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).

Authors' Affiliations: [1]Department of Surgery and Molecular Oncology, Medical Institute of Bioregulation, Kyushu University, Oita, Japan; [2]Department of Surgical Oncology, Tokyo Medical and Dental University, Graduate School of Medical & Dental Science, Tokyo, Japan; [3]Department of Breast Surgery, National Kyushu Cancer Center, Fukuoka, Japan; and [4]Department of Surgical Oncology, Osaka University, Graduate School of Medicine, Osaka, Japan

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Corresponding Author: Masaki Mori, Department of Surgical Oncology, Osaka University, Graduate School of Medicine, 2-2 Yamagaoka, Suita city, Osaka 565-0871, Japan. Phone: 81-6-6879-3251; Fax: 81-6-6879-3259; E-mail: mmori@gesurg.med.osaka-u.jp.

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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

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

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,

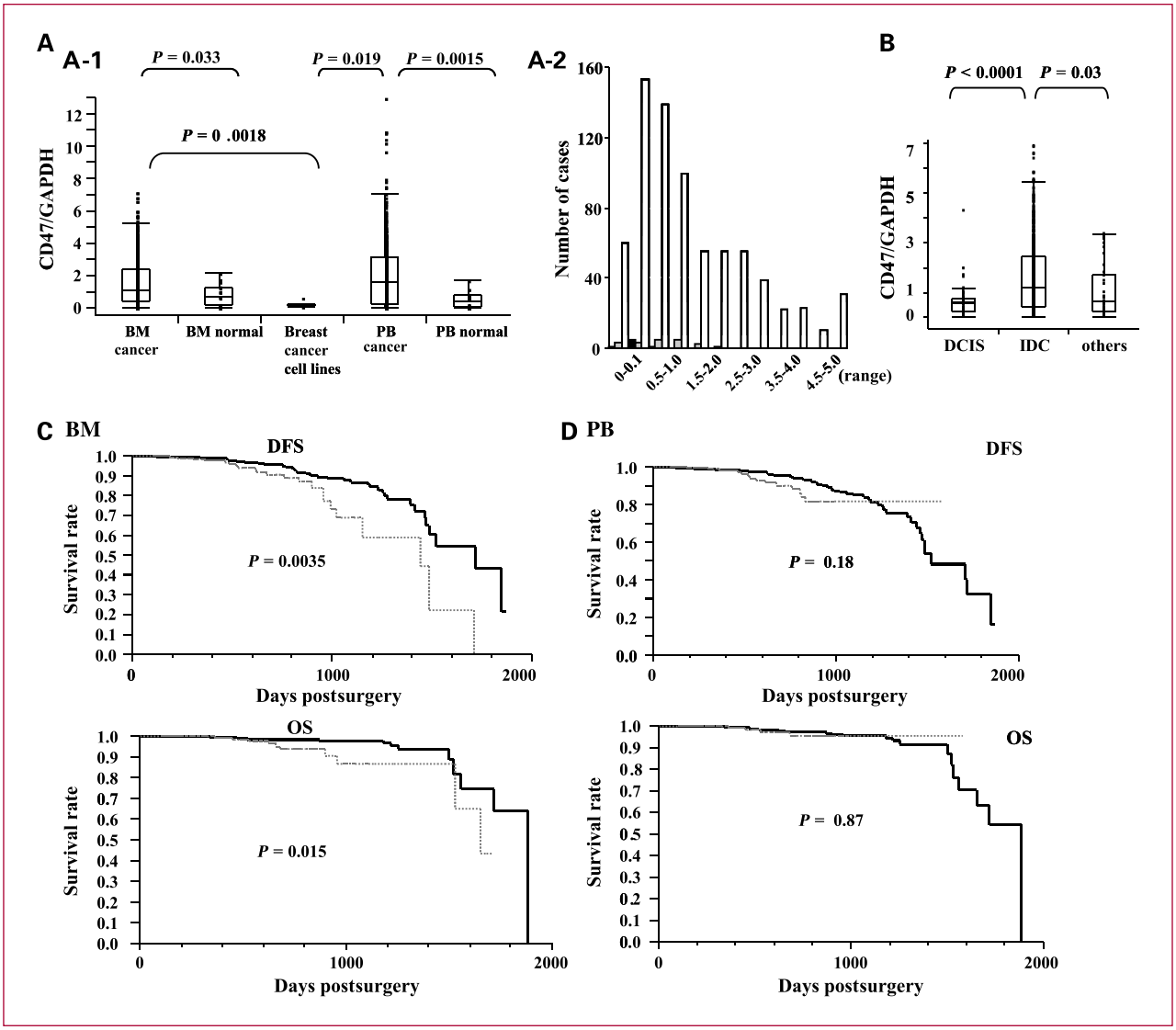

Fig. 1. A-1, comparison of *CD47* mRNA expression in breast cancer, breast cancer cell lines, and control samples. The distribution chart shows each expression ratio of *CD47*/*GAPDH* mRNA derived from cancer cases in bone marrow (BM; BM cancer), control patients in BM (BM normal), breast cancer cell 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.

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,

Table 2. Clinicopathologic significance of the CD47/GAPDH mRNA expression ratio in bone marrow and in peripheral blood

CD47/GAPDH clinicopathologic factors	BM			PB		
	Low ratio (%)	High ratio (%)	*P*	Low ratio (%)	High ratio (%)	*P*
Age, y (mean ± SD)	55.7 ± 11.2	55.1 ± 11.5	n.s.	55.8 ± 10.9	54.9 ± 11.8	n.s.
Menopause status			0.01*			n.s.
Pre	71 (31.4)	96 (42.5)		76 (33.6)	91 (40.3)	
Post	155 (68.6)	130 (57.5)		150 (66.4)	135 (59.7)	
Tumor stage			n.s.			0.0011*
T_1	103 (45.6)	123 (54.4)		96 (42.8)	130 (57.2)	
T_{2-4}	123 (54.4)	103 (45.6)		130 (57.2)	96 (42.8)	
Lymph node metastasis			n.s.			n.s.
Absent	138 (61.1)	137 (60.6)		135 (59.7)	140 (61.9)	
Present	88 (38.9)	89 (39.4)		91 (40.3)	86 (38.1)	
Lymphatic invasion			n.s.			n.s.
Absent	127 (56.2)	146 (64.6)		130 (58.0)	143 (62.3)	
Present	99 (43.8)	80 (35.4)		96 (42.0)	83 (36.7)	
Venous invasion			n.s.			n.s.
Absent	215 (95.1)	208 (92.0)		215 (95.1)	208 (92.0)	
Present	11 (4.9)	18 (8.0)		11 (4.9)	18 (8.0)	
Distant metastasis			n.s.			n.s.
Absent	221 (97.8)	222 (98.2)		221 (98.2)	222 (97.8)	
Present	5 (2.2)	4 (1.8)		5 (1.8)	4 (2.2)	
Stage			n.s.			n.s.
Stage I	78 (34.5)	86 (38.1)		73 (32.3)	91 (40.7)	
Stage II-IV	148 (65.5)	140 (62)		153 (67.7)	135 (59.3)	
ER			n.s.			n.s.
Absent	56 (24.8)	74 (32.7)		68 (30.1)	62 (27.0)	
Present	170 (75.2)	152 (67.3)		158 (69.9)	164 (73.0)	
PgR			n.s.			n.s.
Absent	102 (45.1)	101 (44.7)		95 (42.0)	108 (47.8)	
Present	124 (54.9)	125 (55.3)		131 (58.0)	118 (52.2)	
Her2 score			0.03*			n.s.
0-1	157 (69.5)	135 (59.7)		152 (67.3)	140 (61.9)	
2-3	69 (30.5)	91 (40.3)		74 (32.7)	86 (38.1)	
ER, PgR, Her2 status			0.0097*			n.s.
Triple negative	26 (11.5)	46 (20.4)		26 (11.5)	46 (20.4)	
Either one positive	200 (88.5)	180 (79.7)		200 (88.5)	180 (79.7)	
Recurrence			0.04*			<0.0001*
Absent	189 (83.6)	204 (90.3)		181 (80.1)	212 (93.8)	
Present	37 (16.4)	22 (9.7)		45 (19.9)	14 (6.2)	

Abbreviation: n.s., not significant.
*$P < 0.05$, statistical significance.

venous invasion, estrogen receptor, progesterone receptor, Her2 score, and *CD47* expression. Multivariate analysis indicated that the high expression ratio of *CD47* was found to be an independent and significant prognostic factor for survival ($P = 0.024$). Univariate and multivariate analyses of clinicopathologic factors affecting OS rate in bone marrow are shown in Table 3. Univariate analysis revealed a significant relationship between OS and the following factors: menopause, lymph node metastasis, estrogen receptor, progesterone receptor, recurrence, and *CD47* expression. Multivariate analysis indicated that the high expression ratio of *CD47* was not an independent and significant prognostic factor for survival ($P = 0.41$).

The clinicopathologic factors analyzed in relation to *CD47* mRNA expression in peripheral blood are shown in Table 2. The incidence of recurrence was significantly

Table 3. Univariate and multivariate analyses of clinicopathologic factors affecting disease-free survival and overall survival rate in bone marrow

Clinicopathologic factors	Disease-free survival				Overall survival			
	No. of patients	Univariate analysis HR (CI)	Multivariate analysis		No. of patients	Univariate analysis HR (CI)	Multivariate analysis	
			Relative risk (CI)	*P*			Relative risk (CI)	*P*
Age (years)								
55	233	0.98 (0.58-1.67)	—	—	237	0.55 (0.22-1.32)	—	—
>55	210				215			
Menopause								
Pre	165	0.86 (0.47-1.49)	—	—	167	0.21 (0.03-0.72)	0.25 (0.04-0.92)	0.035*
Post	278				285			
Tumor stage								
T_1	226	0.60 (0.33-1.04)	—	—	226	0.61 (0.22-1.52)	—	—
T_{2-4}	217				226			
Lymphatic invasion								
Present	172	2.08 (1.22-3.70)	1.22 (0.66-2.32)	0.54	179	1.85 (0.78-4.55)	—	—
Absent	271				273			
Lymph node metastasis								
Present	170	3.60 (2.04-6.67)	2.63 (1.31-5.56)	0.0057*	177	3.97 (1.61-11.17)	1.72 (0.07-5.00)	0.27
Absent	273				275			
Venous invasion								
Present	29	3.64 (1.23-8.33)	2.50 (0.84-6.25)	0.1	29	4.00 (0.62-15.02)	—	—
Absent	414				423			
Distant metastasis								
Present	0	—	—	—	9	5.32 (0.83-19.23)	—	—
Absent	443				443			
ER								
Present	318	0.49 (0.29-0.84)	0.80 (0.38-1.69)	0.56	322	0.27 (0.11-0.64)	1.67 (0.48-5.39)	0.40
Absent	125				130			
PgR								
Present	245	0.55 (0.32-0.93)	0.71 (0.35-1.49)	0.36	249	0.21 (0.07-0.54)	0.19 (0.04-0.79)	0.022*
Absent	198				203			
Her2 score								
0-1	285	0.42 (0.25-0.72)	0.69 (0.38-1.26)	0.22	292	0.47 (0.19-1.14)	—	—
2-3	158				160			
Recurrence								
Present	56	—	—	—	59	19.08 (5.26-33.33)	1955	<0.0001*
Absent	387				393			
CD47 expression								
High	221	2.32 (1.28-4.17)	2.00 (1.10-3.61)	0.024*	226	2.99 (1.18-7.61)	1.54 (0.55-4.30)	0.41
Low	222				226			

Abbreviation: HR, hazard ratio.

*$P < 0.05$.

Table 4. Univariate and multivariate analyses of clinicopathologic factors affecting disease-free survival and overall survival rate in peripheral blood

Clinicopathologic variable	Disease-free survival				Overall survival			
	No. of patients	Univariate analysis HR (CI)	Multivariate analysis		No. of patients	Univariate analysis HR (CI)	Multivariate analysis	
			Relative risk (CI)	*P*			Relative risk (CI)	*P*
Age (years)								
55	233	0.98 (0.58-1.67)	—	—	237	0.55 (0.22-1.32)	—	—
>55	210				215			
Menopause								
Pre	165	0.86 (0.47-1.49)	—	—	167	0.21 (0.03-0.72)	0.26 (0.04-0.94)	0.039*
Post	278				285			
Tumor stage								
T_{is-1}	226	0.60 (0.33-1.04)	—	—	226	0.61 (0.22-1.52)	—	—
T_{2-4}	217				226			
Lymphatic invasion								
Present	172	2.08 (1.22-3.70)	1.20 (0.65-2.27)	0.56	179	1.85 (0.78-4.55)	—	—
Absent	271				273			
Lymph node metastasis								
Present	170	3.60 (2.04-6.67)	2.63 (1.32-5.43)	0.0055*	177	3.97 (1.61-11.17)	1.58 (0.63-4.51)	0.34
Absent	273				275			
Venous invasion								
Present	29	3.64 (1.23-8.33)	2.76 (0.92-6.67)	0.07	29	4.00 (0.62-15.02)	—	—
Absent	414				423			
Distant metastasis								
Present	0	—	—	—	9	5.32 (0.83-19.23)	—	—
Absent	443				443			
ER								
Present	318	0.49 (0.29-0.84)	0.69 (0.34-1.41)	0.3	322	0.27 (0.11-0.64)	1.50 (0.45-4.47)	0.49
Absent	125				130			
PgR								
Present	245	0.55 (0.32-0.93)	0.71 (0.36-1.43)	0.35	249	0.21 (0.07-0.54)	0.18 (0.04-0.68)	0.011*
Absent	198				203			
Her2 score								
0-1	285	0.42 (0.25-0.72)	0.69 (0.38-1.24)	0.21	292	0.47 (0.19-1.14)	—	—
2-3	158				160			
Recurrence								
Present	56	—	—	—	59	19.08 (5.26-33.33)	2075	<0.0001*
Absent	387				393			
CD47 expression								
High	221	1.57 (0.79-3.03)	—	—	226	1.10 (0.30-3.22)	—	—
Low	222				226			

*$P < 0.05$.

lower ($P < 0.0001$) in the high-expression group than in the low-expression group. The incidence of tumor stage was significantly lower ($P = 0.0011$) in the high-expression group than in the low-expression group. Conversely, no significant differences were observed regarding age, menopause, lymph node metastasis, lymphatic invasion, venous invasion, distant metastasis, clinical stage, estrogen receptor, progesterone receptor, Her2 score, and ER, PgR, Her2 status. The 5-year DFS and OS rates in patients with high *CD47* mRNA and patients with low *CD47* mRNA are shown in Fig. 1D. The survival difference between these two groups was not statistically significant for DFS ($P = 0.18$, log-rank test) and OS ($P = 0.87$, log-rank test). Univariate and multivariate analyses of clinicopathologic factors affecting DFS rate in peripheral blood are shown in Table 4. Univariate analysis revealed a significant relationship between OS and the following factors: tumor stage, lymphatic invasion, lymph node metastasis, venous invasion, estrogen receptor, progesterone receptor, and Her2 score, but *CD47* expression was not included. Multivariate analysis indicated that the presence of lymph node metastasis was found to be an independent and significant prognostic factor for survival ($P = 0.0055$). Univariate and multivariate analyses of clinicopathologic factors affecting OS rate in peripheral blood are shown in Table 4. Univariate analysis revealed a significant relationship between OS and the following factors: menopause, lymph node metastasis, estrogen receptor, progesterone receptor, and recurrence. Univariate analysis indicated that the high expression ratio of *CD47* was

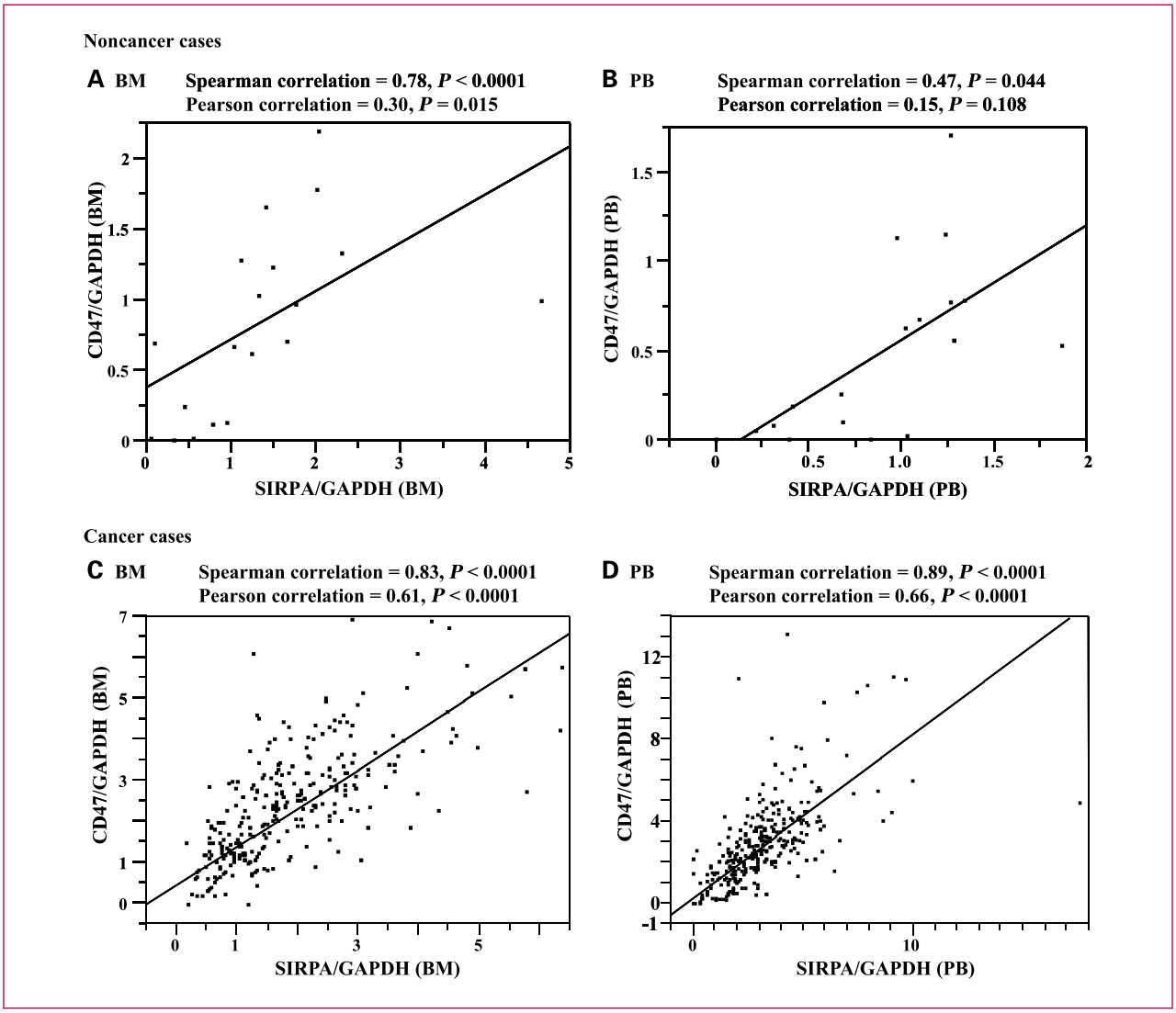

Fig. 2. The correlation between *CD47* expression and *SIRPA* expression. In control patients, *CD47* expression was correlated with *SIRPA* in both bone marrow (A) and peripheral blood (B). In breast cancer cases, *CD47* expression was even more strongly correlated with *SIRPA* in both bone marrow (C) and peripheral blood (D).

not an independent and significant prognostic factor for survival.

Correlation with SIRPA

We investigated *SIRPA* expression in the same breast cancer patients and controls. Figure 2 shows the correlation between *CD47* expression and *SIRPA* expression. In cancer cell lines, *CD47* expression did not correlate with *SIRPA* expression (Spearman correlation = 0.0319; P = 0.95; data not shown). In control patients, *CD47* expression was correlated with *SIRPA* in both bone marrow ($P < 0.0001$) and peripheral blood ($P = 0.0044$; Fig. 2A and B). In 32 noncancer cases, *CD47* expression was correlated with *SIRPA* in both bone marrow ($P = 0.004$) and peripheral blood ($P < 0.0001$; Supplementary Fig. S2-b).

In breast cancer cases, *CD47* expression is more strongly correlated with *SIRPA* in both bone marrow ($P < 0.0001$) and peripheral blood ($P < 0.0001$; Fig. 2C and D).

Discussion

CD47 is expressed on the surface of a wide variety of cells such as hematopoietic cells, keratinocytes, and cells of the brain (17). CD47 is associated with $\alpha_v\beta_3$ integrin and is implicated in the modulation of integrin functions, such as cell adhesion, phagocytosis, and cellular migration (18–20). It is known that CD47 is a marker of self on RBC. CD47 could work as a marker of self on cancer cells, and breast cancer cells may express high levels of CD47 by themselves. Our results showed that the *CD47/GAPDH* expression ratio in breast cancer cell lines was significantly lower than those found in the bone marrow and peripheral blood samples of breast cancer cases. This may indicate that *CD47* has various functions, and that the level of *CD47* expression was affected by the cell environment rather than by the number of cancer cells. Therefore, the high expression of *CD47* in the bone marrow and peripheral blood of breast cancer patients may represent the characteristic appearance of breast cancer and some evidence of a cancer-specific mechanism in the bone marrow and peripheral blood of breast cancer.

Recent reports have shown that CD47 plays a role in inhibiting macrophage phagocytosis of cancer stem cells and tumor-initiating cells (5–7). In the same manner as a cancer stem cell, the cancer cell itself may circumvent immune system surveillance by expressing CD47 as a marker of self, thereby evading natural killer cells (21, 22). In our study, we found that high *CD47* expression had a correlation with high *CK19* expression in the bone marrow and peripheral blood of breast cancer. This result strongly suggests that ITC of breast cancer patients may utilize the function of *CD47* in circulating circumstances such as bone marrow and peripheral blood. Moreover, expression of *CD47* in the bone marrow and peripheral blood of breast cancer patients was significantly higher than in control patients. Because expression of *CD47* in circulating tumor cells increases exponentially with the progress of the cancer stage, *CD47* derived from ITC may be an upregulating factor of breast cancer.

CD47 also promotes apoptosis, and the CD47 ligand thrombospondin (TSP) has been implicated as an antitumor and antimetastatic factor in breast cancer (23–33). Both TSP1 and a CD47 agonist peptide (4N1K, derived from TSP1) can induce a novel form of apoptosis in transformed and activated normal T cells (34, 35), chronic lymphocytic leukemia cells (36), erythroleukemia cells, and primary arterial smooth muscle cells (35). We supposed that CD47 may have a role not only as a marker of self but also as an inducer of apoptosis to inhibit phagocytosis.

In the present study, 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. We suggest that *CD47* may be specifically expressed in the bone marrow and peripheral blood of breast cancer patients and that *CD47* expression may represent an important biomarker in breast cancer patients. As a result of the identification of the clinical significance of *CD47* expression in bone marrow and peripheral blood, we found that overexpression of *CD47* in bone marrow and peripheral blood correlated with the aggressiveness of breast cancer. This result might suggest that the more there are circulating tumor cells expressing increased *CD47* in bone marrow and peripheral blood, the more active the primary immune system is in inducing apoptosis in tumor cells in the circulating systems. Therefore, it is important to clarify the level of *CD47* expression in bone marrow and peripheral blood to indicate whether micrometastasis exists in the breast cancer cases. Thus, *CD47* may be a novel biological marker that predicts the number of highly malignant circulating tumor cells that escape from the immune systems in breast cancer.

To further characterize the function of *CD47* in bone marrow and peripheral blood, we examined *SIRPA* expression in the same breast cancer samples. In doing so, we obtained the novel finding that the expressions of *CD47* and *SIRPA* are markedly associated. The correlation between *CD47* and *SIRPA* was significantly stronger in breast cancer patients than in control cases. In control cases, the CD47-SIRPA signaling system is activated in bone marrow and in peripheral blood, reflecting homeostatic regulation in the hematopoietic system. In the breast cancer cases, carcinogenicity may promote the CD47-SIRPA cell signaling system in bone marrow and in peripheral blood, thereby possibly promoting micrometastases. We suggest that expression of the CD47/SIRPA signal system indicates the presence of cancer-specific microenvironmental areas that support micrometastasis.

In conclusion, our data indicate that *CD47* is a significant prognostic indicator for DFS, and our study is one of the first to report a host factor in bone marrow with prognostic significance. With regard to patient care, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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