are shown in Table 1. Although no significant association was found for pathological factors, serum MMP-1 levels were higher in patients with recurrence of disease. In addition, the serum levels of MT1-MMP (MMP-14) and CEA were compared with clinicopathologic variables. No significant association was found. However, the incidence of recurrence was higher in serum MT1-MMP-positive cases (Table 1, $P < .05$).

Association of Disease-Free Survival and Overall Survival with Serum MMP-1 Levels in CRC Patients Undergoing Curative Resection

The cut-off value for serum MMP-1 levels was set at the average value (49.2 ng/ml), and patients were divided into 2 groups, "MMP-1 high" and "MMP-1 low." The MMP-1 high group consisted of 25 patients, and the low group contained 50 patients. We divided 75 patients by the average expression level of MMP-1. Disease-free survival was 51% in the MMP-1 high group and 81% in the low group ($P < .05$) (Fig. 2). Overall survival was 52% in the

TABLE 1 Serum MMP1 and MT1-MMP levels and correlation with clinicopathologic factors

	n	Serum MMP1 (ng/ml)	*P* value	Serum MT1-MMP (ng/ml)	*P* value	Serum CEA (ng/ml)	*P* value	Serum CA19-9 (ng/ml)	*P* value
Depth of tumor			ns		ns		ns		ns
Shallow	27	46.3 + 7.4		0.56 + 0.14		6.3 + 11.2		18.3 + 24.5	
Deep	48	49.3 + 5.5		0.67 + 0.1		24.4 + 8.4		79.2 + 18.4	
Histology			ns		ns		ns		ns
Well	56	49.2 + 5.1		0.45 + 0.17		13.3 + 7.8		71.4 + 30.0	
Mod	19	45.4 + 8.8		0.70 + 0.10		31.4 + 12.4		52.5 + 17.4	
Tumor size			ns		ns		ns		.001
<5.0 cm	45	53.5 + 5.6		0.51 + 0.11		13.7 + 8.7		18.1 + 18.1	
>5.0 cm	30	40.3 + 6.9		0.82 + 0.13		24.1 + 10.7		116.1 + 22.2	
Lymphatic permeation			ns		ns		ns		ns
Absent	54	50.5 + 5.2		0.60 + 0.10		15.1 + 8.0		57.4 + 17.6	
Present	21	42.4 + 8.3		0.72 + 0.16		24.9 + 12.8		57.1 + 28.6	
Vascular permeation			ns		ns		ns		ns
Absent	40	47.7 + 6.1		0.54 + 0.12		15.1 + 8.0		40.6 + 20.6	
Present	35	48.8 + 6.5		0.73 + 0.12		24.9 + 12.8		76.4 + 21.9	
Lymph node metastasis			ns		ns		ns		.0002
Absent	50	47.8 + 5.4		0.56 + 0.10		14.5 + 8.3		19.6 + 16.9	
Present	25	49.2 + 7.7		0.77 + 0.15		24.5 + 11.7		132.8 + 23.9	
Recurrence			.0013		.018		ns		.0007
Absent	54	39.6 + 4.9		0.51 + 0.10		13.9 + 8.0		26.3 + 16.5	
Present	21	70.4 + 7.8		0.95 + 0.16		28.1 + 12.8		137.0 + 26.4	

n number of cases, *ns* no statistical significance, *shallow* tumor invading within proper muscle, *deep* tumor invading beyond the proper muscle, *well* well differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma

MMP-1 high group and 90% in the low group ($P < .05$) (Fig. 3). In addition, serum MT1-MMP, CEA, and CA19-9 were analyzed in the same way. There were no statistically significant correlations for MT1-MMP and CEA values. However, high CA19-9 activity indicated a higher incidence of recurrence than did CA19-9 low activity (Figs. 2, 3).

Univariate analysis of disease-free survival showed significant differences in MMP-1 level, depth of invasion and lymph node metastasis (Table 2). Univariate analysis for overall survival showed significant differences in MMP-1 level, depth of invasion, lymph node metastasis and venous invasion (Table 3). In multivariate analysis of disease-free survival, MMP-1 level and lymph node metastasis were significant independent prognostic indicators (Table 2). In multivariate analysis of overall survival, MMP-1 level was the only significant independent prognostic indicator (Table 3). Serum MT1-MMP was not a significant independent prognostic factor for disease-free survival or overall survival in univariate and multivariate analyses (Tables 2, 3).

DISCUSSION

In the current study, we examined serum MMP levels to determine which was most suitable to predict the course of disease in CRC cases.[11,17] Microarray analyses showed that *MMP-1*, *MMP-3*, *MMP-7*, *MMP-11*, and *MMP-12* were overexpressed in both cancer cells and stromal cells. Among these, we focused on MMP-1 and demonstrated that high levels of serum MMP-1 correlated with poor survival in patients with CRC after curative resection. Furthermore, serum MMP-1 was an independent and specific prognostic factor for survival in CRC patients. Moreover, we showed that serum MMP-1 level was more useful than more established serum tumor markers, such as CEA and CA19-9, as shown in Figs. 2, 3. There is substantial evidence that MMP-2, MMP-9, and MMP-7 are overexpressed in primary CRC tumors. MMP-7 contributed broadly to each stage of CRC progression: transition from normal mucosa to adenoma, and adenoma to carcinoma, and then to metastasis. Recently, it was reported that serum MMP-7 levels are significantly elevated in patients with

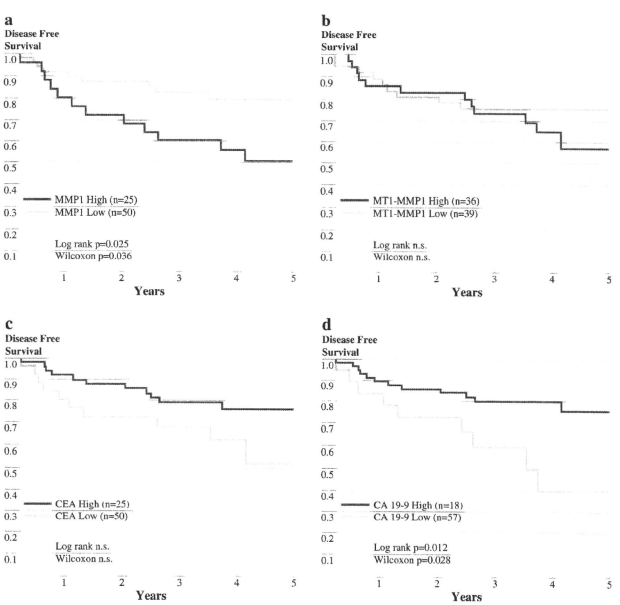

FIG. 2 Disease-free survival rates and correlations with serum markers in 75 cases of CRC following curative surgery. **a** There were significant differences between the 25 cases with high MMP-1 serum activity versus the 50 cases with low MMP-1 serum activity (log rank $P = 0.025$, Wilcoxon $P = .036$). **b** No significant difference was observed between 39 cases with MT1-MMP high activity and 36 cases with low activity. **c** There was no statistical significance in CEA values; however, 18 cases with high CA19-9 activity indicated a higher incidence of recurrence than the 57 cases with low CA19-9 activity (log rank $P = .012$, Wilcoxon $P = .028$)

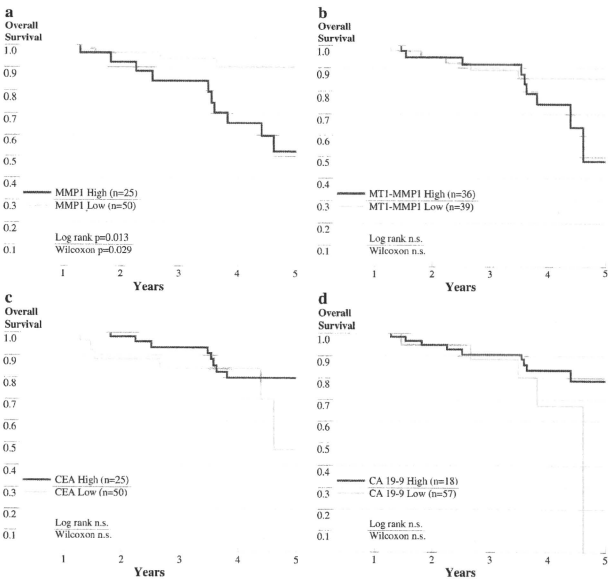

FIG. 3 Overall survival (OS) rates and correlations with serum markers in 75 cases of CRC following curative surgery. **a** 25 cases with high MMP-1 activity showed poorer OS than 50 cases with low MMP-1 activity (log rank $P = .013$, Wilcoxon $P = .029$). However, there were no statistically significant differences in **b** serum MT1-MMP, **c** CEA, or **d** CA19-9

TABLE 2 Univariate and multivariate analysis for disease free survival in CRC cases with curative operation

	Univariate analysis		*P* value	Multivariate analysis		*P* value
	RC	Hazard ratio		RC	Hazard ratio	
Serum MMP1	0.488	1.629	.027*	0.473	1.605	.033*
Serum MT1-MMP	0.628	1.874	.084			
Depth of tumor	−0.744	0.475	.006*	−0.493	0.61	.12
Lymph node metastasis	0.617	1.853	.006*	0.467	1.594	.047*
Histology	0.12	1.128	.625			
Tumor size	−0.315	0.729	.151			
Lymphatic permeation	0.237	1.268	.301			
Vascular permeation	0.368	1.444	.967			

* There is a statistical significance

CRC and serum MMP-7 is an independent prognostic factor for survival in advanced CRC.[11] On the other hand, MMP-2, and MMP-9 may be most important in the early steps of CRC carcinogenesis. Recent studies suggest that serum type IV collagenase (MMP-2 and MMP-9) may be correlated with progression of CRC; however, it has not been proven that serum MMP-2 and MMP-9 are preferred prognostic indicators.

As for the origin of serum MMP-1, there are 2 possibilities. Cancer cells and host stromal cells secrete MMP-1 at the primary cancer site. During tumor progression and invasion, MMP-1 might then drain into the circulation. Alternatively, MMP-1 might be secreted as a response against the invasion of cancer cells, not only at the primary cancer site but also in bone marrow or peripheral blood. The serum MMP-1 level, then, likely represents the total

TABLE 3 Univariate and multivariate analysis for overall survival in CRC cases with curative operation

	Univariate analysis		*P* value	Multivariate analysis		*P* value
	RC	Hazard ratio		RC	Hazard ratio	
Serum MMP1	0.693	1.998	.014*	0.667	1.95	.019*
Serum MT1-MMP	0.182	1.2	.74			
Depth of tumor	−0.721	0.486	.028*	−0.163	0.849	.732
Lymph node metastasis	0.61	1.841	.025*	0.529	1.698	.07
Histology	0.087	1.091	.772			
Tumor size	−0.112	0.894	.68			
Lymphatic permeation	0.386	1.471	.17			
Vascular permeation	0.576	1.78	.039*	0.355	1.426	.28

* There is a statistical significance

amount of MMP-1 secreted from host tissues. On the other hand, Trivedi et al. suggested that MMP-1 might be derived from peripheral blood platelets.[18] Platelets harbor several MMPs that modulate hemostatic function and platelet survival, and they demonstrated that platelet MMP-1 activates protease-activated receptor-1 (PAR1) on the surface of platelets. MMP-1 mediated aggregation through PAR1 activates Rho-GTP pathways, cell shape change, motility, and MAPK signaling.[18] Indeed, we speculate that platelets activated by MMP-1 might play an important role in cancer metastasis. Further investigation will be required to identify the origin of MMP-1 in peripheral blood.

Most MMPs are secreted as inactive zymogens and are activated extracellularly. A portion of the MMP family (MT1-MMP, MMP-16, MMP-17, MMP-24, and MMP-25) is constituted by transmembrane proteases that are activated on the cell surface.[19,20] These MMPs may be good tumor markers because of their tumor specificity.[21] The current microarray expression profile showed that most of these transmembrane *MMPs* were expressed at low levels. Of the transmembrane *MMPs*, MT1-MMP showed the highest gene expression level. However, the serum level of MT1-MMP was not correlated with CRC patient prognosis. It appears that the expression level of MT1-MMP was too low to show significant differences in the serum.

In conclusion, within the MMP family, MMP-1 is not a cancer-specific protease. However, its expression level predicts the course of invasion and progression of malignant cells when assessed in the serum of CRC patients. In this study, the number of enrolled patients was small and the timing of sample measurement was necessarily limited to a single point. Further investigation is needed to validate the reproducibility of the current data.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

The Clinical Significance of Vimentin-Expressing Gastric Cancer Cells in Bone Marrow

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ABSTRACT

Background. Expression of the mesenchymal marker gene vimentin (*VIM*) in gastric cancer is associated with a more aggressive form of the disease and poor prognosis. Because epithelial mesenchymal transition (EMT) plays a critical role in the progression of gastric cancer, *VIM* expression was examined in the bone marrow (BM) of gastric cancer patients.

Methods. BM samples from 437 gastric cancer patients were collected and analyzed by quantitative RT-PCR. Expression of VIM protein in the primary lesions of resected gastric cancers was evaluated using immunohistochemistry. Furthermore, induction of *VIM* expression by TGF-β1 and hypoxia was evaluated in gastric cancer cells.

Results. *VIM* mRNA expression increased concordantly with clinical staging and was significantly associated with tumor invasion and lymph node metastasis ($P < .0001$). Though cancer cells in the primary lesions did not stain with VIM antibody, some of the cells invading the intratumoral vessels were strongly positive for VIM, but were negative for E-cadherin. Hypoxic conditions and treatment with TGF-β1 induced *VIM* expression and repressed E-cadherin in gastric cancer cells, coupled with an alteration of cellular morphology.

Conclusions. We found that gastric cancer cells undergo EMT in BM to survive and metastasize. These findings suggest that isolated tumor cells have the potential to undergo EMT, which could increase the malignancy of gastric cancer.

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Vimentin (VIM) expression has been reported in a variety of carcinomas, such as kidney, breast, lung, and thyroid.[1–4] Utsunomiya et al.[5] reported the significance of VIM expression in solid, poorly differentiated gastric adenocarcinomas, showing that the prognosis of VIM-expressing cases was poorer compared with that of VIM-negative cases. VIM is the predominant intermediate filament protein in mesenchymal cells and is not usually expressed by epithelial cells. Several previous studies indicate that VIM expression induces invasive behavior in human epithelial carcinoma cell lines.[6–8]

Recently, attention has focused on the role of EMT in cancer progression.[9] During epithelial mesenchymal transition (EMT), epithelial cell-cell adhesion is decreased by the downregulation of adhesion molecules such as E-cadherin, and cell morphology becomes fibroblastlike with upregulation of *VIM*.[10] EMT promotes cellular motility, invasiveness, and cytoskeletal rearrangement in a range of cancer cell lines.[11] Furthermore, several studies revealed that transcriptional repressors of E-cadherin such as zinc finger proteins (ZEB1, ZEB2), bHLH protein (twist), and the snail family of zinc finger protein (snail, slug) are associated with EMT.[12–16] Thus, they are useful markers to predict prognosis in various human carcinomas.[17–19] In addition, Kim et al.[20] demonstrated that the expression of EMT-related genes such as E-cadherin, vimentin, N-cadherin, and snail were associated with poor prognosis in gastric cancer.

Development of distant metastases is the final stage of solid cancer progression and is responsible for the majority of cancer-related deaths. Distant metastatic spread has been considered to be a late process in malignant progression, but a recent study suggests that dissemination of primary cancer cells to distant sites might actually be an early event.[21] Disseminated tumor cells can be detected at early stages of tumor progression in lymph nodes, peripheral blood, and in bone marrow (BM) of cancer patients.[22,23] BM may be an important reservoir of tumor cells, from which they recirculate into distant organs such as liver or lungs. Furthermore, recent studies in vivo by Kaplan et al. and Gal et al. revealed that BM-derived cells are required for metastasis.[24,25] Indeed, in a large scale study of gastric cancer cases, we found that the simultaneous expression of epithelial markers (*CEA*, *CK-7*, and *CK-19*) and high levels of *VEGFR-1* expression in BM were significantly associated with hematogeneous metastases.[26]

The present study was designed to examine the clinical magnitude of *VIM* expression in BM and its relation to the progression of gastric cancer. The clinical findings suggest that *VIM* overexpression originates in epithelial cancer cells after the mesenchymal transition. Therefore, we first examined and determined the clinical significance of *VIM* mRNA expression in BM from gastric cancer patients, assessing both isolated tumor cells as well as adjacent host progenitor cells. Second, to provide explanations for clinical findings, we asked whether *VIM* expression was induced in gastric cancer cells by transforming growth factor-β1 (TGF-β1) or hypoxic treatments, both of which are recognized as important inducers of EMT.[10,27] Finally, to determine whether *VIM* expression provides cancer cells the ability to invade tumor vessels and to metastasize to BM, we examined the expression of VIM protein immunohistochemically in poorly differentiated stomach adenocarcinomas including 2 cases of gastric cancer with rhabdoid features.

MATERIALS AND METHODS

Patients

Physicians (T.F. and M.S.) collected BM samples from 437 Japanese gastric cancer patients who underwent surgery from 2001 to 2004 at the Central Hospital, National Cancer Center, Tokyo, Japan. Documented informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee. The average age of the 293 male and 144 female patients was 61.9 years, with a range of 27–86 years (Table 1). Based on the Treaty for Japanese Gastric Cancer Association, 122 cases were classified as stage I, 109 as stage II, 101 as stage III, and 105 as stage IV.[28] Normal negative controls consisted of BM samples collected from 20 patients, with no malignancies (e.g., gallstone and hernia cases), between April 2000 to March 2003.

TABLE 1 *VIM* mRNA expression and clinicopathological factors

Factors	Positive (n = 178)		Negative (n = 259)		P value
	No.	%	No.	%	
Age (mean ± SD)	61.2 ± 11.7		62.4 ± 11.6		.31
Sex					
Male	111	62.4	182	70.3	.08
Female	67	37.6	77	29.7	
Histological grade					.29
Well, moderately	67	37.6	108	41.7	
Poorly, others	111	62.4	151	58.3	
Size	0.15				
<50 mm (small)	68	38.2	117	45.2	
>51 mm (large)	110	61.8	142	54.8	
Depth of tumor invasion					<.0001
m, sm	33	18.5	104	40.2	
mp, ss, se, si	145	71.5	155	59.8	
Lymph node metastasis					<.0001
Absent	38	21.4	117	45.2	
Present	140	78.6	142	54.8	
Lymphatic invasion					.001
Absent	59	33.2	126	48.7	
Present	119	66.8	133	51.3	
Venous invasion					.12
Absent	124	69.7	198	76.5	
Present	54	30.3	61	23.5	
Liver metastasis					.37
Absent	173	97.2	255	98.5	
Present	5	2.8	4	1.5	
Peritoneal dissemination					.92
Absent	161	90.5	235	90.7	
Present	17	9.5	24	9.3	
Distant metastasis					.19
Absent	168	94.4	251	96.9	
Present	10	5.6	8	3.1	
Recurrence					.87
Absent	173	97.2	251	96.9	
Present	5	2.8	8	3.1	
Stage					.01
I–II	81	45.5	150	57.9	
III–IV	97	54.5	109	42.1	

m tumor invasion of mucosa, *sm* submucosa, *mp* muscularis propria, *ss* subserosa, *se* penetration of serosa, *si* invasion of adjacent structures

BM Collection

Aspiration of BM was conducted under general anesthesia immediately before surgery as previously described.[26] The BM aspirate was obtained from the sternum using a BM aspiration needle. The first 1.0 ml of BM was discarded to avoid contamination by the skin. The second 1.0 ml of BM was added to 4.0 ml of Isogen-LS (Nippon Gene, Toyama, Japan), which was shaken vigorously and stored at −80°C until RNA extraction.

Total RNA Extraction and First-Strand cDNA Synthesis

Samples transferred from Tokyo to Beppu remained frozen while in transit. Total RNA was extracted from BM according to the manufacturer's protocol as described elsewhere.[29] The reverse transcriptase reaction (RT) was performed as previously described.[30] The first cDNA strand was synthesized from 2.7 μg of total RNA in 30 μl reaction mixtures containing 5 μl 5× RT buffer (Gibco BRL, Gaithersburg, MD), 200 μM dNTPs, 100 μM of a random hexadeoxynucleotide mixture, 50 units of RNasin (Promega, Madison, WI), 2 μl of 0.1 M dithiothreitol, and 100 units of Maloney leukemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice.

Cell Culture and Oxygen Deprivation

The human gastric cancer cell line NUGC3 was obtained from the Japanese Cancer Research Bank, Tokyo, Japan. The cell line was maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (Equitec-Bio, Ingram, TX) and 100 units/ml penicillin G and streptomycin (Invitrogen). The cells were incubated in 5% CO_2 at 37°C and passaged every 3 days. Oxygen deprivation was carried out in an incubator with 1% O_2, 5% CO_2, and 94% N_2 at 37°C for 5 days.

Induction of EMT by TGF-β1

EMT induction by TGF-β1 was performed using a modification of methods described by Rees et al.[31] Cells were seeded into 6-well plates at 70% confluency and incubated in standard medium for 48 h. The cells were then incubated in serum-free medium supplemented with 5 μg/ml transferrin, 5 μg/ml insulin, 5×10^{-8} mol/l hydrocortisone, and 10 ng/ml endothelial growth factor (EGF) at 37°C in 5% CO_2 for 120 h with 0, 0.05, 0.5, or 5 ng/ml human TGF-β1 (R&D Systems, Inc., Minneapolis, MN). The culture medium was replaced daily.

Quantitative RT-PCR

The primer sequences used to amplify *VIM*, E-cadherin, slug, *ZEB1*, *SIP1*, twist, snail, N-cadherin, and *FN1* mRNA are shown in Supplemental Table 1. Glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as an internal control (Supplemental Table 1). Real-time monitoring of PCR reactions was performed using the LightCycler System (Roche Applied Science, Indianapolis, IN) and SYBR-Green I dye (Roche Diagnostics, Tokyo, Japan). Monitoring was performed according to the manufacturer's instructions, as previously described.[32] In brief, reactions were prepared on ice, containing 1 μl of cDNA, 1× DNA Master SYBR-Green I, 50 ng of primers, and 3 mM $MgCl_2$. The final volume was adjusted to 20 μl with water. After the reaction mixture was loaded into glass capillary tubes, quantitative RT-PCR was performed with the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, annealing at 62°C for 10 s, and extension at 72°C for 10 s. After amplification, the amplicons were subjected to a temperature gradient from 67 to 95°C at 0.2°C/s, under continuous fluorescence monitoring, to produce a melting curve of the products.

Data Analysis for RT-PCR

After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from the baseline. This cycle number was used as the crossing point value. A standard curve was produced by measuring the crossing point of each standard value and plotting it against the logarithmic value concentration. Concentrations of unknown samples were calculated by plotting their crossing points against the standard curve and dividing by *GAPDH* content.

Immunohistochemistry

Immunohistochemical studies for VIM and E-cadherin were performed on formalin-fixed, paraffin-embedded surgical sections obtained from gastric cancer patients. Tissue sections were deparaffinized, soaked in 0.01 M sodium citrate buffer, and boiled in a microwave for 5 min at 500 W to retrieve cell antigens. The primary mouse monoclonal antibodies against VIM (sc-6260 Santa Cruz Biotechnology) and E-cadherin (BD Biosciences) were used at a dilution of 1:50 and 1:100, respectively. Tissue sections were stained using an avidin-biotin-peroxidase protocol (LSAB+ system-HRP; DAKO, Kyoto, Japan) and counterstained with hematoxylin.

Immunocytochemistry

Cells were plated in poly-D-lysine coated 2-well culture slides (BD BioCoat Poly-D-Lysine 2-well culture slide, BD Biosciences) at a density of 1×10^5 cells/well. The cells were incubated in serum-free medium supplemented with 5 μg/ml transferrin, 5 μg/ml insulin, 5×10^{-8} mol/l hydrocortisone, and 10 ng/ml endothelial growth factor (EGF) at 37°C in 5% CO_2 for 120 h with 0, 0.05, or 5 ng/ml human TGF-β1. Culture medium was replaced daily. After treatment with TGF-β1, the cells were fixed in −20°C methanol for 10 min and stained with mouse monoclonal antibody against VIM (sc-6260 Santa Cruz Biotechnology) and E-cadherin (BD Biosciences). All sections were counterstained with hematoxylin.

Statistical Analysis

For continuous variables, data were expressed as the mean ± standard deviation. The relationship between *VIM* mRNA expression and clinicopathological factors was analyzed using a chi-square test and *t* test. Findings were considered significant when the *P* value was $< .05$. All tests were performed using JMP software (SAS Institute Inc., Cary, NC).

RESULTS

Clinicopathological Significance of VIM mRNA in BM of Gastric Cancer Patients

The mean expression level of *VIM* mRNA gradually increased concordantly with advanced clinical staging. Taking into consideration the clinical application of the current study, the 95% confidence interval was used to define the limits for the normal case cutoff values, according to the reference intervals of the Clinical and Laboratory Standards Institute.[33] Levels that were higher or lower than the cutoff values were defined as "positive" or "negative," respectively. The association between clinicopathological features and *VIM* mRNA expression is summarized in Table 1. In the *VIM*-positive group, the depth of tumor invasion, lymph node metastasis, and lymphatic invasion were significantly higher than in the *VIM*-negative group ($P < .0001$, $P < .0001$, and $P = .001$, respectively). In addition, the *VIM*-positive group had more advanced stage cases than the negative group ($P = .01$).

Immunohistochemistry of VIM and E-Cadherin Expression in Intratumoral Vessels of Gastric Cancer Patients

Expression of VIM protein in the primary lesions of resected gastric cancers was evaluated using immunohistochemistry. Cancer cells in the primary lesions did not stain with VIM antibody (Fig. 1a, b). However, some of the cells invading the intratumoral vessels were strongly positive for VIM (Fig. 1c, d), but were negative for E-cadherin. In contrast, the VIM-negative cells stained positively for E-cadherin (Fig. 1e, f).

TGF-β1 Alters Expression of VIM in Gastric Cancer Cells

We examined how TGF-β1 induction of EMT altered VIM expression in a gastric cancer cell line. NUGC3 cells were selected for EMT induction because of their low expression levels of *VIM* mRNA and VIM protein. VIM expression and morphological changes were evaluated using immunocytochemistry. Figure 2a, b show that cell

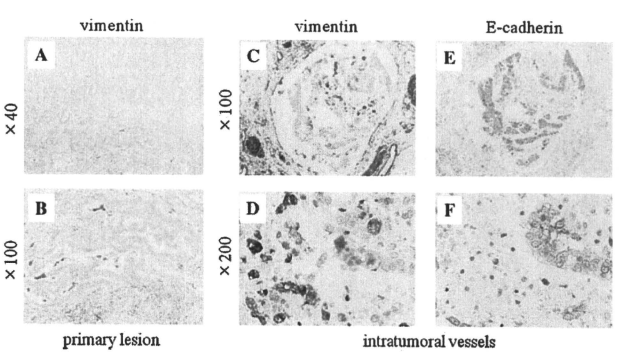

FIG. 1 Expression of VIM and E-cadherin proteins evaluated by immunohistochemistry of primary lesions in gastric cancer. **a, b** Cancer cells in the primary lesion did not express VIM. **c, d** Cells invading intratumoral vessels strongly expressed VIM. **e, f** VIM-positive cells in the intratumoral vessels were negative for E-cadherin. Conversely, VIM-negative cells were positive for E-cadherin

shape changed from round to fibroblastlike as VIM expression increased with the concentration of TGF-β1. In contrast, Fig. 2c, d shows that expression of E-cadherin was decreased by the addition of TGF-β1. The expression of *VIM* mRNA increased 5-fold after the addition of 5 ng/ml TGF-β1 (Fig. 2e). Levels of fibronectin and N-cadherin mRNA (mesenchymal markers) (data not shown) and slug mRNA (transcriptional repressor of E-cadherin) also rose as the concentration of TGF-β1 increased. Other transcriptional repressors of E-cadherin, such as snail, twist, SIP1, and ZEB1 mRNA, were not altered by TGF-β1 (data not shown). In contrast, the expression of E-cadherin mRNA decreased with increasing concentrations of TGF-β1 (Fig. 2e).

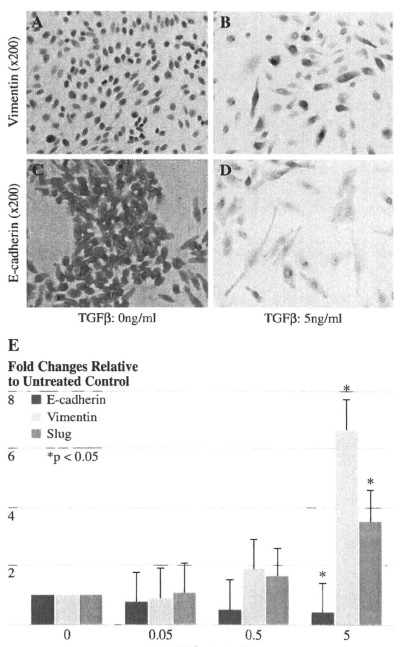

FIG. 2 Alteration of *VIM* expression in NUGC3 cells following addition of TGF-β1. **a, b** The shape of cells changed from round to fibroblast-like with increasing VIM expression after the addition of TGF-β1. **c, d** The expression of E-cadherin decreased after the addition of TGF-β1. **e** *VIM* and slug mRNA expression increased as the concentration of TGF-β1 increased, conversely, E-cadherin decreased. Data are expressed as the fold-change relative to the untreated control (TGF-β1: 0 ng/ml), * $P < .05$

Alteration of VIM Expression in Gastric Cancer Cells Under Hypoxic Conditions

After verifying VIM protein expression in NUGC3 cells under hypoxic conditions, VIM expression and morphological changes were evaluated by immunocytochemical analysis. Hypoxia stimulated an increase in VIM protein levels in NUGC3 cells and a change in shape from round to fibroblast-like (Fig. 3a, b). The loss of E-cadherin protein expression was observed with the alteration of cellular morphology (Fig. 3c, d). The expression of *VIM* mRNA under hypoxic conditions was significantly higher than that observed under normoxia, while E-cadherin mRNA decreased (Fig. 3e). Furthermore, slug mRNA also

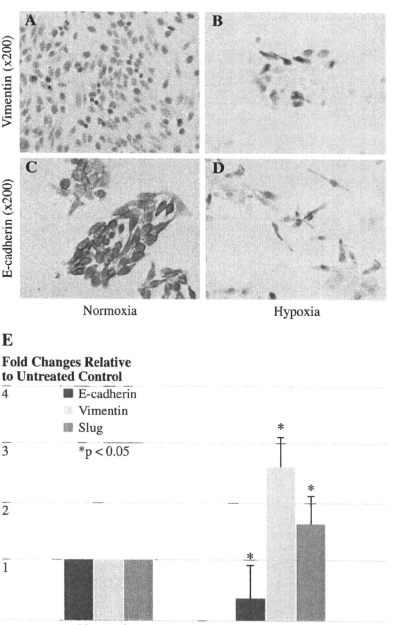

FIG. 3 Alteration of *VIM* expression in NUGC3 cells induced by hypoxia. **a, b** VIM expression in NUGC3 cells increased under hypoxic conditions with a change in shape from round to fibroblast-like. **c, d** E-cadherin expression decreased with an alteration of cellular morphology. **e** *VIM* and slug mRNA expression under hypoxic conditions was higher than under normoxia, conversely, E-cadherin decreased. Data are expressed as the fold-change relative to the untreated control (under normal conditions), * $P < .05$

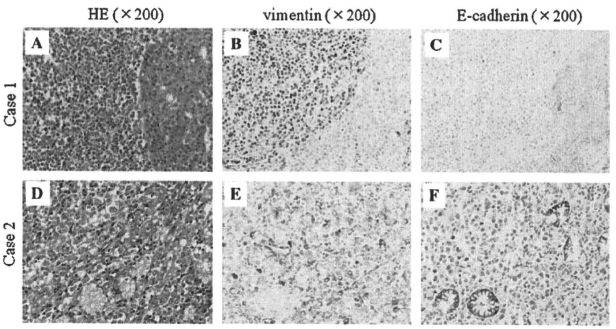

FIG. 4 Two cases of gastric cancer with rhabdoid features. In both cases (**a**, **b**, **c** Case 1; **d**, **e**, **f** Case 2), HE stained sections showed VIM-positive polygonal tumor cells had vesicular nuclei and abundant eosinophilic cytoplasm in an alveolar arrangement. VIM-positive tumor cells were negative for E-cadherin (**a**, **c** HE; **b**, **e** VIM; **c**, **f** E-cadherin: ×200)

increased under hypoxia as well as after addition of TGF-β1 (Fig. 3e).

Two Cases of Gastric Cancer with Rhabdoid Features

We examined the expression of VIM protein immunohistochemically in 30 poorly differentiated stomach adenocarcinomas. Two of these cases contained VIM-positive primary lesions. In both cases, hemotoxylin-eosin (HE) stained sections showed that the VIM-positive polygonal tumor cells had vesicular nuclei and abundant eosinophilic cytoplasm in an alveolar arrangement, as seen in malignant rhabdoid kidney tumors (Fig. 4a, b, d, e).[34] The VIM-positive tumor cells were E-cadherin-negative (Fig. 4c, f).

DISCUSSION

In advanced clinical cases of gastric cancer with invading tumor, lymph node metastasis, and lymphatic permeation, we found that the average expression level of *VIM* mRNA in BM was significantly higher than in the *VIM* negative group (Table 1). The current findings indicate that the abundant expression of *VIM* in BM primarily originates from cancer cells that have undergone mesenchymal transition in gastric cancer. Recent studies have shown that EMT plays a critical role in cancer progression and metastasis in epithelial malignancies other than gastric cancer.[9] VIM overexpression in cancer cells has become a focus of research efforts because it is a well-recognized representative mesenchymal marker.[35–37] In experimental models, EMT can be induced by TGF-β1 and hypoxia, which can produce metastatic phenotypes. However, there have been few studies of the induction of EMT by TGF-β1 and hypoxia in gastric cancer cells.[27,31] Our data show that the upregulation of VIM is induced by TGF-β1 and hypoxia with a concomitant alteration of cellular morphology (Figs. 2, 3). Importantly, among transcriptional repressors of E-cadherin, such as snail, twist, SIP1, and ZEB 1, we showed that in gastric cancer cells only slug mRNA rose along with the increasing concentration of TGF-β1 under hypoxic conditions. This finding may be supported by the previous report by Castro Alves et al.[38] that slug upregulation is associated with E-cadherin downregulation in diffuse and intestinal-type carcinoma.

With regard to the development of metastasis in solid cancer, isolated tumor cells detached from the primary site are able to survive against the hypoxic condition with the abundant expression of TGF-β1 in the circulating and disseminating systems, such as peripheral blood and bone marrow, by avoiding anoikis. Circulating gastric cancer cells in circulating system can overcome the deadly circumstance by the acquisition of a dynamic morphologic change, named EMT. In vitro, pancreatic cancer cells continuously exposed to TGF-β1 resist proliferation arrest and apoptosis following transfection of hypoxia inducible factor 1α (HIF-1α).[24,39] In breast cancer, Mehes et al.[40] reported that almost all breast cancer cells in the peripheral circulation were apoptotic. Therefore, our current findings imply that VIM-positive tumor cells can survive in the peripheral circulation and in BM and that VIM-positive cancer cells that invade intratumoral vessels must have undergone mesenchymal transition (Fig. 1).

To better understand the impact of the acquired mesenchymal phenotype on the progression of gastric cancer cases, we analyzed 2 well-defined cases. Carcinomas with rhabdoid features, which are extremely rare in gastrointestinal tract neoplasms and are highly malignant, are characterized by an aggressive clinical course.[41] Interestingly, 2 primary cases in

this study were VIM positive (Fig. 4). Therefore, aggressive VIM-positive rhabdoid gastric tumors have, like more typical gastric cancer that has metastasized to BM, mesenchymal features.

As for the origin of VIM-positive cells in BM, these are likely disseminated tumor cells. Immunochemistry showed that the cancer cells that invaded the intratumoral vessels were positive for VIM (Fig. 1). This finding in clinical samples suggests that circulating VIM-positive tumor cells not only survive in the peripheral circulation but can implant at metastatic sites. Here, we need to discuss the differences among ITC detection methods. The two main approaches for the detection of ITC are immunological assays using monoclonal antibodies and PCR-based molecular assays exploiting tissue-specific transcripts.[23] Immunocytochemical detection of epithelial or tumor-associated antigens is widely accepted.[42] We previously reported that RT-PCR assays targeting tumor-specific genes such as CEA and CK are useful to detect ITCs in various carcinomas.[29,30,43,44] However, in gastric cancer, there were no definitive data demonstrating that the detection of ITC was a useful prognostic marker (with either method) until our large-scale study.[26] We showed that > 50% of early gastric cancer cases expressed at least 1 ITC marker in PCR-based assays. Therefore, in the current study, we assume that not all detected ITCs but rather only a few that have undergone EMT, could give rise to metastasis or recurrence. On the other hand, one cannot neglect the enormous number of normal, VIM-positive cells in BM, such as precursors for fibroblasts, endothelial cells, macrophages, and lymphocytes that are associated with cancer progression. Recently, we described the importance of the synergistic relationship between tumor cells and BM-derived hematopoietic progenitor cells (HPCs) and endothelial progenitor cells (EPCs) in BM, as reported by Kaplan and Gao et al. previously.[25,26,45,46] In the current work, it is difficult to specify which cells might have expressed VIM protein. Therefore, it is possible that VIM expression originated from cancer cells and/or from normal cells in the BM.

In conclusion, we found that TGF-β1 and hypoxia upregulated VIM and inhibited E-cadherin in gastric cancer cells. The current data suggest that the abundant VIM expression, mainly from mesenchymal transited cancer cells in BM, might be associated with cancer progression and metastasis in gastric cancer.

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Clinical Cancer Research

Correlated Expression of *CD47* and *SIRPA* in Bone Marrow and in Peripheral Blood Predicts Recurrence in Breast Cancer Patients

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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Correlated Expression of *CD47* and *SIRPA* in Bone Marrow and in Peripheral Blood Predicts Recurrence in Breast Cancer Patients

Makoto Nagahara[1,2], Koshi Mimori[1], Akemi Kataoka[3], Hideshi Ishii[4], Fumiaki Tanaka[1], Tsuyoshi Nakagawa[2], Takanobu Sato[2], Shinji Ono[3], Kenichi Sugihara[2], and Masaki Mori[1,4]

Abstract

Purpose: CD47 plays a variety of roles in intercellular signaling. Herein, we focused on the clinicopathologic significance of *CD47* expression in human breast cancer. Our data suggest that the correlation between *CD47* and signal regulatory protein α (*SIRPA*) expression may play a key role in the progression of breast cancer.

Experimental Design: Quantitative real-time PCR was used to evaluate *CD47* mRNA and *SIRPA* mRNA expression in bone marrow and in peripheral blood from 738 cases of breast cancer.

Results: In patients with high levels of *CD47* expression in the bone marrow, survival was significantly poorer compared with patients with low levels of *CD47* expression [disease-free survival (DFS), $P = 0.0035$; overall survival (OS), $P = 0.015$]. Furthermore, high *CD47* expression group in a multivariate analysis showed significance as an independent variable for poorer prognosis in DFS ($P = 0.024$). In the peripheral blood, however, high *CD47* expression in patients was not an independent and significant prognostic factor for DFS and OS in a multivariate analysis. *CD47* expression was strongly correlated with *SIRPA* expression in both the bone marrow ($P < 0.0001$) and peripheral blood ($P < 0.0001$) of breast cancer patients.

Conclusions: This is one of the first studies to show that a host factor in bone marrow confers prognostic importance. CD47 is an important biomarker in breast cancer, and functions as a prognostic factor for DFS. Moreover, we suggest that the poor prognosis of breast cancer patients with high expression of *CD47* is due to an active CD47/SIRPA signaling pathway in circulating cells. *Clin Cancer Res; 16(18); 4625–35. ©2010 AACR.*

The numerous efforts in breast cancer research and care have improved early detection and treatment. However, breast cancer prevalence and mortality remain at a high level every year. The prevention and therapy of breast cancer among Japanese women is a crucial public health concern. The most recent statistics for Japan document over 55,000 cases per year (1), with a mortality surpassing 12,000 per year (2). Even after apparently successful localized treatments, there are long-term risks of recurrence and metastasis. To evaluate the postsurgical risk of recurrence of breast cancer, mammography, echogram, computer tomography, and magnetic resonance imaging are utilized for diagnostic imaging, and carcinoembryonic antigen (CEA), CA15-3, and NCC-ST439 are evaluated in peripheral blood as tumor markers. However, the long-term risk of relapse is largely due to clinically occult microrecurrences and micrometastases that are currently beyond detection by current conventional screening strategies. Therefore, it is important to exploit novel tumor markers that could predict recurrence and metastasis with greater reliability.

Kaplan et al. have shown that bone marrow–derived hematopoietic progenitor cells play an important role in the accumulation of premetastatic niches and the promotion of carcinogenesis and metastasis (3). We confirmed their findings by using clinical samples in which hematogenous metastasis occurred, and within these metastases, hematopoietic progenitor cells and isolated tumor cells (ITC) coexisted. This study showed the necessity of identifying metastasizing cancer cells as well as normal host side factors, such as bone marrow–derived cells and endothelial cells (4).

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Translational Relevance

The association between cancer cells and normal host side factors is thought to be important in promoting cancer progression and metastasis. Herein, we focused on the clinicopathologic significance of *CD47* gene expression in bone marrow and peripheral blood of breast cancer. Our data strongly suggest that CD47 is a significant prognostic indicator for disease-free survival (DFS). Moreover, *CD47* expression is strongly correlated with *SIRPA* expression in both the bone marrow and peripheral blood of breast cancer, and it indicates that the poor prognosis of breast cancer with high expression of *CD47* is due to an active CD47/SIRPA signaling pathway in circulating cells. Regarding patient care after surgery, many cases require postoperative adjuvant chemotherapy. Due to the associated adverse effects of such treatment, reliable prognostic markers for recurrence and metastasis would greatly improve patient management. We suggest that this biomarker may fill that need for enhanced patient care.

Recent studies showed that CD47 specifically inhibits phagocytosis and that there was a significant correlation between gene expression of *CD47* and leukemia, hematopoietic stem cells, and tumor-initiating cells of bladder cancer (5–7). It can be inferred from the association between CD47 and cancer stem cells that ITC would elude the immune system by taking advantage of activation and initiation of the signal transduction cascade of CD47, resulting in inhibition of phagocytosis.

CD47 was originally identified in association with the integrin $\alpha_v\beta_3$, hence its alternative name of integrin-associated protein. It is also a member of the Ig superfamily, possessing a V-type Ig-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail (8). CD47 seems to carry out several functions. For instance, CD47 functions as a marker of "self" on murine RBC. Erythrocytes lacking CD47 expression are rapidly removed from the bloodstream by splenic red pulp macrophages (9, 10). Signal regulatory protein α (SIRPA), a transmembrane glycoprotein, is a novel intracellular signal transducer when it is engaged by its ligand, CD47. CD47 on normal peripheral blood red cells circumvent elimination by binding to SIRPA (10). The interaction of CD47 with SIRPA occurs between host-derived cells, and is mostly related to cell signaling in the immune and nervous systems (11).

In the present study, we confirmed that high expression of *CK19*, a marker for ITC, had correlation with high *CD47* expression. Therefore, we focused on the clinicopathologic significance of *CD47* gene expression in the bone marrow and peripheral blood of breast cancer and its potential utility as a novel and specific biological marker for recurrence and/or overall survival (OS) in breast cancer patients. Investigating the characteristics of *CD47* mRNA expression in bone marrow and peripheral blood in this study, we evaluated the correlation between *CD47* and clinicopathologic factors in 738 breast cancer cases, and showed that the magnitude of *CD47* expression could be used as a new prognostic marker for recurrence and metastasis. Moreover, we found that expression of *CD47* and *SIRPA* were correlated in bone marrow and peripheral blood. This association has potentially important implications for clinicopathologic outcome. We suggest that the correlated expression of *CD47* and *SIRPA* represents a dynamic process involved in the progression of breast cancer cells. This report is one of the first to show that a host factor in bone marrow confers prognostic importance.

Materials and Methods

Patients

A total of 738 breast cancer patients were identified based on their pathologic diagnosis before surgery at Kyushu Cancer Center from July 2000 to August 2005. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board. Patients ranged in age from 24 to 89 years, with a mean age of 55.1 years. No patients received antihormonal treatment, chemotherapy, or radiotherapy before surgery. All patients were closely followed after surgery at regular 3- to 6-month intervals, and the follow-up periods ranged from 2 months to 6 years, with a median of 3.0 years. After surgery, all patients were clearly classified into the category of breast cancer based on the clinicopathologic criteria described by the Japanese Society for Breast Cancer. All data, including age, menopause, tumor stage, lymphatic invasion, lymph node metastasis, vascular invasion, distant metastasis, clinical stage, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (Her2) score, and recurrence were obtained from the clinical and pathologic records. ER, PgR, and Her2 scores were obtained from immunohistochemistry staining conducted by two well-trained pathologists. Her2 status was scored using the Her2 expression criteria (Supplementary Table S1). These criteria changed in May 2009, but we applied the previous criteria to evaluate Her2 status. The primary tumors with Her2 score (2+) had their immunohistochemistry results additionally validated by fluorescence *in situ* hybridization. After surgical therapy, all patients were individually treated by antihormonal treatment, chemotherapy, and/or radiotherapy according to breast cancer treatment guidelines in Japan, which were based on American Society of Clinical Oncology and National Comprehensive Cancer Network recommendations. Regarding noncancer patients, we considered that inflammatory and neoplastic diseases, including benign tumors, might affect the result of our experiments, and we therefore excluded those patients from this group. We selected 19 patients who underwent surgery for elective cholecystolithiasis at the Medical Institute of Bioregulation Hospital, Kyushu University, between 1999 and 2003; all patients had a blood test before surgery, and they were

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confirmed to be without inflammatory symptoms. Written informed consent was obtained from all patients. All control patients had a whole body computer tomography examination to determine whether they had cancer, and their status was confirmed by assessing tumor markers in the peripheral blood. After surgery, they were followed at regular 6-month intervals, and the absence of cancer was confirmed over 3 years following surgery.

Sample collection

Bone marrow and peripheral blood samples were obtained from patients under anesthesia before surgery. Peripheral blood samples were taken from superficial veins on the opposite side of the breast cancer, and bone marrow samples were taken from the sternum with a 15G needle. As there was a potential for contamination by skin, bone marrow specimens were collected with another syringe after the first 2 to 3 mL were aspirated. ISOGEN-LS (Nippon Gene Co., Ltd., Japan) was added and mixed, stored for 5 minutes at room temperature, and was immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Samples from noncancer patients were obtained with the same procedure.

Cell lines

The breast cancer cell lines CRL1500, MCF7, MRK-nu1, YMB1, YMB1E, SKBR3, and MDA-MB-231 were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Japan).

RNA preparation and reverse transcription

Total RNA from bone marrow and peripheral blood was extracted from control and breast cancer patients using the ISOGEN-LS method followed by Isogen-chloroform extraction and isopropanol precipitation (12). As described previously, cDNA was synthesized from 8.0 μg of total RNA (13).

Evaluation of *CD47* and *SIRPA* expression in clinical samples

The sequences of *CD47* primers were as follows: sense primer, 5′-GGCAATGACGAAGGAGGTTA-3′; antisense primer, 5′-ATCCGGTGGTATGGATGAGA-3′. The sequences of *SIRPA* primers were as follows: sense primer, 5′- GTTTAAGTCTGGAGCAGGCACT-3′; antisense primer, 5′-GCAGATGACTTGAGAGTGAACG-3′. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control and the sequences of *GAPDH* primers were as follows: sense primer, 5′-TTGGTATCGTGGAAGGACTCTA-3′; antisense primer, 5′-TGTCATATTTGGCAGGTT-3′. cDNA was synthesized from 8.0 μg of total RNA. Real-time monitoring of PCR reactions was done using the LightCycler system (Roche Applied Science) and SYBR-Green I dye (Roche Applied Science). Monitoring was done according to the manufacturer's instructions. Quantitative reverse transcriptase-PCR (RT-PCR) was done with the following cycling conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds. After amplification, products were subjected to a temperature gradient from 68°C to 95°C at 0.2°C/second, under continuous fluorescence monitoring, to produce a melting curve of the products. All concentrations were calculated relative to the concentration of cDNA from Human Universal Reference total RNA (Takara Bio Inc., Japan). The concentrations of *CD47* and *SIRPA* mRNAs were then divided by the concentration of the endogenous reference (*GAPDH*) to obtain normalized expression values (14–16). Each assay was done twice to verify the results, and the mean normalized value of mRNA expression was used for subsequent analyses.

Statistics

For continuous variables, data were expressed as means ± SD. The relationship between *CD47*, *SIRPA* mRNA expression and clinicopathologic factors was analyzed using the χ^2 test and Student's *t*-test. In addition, the data were also analyzed using the nonparametric Wilcoxon rank-sum test. Survival curves were plotted according to the Kaplan-Meier method, and the generalized log-rank test was applied to compare the survival curves. Variables with a value of $P < 0.05$ in univariate analysis were used in a subsequent multivariate analysis using the Cox regression. All tests were analyzed using JMP 7 software (SAS version 7.0.1, SAS Institute, Inc.), and the findings were considered significant when $P < 0.05$.

Results

Comparison between *CK19* and *CD47* expression

In our study, we assessed *CK19* expression in the bone marrow and peripheral blood of 738 breast cancer patients with quantitative real-time RT-PCR analysis. We detected 57 *CK19*-positive cases in bone marrow and 57 cases in peripheral blood. We divided patients into two groups: the *CK19*-negative group and the *CK19*-positive group. We then evaluated *CD47* expression in the bone marrow and peripheral blood of 738 breast cancer patients with quantitative real-time RT-PCR and compared *CD47* expression with *CK19* status. *CD47* expression in the *CK19*-positive group was higher than that in *CK19*-negative group (bone marrow, $P = 0.04$; peripheral blood, $P = 0.02$; Supplementary Fig. S1). *CD47* expression (mean ± SD) in bone marrow was 1.91 ± 2.06 [confidence interval (CI), 1.54-2.28] in the *CK19*-positive group and 1.51 ± 1.34 (CI, 1.39-1.63) in the *CK19*-negative group. CD47 expression in peripheral blood was 2.65 ± 3.03 (CI, 2.12-3.18) in the *CK19*-positive group and 1.97 ± 1.74 (CI, 1.74-2.19) in the *CK19*-negative group.

High expression levels of *CD47* in bone marrow and peripheral blood of breast cancer patients

In this study, we selected 19 patients with cholecystolithiasis as control cases. Quantitative real-time RT-PCR

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analysis showed higher expression of *CD47* mRNA in breast cancer cases than in control cases (Fig. 1A-1). In bone marrow, the mean expression ratio (mean ± SD) of *CD47/GAPDH* mRNAs in breast cancer, 1.52 ± 1.43 (CI, 1.42-1.63), was significantly higher than that in noncancer cases, 0.80 ± 0.65 (CI, 0.49-1.11; Wilcoxon Rank-Sum test, $P = 0.033$). In peripheral blood, the mean expression ratio (mean ± SD) of *CD47/GAPDH* mRNAs in breast cancer, 1.83 ± 1.69 (CI, 1.67-1.99), was significantly higher than that in noncancer cases, 0.48 ± 0.49 (CI, 0.24-0.73; $P = 0.0015$). In Fig. 1A-2, the histogram shows the number of cases within each range of expression ratios of CD47/GAPDH. We also evaluated *CD47* mRNA in 32 noncancer patients; the results of *CD47* expression were similar to that from 19 patients as normal controls (Supplementary Fig. S2-a).

We assessed *CD47* expression in breast cancer cell lines to determine whether the expression ratio of *CD47* is affected by the number of ITC in bone marrow or peripheral blood. The human breast cancer cell lines CRL1500,

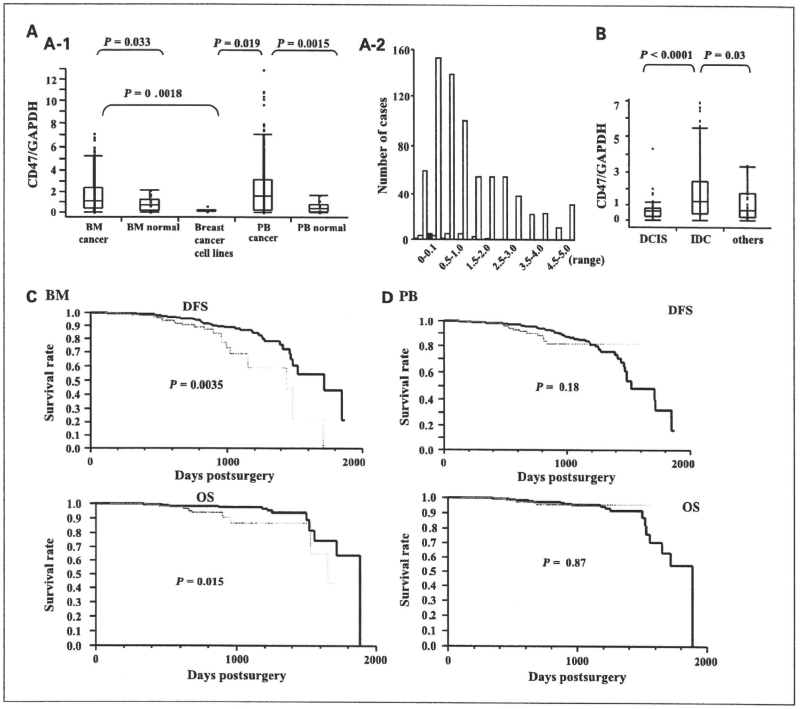

Fig. 1. A-1, comparison of *CD47* mRNA expression in breast cancer, breast cancer cell lines, and control samples. The distribution chart shows each expression ratio of *CD47/GAPDH* mRNA derived from cancer cases in bone marrow (BM; BM cancer), control patients in BM (BM normal), breast cancer cell lines, cancer cases in peripheral blood (PB; PB cancer) and normal patients in PB (PB normal). A-2, number of cases within each range of expression ratios of *CD47/GAPDH* mRNA in bone marrow derived from breast cancer patients (white bar), breast cancer cell lines (black bar), and normal cases (grey bar). B, comparison of CD47 expression among histologic types of breast cancer. The distribution chart shows each expression ratio of *CD47/GAPDH* mRNA derived from ductal carcinoma *in situ* (DCIS), invasive ductal carcinoma (IDC), and other types of breast cancer (other). C and D, the 5-year disease-free survival (DFS) and overall survival (OS) rates in patients with high *CD47* mRNA (dash line) and patients with low *CD47* mRNA (solid line) in BM and in PB. In DFS, excluding 9 metastatic breast cancer patients, the numbers of patients with high *CD47* and low *CD47* are 221 and 222, respectively in BM and PB. In OS, 226 patients with high *CD47* expression and 226 patients with low *CD47* expression are displayed with dash and solid lines, respectively.

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Table 1. Clinical magnitude of *CD47/GAPDH* in breast cancer cases prior to surgery, and comparison with known serum tumor markers

	Normal value	Average (SD)	CI
CEA (serum)	<5.0 ng/mL	2.01 (3.37)	1.69-2.33
CA15-3 (serum)	<25.0 U/mL	12.19 (11.19)	11.13-13.26
NCC-ST439 (serum)	<7.0 U/mL	7.45 (31.50)	1.06-13.83
CD47/GAPDH (BM)	(0.80, average of BM in noncancer cases)	1.52 (1.43)	1.42-1.63
High-expression group		2.95 (1.11)	2.80-3.09
Low-expression group		0.62 (0.42)	0.56-0.67
CD47/GAPDH (PB)	(0.48, average of PB in noncancer cases)	1.83 (1.69)	1.67-1.99
High-expression group		3.86 (1.86)	3.60-4.12
Low-expression group		0.58 (0.63)	0.50-0.66

Abbreviations: BM, bone marrow; PB, peripheral blood.

MCF7, MRK-nu1, YMB1, YMB1E, SKBR3, and MDA-MB-231 were assessed. The mean expression ratio of *CD47/GAPDH* mRNA was 0.24 ± 0.18 (CI, 0.07-0.40) in breast cancer cell lines, and was significantly lower than those found in the bone marrow and peripheral blood samples of breast cancer cases. Next, we compared the *CD47/GAPDH* ratio of each histologic type of breast cancer in bone marrow (Fig. 1B). We divided patients into three types: ductal carcinoma *in situ* (DCIS; $n = 53$), invasive ductal carcinoma (IDC; $n = 647$), and other types of breast cancer (other; $n = 38$). The mean expression ratios of *CD47/GAPDH* mRNAs were as follows: DCIS, 0.68 ± 0.71 (CI, 0.47-0.88); IDC, 1.66 ± 1.47 (CI, 1.54-1.77); others, 1.09 ± 1.31 (CI, 0.69-1.49). The *CD47/GAPDH* expression ratio in IDC was significantly higher than in DCIS ($P < 0.0001$) and others ($P = 0.03$). Such variations in the CD47/GAPDH expression ratios were likely to be reflected in the bone marrow of IDC, so we focused on the IDC subtype for subsequent analyses.

Comparison with tumor markers

Table 1 shows the results of tumor markers that were analyzed in peripheral blood samples taken before surgery. The mean values (mean ± SD) of tumor markers in breast cancer were as follows: CEA, 2.01 ± 3.37; CA15-3, 12.19 ± 11.19; and NCC-ST439, 7.45 ± 31.50. NCC-ST439 was only slightly higher than normal levels, whereas the values of CEA and CA15-3 were within normal levels. As a result, these tumor markers may not be useful as biomarkers in breast cancer cases before surgery.

Next, we divided breast cancer patients into two groups according to their *CD47/GAPDH* ratios. Thus, bone marrow and peripheral blood values were divided into those greater or less than the median *CD47/GAPDH* ratio: the high-expression group ($n = 226$) and the low-expression group ($n = 226$). In both bone marrow and peripheral blood, the mean expression ratios of *CD47/GAPDH* mRNAs in cancer cases were significantly higher than in control patients. Furthermore, the mean ratio of *CD47/GAPDH* mRNAs in the high-expression group of cancer cases was three to five times higher than in noncancer cases.

Clinicopathologic factors, disease-free survival, and overall survival of breast cancer patients

In this study, all the data were obtained from clinical and pathologic records. However, due to lack of data from breast cancer patients, particularly Her2 scores, we limited the analysis to the 452 clinical cases in which data were complete. Clinicopathologic significance of the *CD47/GAPDH* mRNA expression ratio in bone marrow is shown in Table 2. The incidence of triple negatives for ER, PgR, and Her2 status was significantly higher ($P = 0.0097$) in the high-expression group than in the low-expression group. The incidence of premenopausal patients was significantly higher ($P = 0.01$) in the high-expression group than in the low-expression group. The incidence of Her2 score was significantly higher ($P = 0.03$) in the high-expression group than in the low-expression group. The incidence of recurrence was significantly lower ($P = 0.04$) in the high-expression group than in the low-expression group. Conversely, no significant differences were observed regarding age, tumor stage, lymph node metastasis, lymphatic invasion, venous invasion, distant metastasis, clinical stage, estrogen receptor, and progesterone receptor.

The 5-year disease-free survival (DFS) and OS rates in patients with high *CD47/GAPDH* mRNA in bone marrow and patients with low *CD47/GAPDH* mRNA in bone marrow are shown in Fig. 1C. The survival difference between these two groups was statistically significant (DFS, $P = 0.0035$, log-rank test; OS, $P = 0.015$, log-rank test). The patients received at least one postoperative therapy (antihormonal treatment, chemotherapy, or radiotherapy). Univariate and multivariate analyses of clinicopathologic factors affecting DFS rate in bone marrow are shown in Table 3. Univariate analysis revealed a significant relationship between DFS and the following factors: lymphatic invasion, lymph node metastasis,