ORIGINAL ARTICLE

Potential of Reduction in Total Tumor Volume Measured with 3D-MRI as a Prognostic Factor for Locally-Advanced Breast Cancer Patients Treated with Primary Chemotherapy

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■ **Abstract:** For accurate assessment of the response to primary chemotherapy (PCT) for locally advanced breast cancer, we measured reduction in total tumor volume (TTV) by using three-dimensional magnetic resonance imaging (3D MRI), and examined the relationship between this reduction and patient prognosis. Fifty-one patients with locally advanced breast cancer were treated with four cycles of docetaxel (60 mg/m^2) before surgery. Tumor size was measured with calipers, ultrasonography (US) and conventional two-dimensional (2D) MRI before and after chemotherapy. TTV was measured with 3D MRI. These and other clinicopathological parameters were statistically analyzed to determine the prognosis for the patients. Median follow-up time was 46 months (1–64 months). Of the 51 patients, 25 developed distant recurrences. Patients whose TTV decreased by 75% or more after PCT showed significantly better prognosis than others, while tumor size measured with calipers, US and 2D MRI showed no significant relationship with patient prognosis. Of the clinicopathological parameters, only reduction in TTV and histological grade showed a significant association with distant recurrence-free survival ($p = 0.03$ and 0.02, log-rank test), while stepwise multivariate Cox's proportional hazards analysis identified TTV as the strongest independent prognostic factor. Reduction in TTV measured with 3D MRI can be a useful prognostic factor for patients with locally advanced breast cancer treated with PCT. ■

Key Words: breast cancer, primary chemotherapy, prognostic factor, three-dimensional magnetic resonance imaging

Since Fisher et al. (1) reported that their study of the results of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 showed that primary chemotherapy (PCT), also known as neoadjuvant chemotherapy, could increase the rate of successful breast-conserving surgery, PCT has often been used for locally advanced breast cancer. However, 5-year disease-free survival and 5-year overall survival were not improved by PCT, so that until recently the only benefit of PCT was considered to be improvement in the rate of breast conservation for patients with a large tumor. However, analysis of subsets of the NSABP B-18 data showed that the prognosis for patients with pathologic complete response (pCR) was significantly better than for those with partial response to PCT (2). Several new agents, such as taxanes and trastuzumab, have recently been used for PCT with reported pCR rates of 4.2–26%, which are expected to improve further with more intensive PCT regimens (3,4).

The question remains how PCT affects the prognosis for patients with pathologic partial response (pPR), especially those with high pPR approaching pCR. Higher response to PCT can be expected to result in better prognosis, because some patients with very high pPR may achieve pCR with longer treatment. Accurate assessment of response to PCT is therefore essential for determining patient prognosis.

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Response to PCT is generally evaluated by measuring tumor size with calipers, mammography and ultrasound. However, accurate measurement of the tumor size after PCT is reportedly difficult because of pathologic degeneration of the tumor and increased fibrous scar tissue (5–7). To overcome this problem, we used three-dimensional magnetic resonance imaging (3D MRI) for assessment of tumor size after PCT and were able to show that 3D MRI is superior to calipers and ultrasound for measurement of residual tumor size (8). After PCT, however, the remnant tumor often shows a scattered pattern with many small foci, which makes the determination of tumor size fuzzy. That is, the tumor size can be interpreted as the largest of the scattered area, the total size of all foci or the largest focus of the tumor. To avoid this problem, we decided that measurement of total tumor volume (TTV) of all foci of the cancer may be useful for attaining a more accurate assessment of response to PCT. We therefore measured TTV with 3D MRI before and after PCT to determine the rate of reduction.

In the study reported here, we examined the relationship between clinicopathological factors including TTV and prognosis for patients treated with PCT.

MATERIALS AND METHODS

Patients and Primary Chemotherapy Regimen

The study subjects were 51 women with breast cancer, who were treated with PCT and then underwent radical surgery at Osaka University Hospital between September 2000 and October 2002. The criteria for patient' eligibility for PCT was T2 or T3 tumors with or without regional lymph node involvement, but without distant metastasis. The median age was 53 years (28–75 years). All tumors were confirmed to be invasive breast cancer by pathologic examination of specimens obtained by means of vacuum-assisted breast biopsy before PCT.

The PCT regimen consisted of four cycles of 60 mg/m^2 of docetaxel every 3 weeks, after which all patients underwent radical mastectomy or partial mastectomy with complete axillary dissection. Patients, who showed response to PCT detected with 2D MRI, received an additional four cycles of 60 mg/m^2 of docetaxel after surgery. Patients, who showed no response to PCT or showed progression of the disease, received four cycles of CE (600 mg/m^2 of cyclophosphamide and 60 mg/m^2 of epirubicin) every 3 weeks as postoperative adjuvant treatment. Patients with hormone-receptor positive tumors received hormonal therapy following postoperative adjuvant chemotherapy. Patient' characteristics are shown in Table 1.

Table 1. Patient Characteristics

		No. patients
Age (median, years)		28–75 (53)
Tumor size before PCT	T1	1
	T2	30
	T3	20
Nodal status before PCT	N0	16
	N1-2	35
Histological grade before PCT	1	13
	2	30
	3	8
Estrogen receptor	Positive	28
	Negative	23
Progesterone receptor	Positive	17
	Negative	34
HER-2 amplification	Positive	10
	Negative	33
	unknown	8
Clinical response	CR	1
	PR	36
	NC	13
	PD	1
Surgery	MST	42
	BCS	9
Pathologic assessment of PCT efficacy	Grade 0	3
	Grade I	35
	Grade II	10
	Grade III	3
Axillary nodal status after surgery	n1–2	30
	n0	21
Additional chemotherapy after surgery	DOC	37
	CE	14

PCT, primary chemotherapy; CR, complete response; PR, partial response; PD: progressive disease; DOC, docetaxel; CE, cyclophosphamide and epirubicin; NC, no change; MST, mastectomy; BCS, breast conserving surgery.
Clinical response was determined by measure calipers, and assessed according to the WHO criteria.

Patients were followed-up with interviews, physical examinations using palpation and laboratory examinations including tumor markers every 3 months, and with chest x-ray examinations every 6 months. When abnormal findings were detected, further examinations with CT, ultrasound, MRI and bone scintigraphy were performed for detection of possible recurrence.

Histopathological Examination of Tumors and Evaluation of Treatment Efficacy

All tumors were examined histopathologically by using specimens obtained before chemotherapy, and their histological type, histological grade, hormone receptor status, and HER-2 amplification were recorded. Tumor size was measured with calipers and ultrasound before and after chemotherapy. The maximum length and width of the tumor were recorded, and

clinical response to PCT was assessed by multiplication of the two measurements in accordance with the criteria of the World Health Organization (WHO) (9).

After the operation subsequent to PCT, efficacy of chemotherapy was evaluated histopathologically according to the 15th edition of the General Rules for Clinical and Pathological Recording of Breast Cancer published by the Japanese Breast Cancer Society. The status of axillary lymph nodes was also examined.

MRI Examination

The breast MRI was obtained by means of a Signa Horizon LX with a field strength of 1.0 T unit (GE Yokogawa Medical Systems, Tokyo, Japan) using a breast phased array coil. The conditions for image sequences were similar for all patients and for the pre- and post-chemotherapy examination. Before a dynamic examination, a sagittally oriented T1-weighted fat-suppressed spin-echo sequence (TE 18, TR 500, matrix size 256 × 224mm, field of view [FOV] 16 × 16mm, 5.0 mm-slice thickness, 1.5 gap) and a coronally oriented T1-weighted fat suppressed spin-echo sequence (TE 11, TR500, matrix size 512 × 224mm, FOV 16 × 16mm, 5.0 mm-slice thickness 1.7 gap) were obtained, followed by a sagittally oriented T2-weighted fast spin-echo sequence (TE 102, TR 4000, FOV 16 mm, matrix size 512 × 512 mm, 5.0 mm-slice thickness 1.7 gap) and a coronally oriented T2-weighted fast spin-echo sequence (TE 102, TR 4000, FOV 16 mm, matrix size 512 × 512 mm, 5.0 mm-slice thickness 1.8 gap). The dynamic study was then performed in the same manner as that for the sagittally oriented T1-weighted fat suppressed spin-echo sequence using an EFGRE 3D sequence (TE 3.6, Prep Time 32, flip angle [FA] 30°, FOV 16 mm, matrix size 256 × 128, acquisition time 50 milliseconds, 4.0 mm-slice thickness, no gap). Data acquisition from the dynamic contrast-enhanced series was performed precontrast, immediately (early phase), and 1, 2, 4, and 7 minutes after intravenous (IV) injection of 0.1 mol/kg body weight Gd-DPTA, Magnevist (Schering, Berlin, Germany) into the cubital vein by rapid injection through an IV catheter. The fat-suppression method was used to distinguish enhanced lesions from the bright fat signal on T1-weighted images.

Measurement of Tumor Size and Volume with MRI

Three-dimensional MR images were created by means of the volume rendering method from the original two-dimensional (2D) MR images with the aid of the 3D-image analysis software, Virtual Place (Medical Imaging Laboratory, Tokyo, Japan). A 3D subtracted maximum intensity projection (3D MIP) image was obtained by subtraction of the pre-enhanced 3D MR image from the early-phase contrast-enhanced 3D image. The resultant 3D MIP image was observed from various angles by rotating the image manually, and the extent of the tumor was determined as described previously (8,10). The maximum length and width were measured on the anterior-posterior view of the MIP image, and clinical response was assessed using the WHO criteria. The tumor volume was also measured by using the same software. Residual tumor images were obtained after trimming off surrounding tissue with the masking method at various angles, and the volume was then calculated with the software. When multiple tumor nests were detected, the volume of each nest was measured individually and then summed (Fig. 1).

Statistical Analysis

Dr. SPSS II (SPSS Japan Inc, Tokyo, Japan) was used for statistical analysis. The factors examined were patient age, histological grade, hormone receptor status, HER-2 amplification, axillary lymph node status after surgery, maximum diameter of tumor measured before and after PCT, rate of tumor size reduction examined in two directions according to the WHO criteria and rate of tumor volume reduction measured with 3D MRI. To examine the relationship between tumor reduction and distant recurrence-free survival (DRFS), patients were divided into two groups according to the reduction rate with a range from 0% to 100% at increments of 5%, and DRFS was compared between each pair of groups. The relationships between other clinicopathological parameters as well as the reduction rate and DRFS were examined using the Kaplan–Meier method and tested with the log-rank test. Parameters with significant correlation to DRFS ($p \leq 0.05$) were then analyzed using multivariate analysis with the stepwise Cox proportional hazards analysis, and the results were displayed as Kaplan–Meier curves.

RESULTS

Clinicopathological Parameters

Patient' characteristics are summarized in Table 1. The mean follow-up period for the 51 patients was 46 months (range, 1–64 months), during which 25 patients developed distant recurrences, with a median

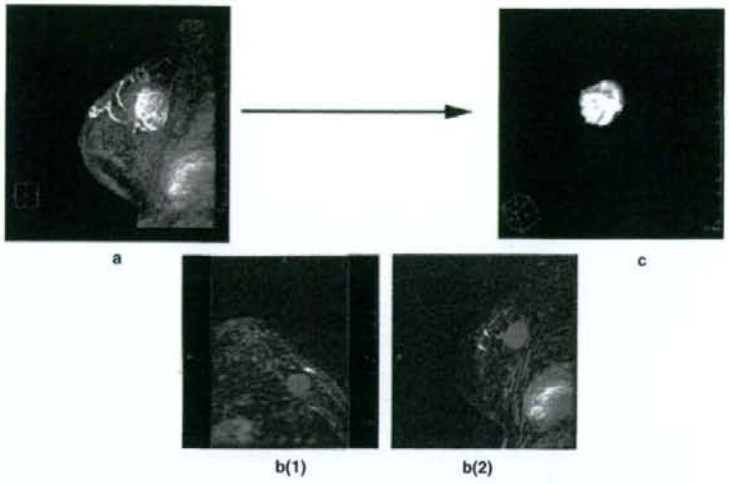

Figure 1. Tumor volume was measured on 3D tumor MR images. Three-dimensional (3D) tumor image (c) was obtained after trimming off tissue surrounding the tumor in 3D maximum intensity projection (MIP) image (a) by using masking method at various angles (b-1, b-2).

time to recurrence of 35.5 months (range, 1–62 months). The median initial tumor size before PCT was 4.5 cm when measured with calipers, 3.6 cm with US and 4.6 cm with MRI. Estrogen receptors showed positivity in 28 patients, and progesterone receptors in 17 patients. Ten patients were positive for HER-2 overexpression, 33 negative, and for eight patients it was not examined. Forty-two patients underwent total mastectomy after PCT, and nine partial mastectomy with a completion of axillary clearance.

Clinical responses examined by calipers and assessed according to the WHO criteria were one CR, 36 PR, 13 no change, and one progressive disease. According to histopathological evaluation criteria of efficacy of chemotherapy based on the 15th edition of General Rules for Clinical and Pathological Recording of Breast Cancer edited by the Japanese Breast Cancer Society, three patients were classified as grade III with no residual tumor cells, 10 as grade II with major pathologic changes in more than two-thirds of the tumor, 35 as grade I with major changes in less than two-thirds of the tumor or minor change regardless of response area, and three as grade 0 with no response. Axillary lymph node metastases were observed in 30 patients.

Correlation of Parameters with Recurrence-free Survival

Results of univariate analysis of correlation between each of the parameters and DRFS are summarized in Table 2. Preoperative histological grade correlated significantly with DRFS ($p = 0.02$), but other parameters, such as menopausal status, hormone receptors, HER-2 amplification and axillary lymph node status, showed no significant correlation with DRFS. No significant correlation was also observed between DRFS and initial tumor diameter, when the patients were divided into two groups (tumor diameter ≥5 and <5 cm) measured by calipers ($p = 0.96$), by US ($p = 0.19$), by 2D MRI ($p = 0.18$), and also between DRFS and initial tumor area determined by the WHO criteria measured by the tree modalities when the patients were divided into two groups (tumor area ≥20 and <20 cm^2). Initial tumor volume measured with 3D MRI also showed no significant correlation with DRFS.

Correlation between tumor reduction rate measured with each of the modalities and DRFS are summarized in Table 3. Volumetric reduction rate measured by 3D MRI correlated significantly with DRFS, when patients were divided into two groups (reduction rate ≥75% and <75%; $p = 0.03$). Twenty-three of the 40 patients (57.5%) with TTV reduction rates of <75% developed distant recurrences, while only two of the 11 patients (18.2%) with TTV reduction rates of 75% or more did. The Kaplan–Meier curves are shown in Table 3. On the other hand, reduction in tumor size measured by ultrasound and MRI according to the WHO criteria showed no significant correlation with DRFS.

Table 2. Relationship between Clinicopathological Parameters and Distant Recurrence-free Survival

Variables		Number of patients			p-value*
		With recurrence	Without recurrence	Total	
Menopausal status[1]	Premenopausal	10	11	21	0.88
	Postmenopausal	15	15	30	
Estrogen receptor	Positive	15	13	28	0.48
	Negative	10	13	23	
Progesterone receptor	Positive	6	11	17	0.27
	Negative	19	15	34	
HER-2 amplification	Positive	7	3	10	0.24
	Negative	16	17	33	
Histological grade[2]	1 + 2	19	24	43	**0.02**
	3	6	2	8	
Nodal metastasis[3]	Positive	17	13	30	0.25
	Negative	8	13	21	
Pathologic response[4]	Grade 0	2	1	3	0.42
	Grade I	15	17	32	
	Grade II	7	6	13	
	Grade III	1	2	3	
Initial tumor size					
Tumor diameter[5]					
Calipers	<5 cm	14	17	31	0.96
	≥5 cm	11	9	20	
Ultrasound	<5 cm	19	23	42	0.19
	≥5 cm	6	3	9	
2D MRI	<5 cm	11	18	29	0.18
	≥5 cm	14	8	22	
Tumor area[6]					
Calipers	<20 cm^2	19	22	41	0.36
	≥20 cm^2	6	4	10	
Ultrasound	<20 cm^2	20	24	44	0.15
	≥20 cm^2	5	2	7	
2D MRI	<20 cm^2	21	18	39	0.10
	≥20 cm^2	4	8	12	
Tumor volume[7]					
3D MRI	<20 cm^3	13	13	26	0.87
	≥20 cm^3	12	13	25	
	<30 cm^3	18	20	38	0.40
	≥30 cm^3	7	6	13	
	<40 cm^3	21	23	44	0.26
	≥40 cm^3	4	3	7	

MRI, magnetic resonance imaging.
[1]Menopausal status of patients at the start of chemotherapy.
[2]Histological grade of tumors determined before primary chemotherapy.
[3]Axillary lymph node metastasis examined after surgery.
[4]Pathologic assessment of response to primary chemotherapy based on the 15th edition of the General Rules for Clinical and Pathological Recording of Breast Cancer published by the Japanese Breast Cancer Society.
[5]Maximum length of tumor before chemotherapy.
[6]Results of multiplication of maximum length and width of tumor before chemotherapy according to the criteria of World Health Organization.
[7]Total tumor volume measured with 3D MRI before chemotherapy.
*Log-rank test.
Bold value indicates the statistically significant value.

Multivariate Analysis of Parameters

Multivariate analysis showed that volumetric reduction measured by 3D MRI was the only prognostic factor for DRFS (Table 4). In particular, patients with a TTV reduction of 75% or more showed significantly better prognosis than those with reduction rates of <75% (hazard ratio = 0.122, confidential interval = 0.053–0.949, p = 0.042). The preoperative histological grade, which was identified as significant by univariate analysis, showed no significance for DRFS in multivariate analysis.

DISCUSSION

Evaluation of the efficacy of PCT and assessment of prognosis for a patient with breast cancer are important for deciding treatment strategies including surgery and postoperative adjuvant therapy. Various new

Table 3. Relationship between reduction rate of tumor size measured with three modalities and distant recurrence-free survival

Modality	Reduction rate (%)	Number of patients: With recurrence	Without recurrence	Total	Median DFRI	p-value[‡]
Ultrasound*	<50	10	7	17	50	0.25
	≥50	15	19	34	41	
	<60	15	16	31	53	0.70
	≥60	10	10	20	50	
	<70	17	19	36	53	0.89
	≥70	8	7	15	45	
	<75	20	23	43	53	0.37
	≥75	5	3	8	21	
	<80	22	24	46	50	0.72
	≥80	3	2	5	51	
2D MRI*	<50	12	13	25	53	0.81
	≥50	13	13	26	45	
	<60	15	16	31	53	0.66
	≥60	10	10	20	45	
	<70	19	19	38	50	0.88
	≥70	6	7	13	50	
	<75	21	20	41	50	0.43
	≥75	4	6	10	50	
	<80	23	23	46	50	0.52
	≥80	2	3	5	50	
3D MRI†	<50	7	7	14	45	0.86
	≥50	18	19	37	50	
	<60	17	17	34	45	0.83
	≥60	8	9	17	50	
	<70	22	17	39	45	0.07
	≥70	3	9	12	48	
	<75	23	17	40	37	0.03
	≥75	2	9	11	50	
	<80	24	24	48	50	0.63
	≥80	1	2	3	45	

DFRI, distant recurrence-free survival; MRI, magnetic resonance imaging.
*Tumor size was calculated by multiplication of maximum length and width of tumor according to the criteria of the World Health Organization.
†Tumor size was determined as total tumor volume measured with 3D MRI.
‡Log-rank test.

Table 4. Results of multivariate Cox's proportional hazards analysis

		Hazard ratio	p-value	95% confidential interval
Reduction rate of total tumor volume	<75	1	**0.042**	0.053–0.949
	≥75	0.122		

agents, such as taxanes and trastuzumab, have been used for PCT recently, while treatment has become longer and dosages higher to achieve better clinical outcome (11–16). Furthermore, even patients with a small breast cancer can be candidates for PCT when they show clinical evidence of axillary lymph node metastases. In such cases, an accurate evaluation of PCT efficacy becomes essential.

Measurement of tumor size and pathologic examination are generally used for assessment of the response to chemotherapy. For evaluation of change in tumor size, the WHO criteria, which involve multiplication of the maximum length and width, are widely used. In PCT cases, however, accurate measurement of the tumor is often difficult, because major histological changes, such as tumor necrosis and fibrosis, occur in and around the tumor (6,7). Since evaluation with conventional methods, such as calipers, mammography and ultrasound, is thus often not accurate (5,17,18), we used 3D MRI, and the utility of which was reported previously (8). However, breast cancer does not always shrink to a single focus, so that some cases show many small tumor nests after PCT, thus making measurement of tumor size according to the WHO criteria often difficult (Fig. 2). In fact, the two-dimensionally measured rate of tumor size reduction was not a significant predictor of DRFS in the current study. We therefore measured TTV as a parameter of tumor size. With this method, multiple residual small foci of breast cancer could be evaluated accurately, and the reduction in TTV was found to be

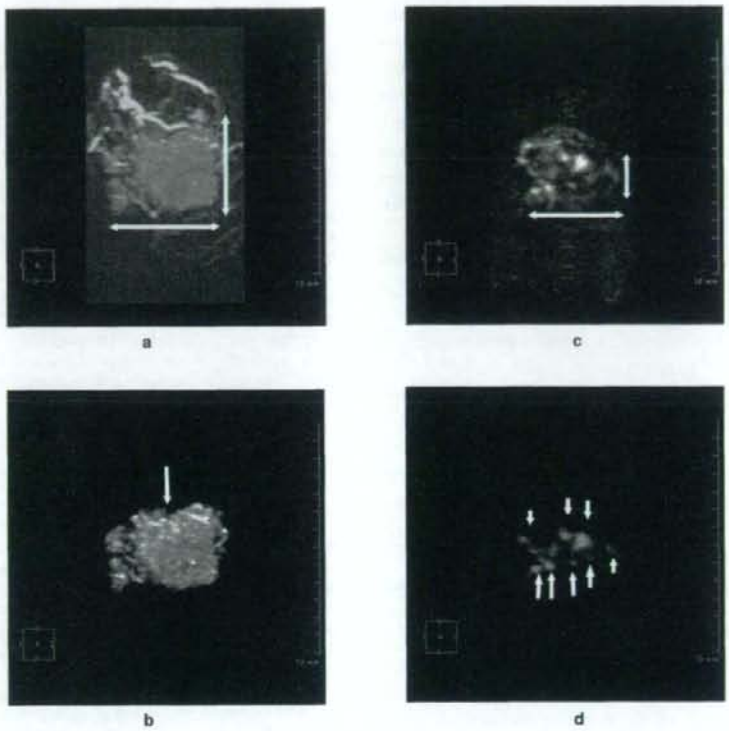

Figure 2. Upper row: Anterior-posterior views of 3D MRI of the right breast of a 63-year-old woman with invasive ductal carcinoma before (a) and after (b) primary chemotherapy (PCT). Tumor size before PCT, measured according to the WHO criteria, was 7.7 × 4.6 cm, and after PCT it was 5.4 × 4.2 cm, for a reduction rate of 35.9%. Lower row: Three-dimensional images of the tumor before PCT (c) and the residual tumors (small arrows) after PCT (d). Total tumor volume (TTV) was 66.0 cm^3 before and 15.8 cm^3 after PCT for a reduction rate of TTV of 76.1%. The patient was alive without recurrences at the end of the study.

a significant prognostic factor for DRFS. When the patients were divided into two groups with a tumor reduction rate of 75% as the demarcation point, those with a TTV reduced to 25% or less of the initial value showed significantly better prognosis than others. Five-years DRFS of those showing 75% or more reduction of TTV was 81.8%, and others was 42.5% in the current study. This means that the former might have attained pCR, if they had received further PCT treatment. Therefore, assessment of the efficacy of PCT by measuring TTV should prove useful for deciding whether the PCT regimen should be continued or changed (Fig. 3).

As for evaluation of therapeutic effect, only a few reported studies have used 3D imaging. Partridge et al., reporting on the usefulness of 3D MRI for evaluation of PCT, showed that the initial tumor size was the most significant variable for relapse-free survival

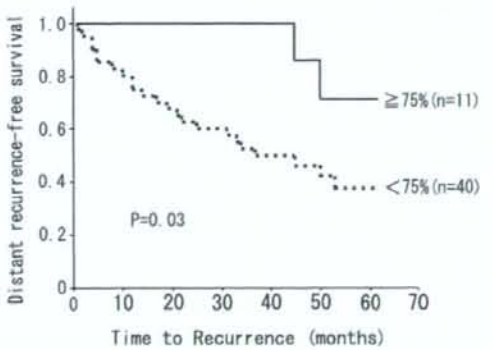

Figure 3. Association between distant recurrence-free survival (DRFS) and reduction in total tumor volume (TTV). Comparison of DRFS of two patient groups (TTV reduction rate ≥75% and <75%) showed significantly better prognosis for DRFS for the group with TTV reduction ≥75% ($p = 0.03$).

(19). The final change in MRI tumor volume was also found to be a significant prognostic factor when the patients were divided into two groups with a tumor volume reduction of 50% as the demarcation point. The median follow-up of the patients in that study was 32.5 months, shorter than that of the subjects in our study, while the recurrence rate was lower, even though both local and distant recurrences were counted, so that the difference was probably due to differences in the PCT regimen. In spite of these slight differences, however, the finding our studies have in common is that the rate of reduction in TTV measured by 3D MRI is a significant and useful prognostic factor for DRFS of patients treated with PCT.

On the other hand, histological grade is well known as a prognostic factor for early breast cancer patients as well as for advanced breast cancer patients treated with PCT (20–22). In our study, preoperative histological grade was also identified as an independently significant prognostic factor for DRFS by the Kaplan–Meier analysis with the log-rank test. However, this variable showed no significant association with DRFS when analyzed with the stepwise multivariate Cox proportional hazards analysis. Reduction in TTV thus remained as the only significant prognostic factor for DRFS. How effectively PCT can control the main tumor is therefore the most important factor for control of the disease, although the risk of distant metastases may increase as the histological grade of the tumor deteriorates. The entry criteria for PCT in our study, however, were T2 or T3 tumor regardless of the presence or absence of regional lymph node metastasis, so that there were only eight patients (16%) whose tumor was assessed as histological grade III. Furthermore, none of the patients showed pCR to the PCT with four cycles of docetaxel, while a number of patients showed pCR to more aggressive chemotherapy. These factors may also have affected our results.

In conclusion, reduction in TTV can be a powerful prognostic factor for DRFS of breast cancer patients treated with PCT. Furthermore, 3D MRI proved to be a useful modality for accurate evaluation of tumor volume after chemotherapy. However, these findings were obtained from a limited number of patients treated with a single regimen of chemotherapy, so that our findings need to be verified with a larger number of patients with longer follow-up as well as with patients treated with other chemotherapy regimens.

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Association of GSTP1 expression with resistance to docetaxel and paclitaxel in human breast cancers

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Abstract

Aims: It has been reported that glutathione S-transferase P1 (GSTP1) expression is implicated in resistance to taxanes (docetaxel and paclitaxel) in human breast cancer cells in vitro. In the study presented here, we examine whether GSTP1 expression is associated with resistance to docetaxel or paclitaxel in human breast cancers. We also investigated the relationship between GSTP1 methylation status and response to these taxanes.

Material and methods: Sixty two primary breast cancer patients were treated with docetaxel or paclitaxel as primary systemic treatment (PST). GSTP1 expression was detected immunohistochemically and the hypermethylation status GSTP1 gene was identified with a methylation specific primer assay.

Results: The mean tumor reduction rate for all patients ($n = 62$) was significantly ($p < 0.001$) higher in GSTP1 negative (0.73 ± 0.04; mean $\pm$ standard error) than GSTP1 positive (0.31 ± 0.09) tumors. The subset analysis showed that the mean reduction rate was significantly ($p = 0.005$) higher in GSTP1 negative (0.59 ± 0.06) than GSTP1 positive (0.11 ± 0.13) tumors in the docetaxel group as well as in the paclitaxel group ($p = 0.006$; GSTP1 negative tumors: 0.84 ± 0.05; GSTP1 positive tumors: 0.56 ± 0.08). On the other hand, GSTP1 methylation showed no significant association with the reduction rate.

Conclusion: Our present study has suggested that GSTP1 protein expression, but not GSTP1 methylation status, might be associated with response to docetaxel and paclitaxel. This suggests that GSTP1 immunohistochemical expression might be a potentially clinically useful predictive factor for response to docetaxel and paclitaxel.

Keywords: GSTP1; Docetaxel; Paclitaxel; Primary systemic treatment; Breast cancer

Introduction

Taxanes including docetaxel and paclitaxel are some of the most effective anticancer drugs for breast cancer. A growing number of breast cancer patients have recently been treated with taxanes not only in the metastatic setting but also in the adjuvant and neoadjuvant settings. Since the response rate to taxanes ranges from 22.9% to 43%[1–3] and not all patients benefit from taxane therapy, it is very important to select those patients who are likely to respond to taxanes in order to avoid unnecessary treatment. For this purpose, a reliable predictive factor for response to taxanes in human breast cancers needs to be developed.

Glutathione S-transferase P1 (GSTP1) belongs to a family of phase II metabolic enzymes that can detoxify several anticancer drugs by conjugating them with glutathione.[4] GSTP1 is therefore thought to confer resistance to chemotherapy. In fact, several studies have reported on the impact of GSTP1 expression on response to chemotherapy in breast tumor tissues.[5–8] Su et al. found that breast tumors with GSTP1 expression are resistant to anthracycline-containing chemotherapy in the neoadjuvant setting.[8] Huang et al. reported that patients with GSTP1 positive breast tumors showed a poorer prognosis than those with GSTP1 negative breast tumors when all the patients were

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

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treated with adjuvant chemotherapy, consisting of 49% anthracycline-containing regimen,[7] which suggests that GSTP1 can serve as a predictive factor for resistance to anthracycline-containing chemotherapy. Although contradictory results have also been reported,[9] a vast majority of the studies appear to indicate a significant relationship between GSTP1 expression and resistance to anthracycline-containing chemotherapy.[10]

On the other hand, the relationship between GSTP1 expression and response to taxanes has rarely been the subject of clinical studies of breast tumors. Recently, we were able to show that transfection of the GSTP1 expression vector into a human breast cancer cell line (MCF-7 cells) results in the acquisition of resistance to docetaxel.[11] Moreover, Mathieu et al. used an orthotopic model of a human non-small cell lung carcinoma cell line (A549 cells) to show that GSTP1 overexpression is associated with resistance to paclitaxel.[12] These results seem to suggest that GSTP1 plays a definite role in the acquisition of resistance to taxanes and that the identification of GSTP1 expression may thus be clinically useful. We therefore attempted to clarify the relationship between immunohistochemically identified GSTP1 expression and response to taxanes (docetaxel and paclitaxel) in the neoadjuvant setting. We also studied the GSTP1 methylation status and its association with response to taxanes since GSTP1 expression is often silenced by DNA promoter hypermethylation.

Materials and methods

Patients

Sixty two primary breast cancer patients (stage II, $n = 20$; stage III, $n = 35$; stage IV, $n = 7$) who were treated with docetaxel or paclitaxel monotherapy as primary systemic therapy (PST) at Osaka University Hospital between December 1999 and November 2005 were recruited for this study. Tumor tissue samples were obtained from primary breast tumors by means of core needle biopsy or vacuum-assisted core biopsy before PST. All patients were histologically diagnosed as invasive breast cancer, and no patients had been treated with chemotherapy and/or hormonal therapy before biopsy.

After informed consent had been obtained from all patients, 31 were treated with docetaxel (60 mg/m^2 every 3 weeks for 4 cycles), and the other 31 were treated with paclitaxel (80 mg/m^2 every week for 12 weeks) as PST. Of these 62 patients, 35 patients received anthracycline-containing chemotherapy after docetaxel ($n = 5$) or paclitaxel ($n = 30$), and all patients underwent breast conservative surgery or mastectomy after PST.

Assessment of clinical response

Bi-dimensional (cm^2) breast tumor measurements were made before and after taxane (docetaxel or paclitaxel) treatment and mostly with MRI since we have shown that MRI is the most accurate modality for measuring breast tumor size.[13] Reduction rate was calculated as follows: (area before taxane − area after taxane)/area before taxane.

Immunohistochemical assay

A total of 3 μm sections were cut and placed on silanized slides (DakoCytomation Inc., Carpinteria, CA). After dewaxing of the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 10 min. The sections were then immediately incubated at 95 °C in citrate buffer (pH 6) for 15 min. Immunostaining was performed by using an immunoperoxidase method according to the manufacturer's instructions (EnVision+ Dual Link System Peroxidase; DakoCytomation Inc.). The incubation of the primary rabbit anti-GST-Pi polyclonal antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was performed overnight at 4 °C at a dilution of 1:1000 in 1% BSA in PBS. After incubation, the secondary antibody was added for 45 min to amplify the specific binding of the primary antibody. The sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in PBS), counterstained with hematoxylin, dehydrated, and mounted. Appropriate control slides, positive and negative cases, were included in each series. Expression was considered to be positive when >10% of the tumor cells exhibited cytoplasmic or nuclear staining.[7]

Sodium bisulfite treatment

Genomic DNA was extracted from frozen tumor specimens with the phenol/chloroform method. Sodium bisulfite conversion of 2 μg of genomic DNA was performed with a modified version of a method as described previously.[14] Briefly, DNA was denatured with 0.2 M NaOH and incubated for 30 min at 37 °C. A volume of 520 μl of freshly made bisulfite solution [3 M sodium metabisulfite and 10 mM hydroquinone (pH = 5.0)] was added to each sample, and the mixture was then incubated at 55 °C for 16 h in the dark. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer's instructions (Promega Corp., Madison, WI) and eluted into 50 μl of water. Modification was completed by NaOH (final concentration: 0.3 M) treatment for 20 min at 37 °C, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20 °C.

Methylation specific PCR (MSP) assay

For PCR amplification, 1 μl of bisulfite-modified DNA was added to a final volume of 20 μl PCR mix containing 1 × PCR buffer [18 mM ammonium sulfate, 60 mM Tris (pH 8.9)], deoxynucleotide triphosphates (0.2 mM each), 1 unit Platinum TaqDNA polymerase (Invitrogen, Carlsbad,

CA), and primers. The primer sequences for GSTP1 for the unmethylated reaction were 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ (upper primer) and 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′ (lower primer) and for the methylated reaction 5′-TTC GGG GTG TAG CGG TCG TC-3′ (upper primer) and 5′-GCC CCA ATA CTA AAT CAC GAC G-3′ (lower primer). PCR amplifications were carried out under the following conditions: 1 cycle at 95 °C for 5 min, and 38 cycles at 95 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min. The final extension was performed for 5 min at 70 °C.[15] DNA from MCF-7 breast cancer cells was used as a positive control for methylated alleles, and DNA from normal lymphocytes as a negative control for methylated genes.[16] PCR reactions were analyzed with 3% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis

The relationship between GSTP1 expression and clinicopathological parameters were examined by χ^2 test or Fisher's exact test. Correlations between quantitative reduction rate and GSTP1 expression or methylation status were evaluated with the Mann–Whitney *U*-test. Statistical significance was defined as $p < 0.05$.

Results

GSTP1 expression and clinicopathological parameters of breast cancers

Of the 62 breast tumors, 35 were found to be GSTP1 positive. There was a significant ($p < 0.05$) association between GSTP1 positive expression and large tumor size or clinical cancer stage, as well as between GSTP1 methylation and GSTP1 negative expression. No other clinicopathological parameters, such as age, menopausal status, histological grade, ER, PR, HER-2, were significantly associated with GSTP1 expression.

Relationship between GSTP1 expression and response to docetaxel or paclitaxel

For all the patients ($n = 62$) treated with docetaxel or paclitaxel, the mean reduction rate (0.73 ± 0.04) in the GSTP1 negative tumors was significantly higher than that (0.31 ± 0.09) in the GSTP1 positive tumors, as it was for the patients ($n = 31$) treated with docetaxel alone (corresponding mean reduction rates 0.59 ± 0.06 and 0.11 ± 0.13). And for those treated with paclitaxel alone ($n = 31$) (corresponding values: 0.84 ± 0.05, 0.56 ± 0.08). Tumor size and clinical stage were not significantly associated with reduction rate in all patients, in those treated with docetaxel, or in those treated with paclitaxel, while there was a significant association between GSTP1 expression and tumor size or clinical stage.

Relationship between GSTP1 methylation status and response to docetaxel or paclitaxel

Genomic DNA could be obtained from 48 breast tumors and was subjected to MSP assay. There ware 10 tumors (21%) with GSTP1 methylation. Of all the patients ($n = 48$) treated with docetaxel or paclitaxel, the reduction rate (0.68 ± 0.08) for the GSTP1 methylated tumors was not significantly different from that (0.47 ± 0.07) for the GSTP1 unmethylated tumors, nor was any significant difference found when the patients treated with docetaxel or paclitaxel were considered separately.

Discussion

Association of GSTP1 expression with resistance to docetaxel and paclitaxel

We were able to show herein that GSTP1 negative breast tumors are significantly more closely associated with a higher reduction rate to docetaxel or paclitaxel than are GSTP1 positive breast tumors. Although several in vitro studies have suggested that GSTP1 expression is implicated in the acquisition of resistance to taxanes,[11,17,18] the significance of GSTP1 expression for resistance to taxanes has rarely been studied clinically in human breast cancers. Our study is thus the first to investigate the clinical significance of GSTP1 expression for taxane resistance in the PST setting. Until now, only one such study has been reported by Schmidt et al., who investigated the relationship between GSTP1 expression determined by immunohistochemistry and response to paclitaxel in metastatic breast cancers, but they could not show a significant association.[19] This discrepancy between their study and ours may be explained, at least in part, by the prior use of hormonal therapy and/or chemotherapy in Schmidt et al.'s study (73% of their patients had undergone therapy before paclitaxel administration) and the absence of such therapies in our study. Such prior therapies may have affected the GSTP1 expression. Our study, based on primary breast cancer patients treated with PST, seems to have an advantage over than based on metastatic breast cancer patients, who often have a history of prior therapy. When tumors are naïve to chemotherapy, the significance of a candidate predictive factor for response to chemotherapy can be evaluated under more relevant conditions not affected by prior therapy.

GSTP1 and metabolism of docetaxel and paclitaxel

CYP3A4 is involved in the inactivation of docetaxel and paclitaxel, and we were recently able to show that CYP3A4 expression in breast tumor tissues is associated with resistance to docetaxel.[20] GSTP1 is involved in the second phase of metabolism of docetaxel and paclitaxel, i.e., conjugation of docetaxel metabolites and paclitaxel metabolites with glutathione, suggesting a possibility that the

enhanced metabolism of these agents induced by GSTP1 up-regulation may lead to decreased anti-tumor activity.[17] The relationship of intra-tumoral concentrations of taxanes and their metabolites with GSTP1 expression thus need to be investigated in order to clarify the role of enhanced metabolism by GSTP1 in resistance to taxanes.

GSTP1 and c-Jun N-terminal kinase (JNK)-mediated apoptosis

Another reason which may explain the association of GSTP1 expression with resistance to taxanes is the anti-apoptotic effect of GSTP1. It has recently been shown[21] that monomeric GSTP1 can form a complex with JNK, which has a very important function in the control of cell survival and death pathways, while GSTP1 overexpression inhibits apoptosis induced by JNK.[22] Since both docetaxel and paclitaxel reportedly activate JNK in a dose-dependent manner and induce apoptosis,[23] we speculate that GSTP1 up-regulation may inhibit this JNK-mediated apoptosis, resulting in resistance to docetaxel and paclitaxel.

GSTP1 methylation and response to docetaxel and paclitaxel

It has been well established that GSTP1 gene expression is silenced by methylation of its promoter region in about 13–30% of breast cancers.[24–26] In our study, 10 tumors were found to have GSTP1 methylation. Although the reduction rate in tumors with GSTP1 methylation (0.68 ± 0.08) was higher than in those without GSTP1 methylation (0.47 ± 0.07), there was no statistically significant difference. In eight of the 10 tumors with GSTP1 methylation, immunohistochemistry detected no GSTP1 protein expression. On the other hand, in the 38 tumors without GSTP1 methylation, GSTP1 protein expression was present in 24 tumors and absent in 14 tumors, indicating that GSTP1 methylation is strictly associated with negative GSTP1 expression but that unmethylated GSTP1 does not necessarily mean positive GSTP1 expression. Consequently, the weak relationship ($p = 0.162$) between GSTP1 methylation status and its protein expression seems to explain why GSTP1 methylation status is not significantly associated with a response to taxanes.

Limitations of the present study

Since pathological complete response (pCR) achieved by PST is associated with a favorable patient prognosis,[27] pCR has recently been used more and more often as a marker of response in the PST setting. However, pCR rates achieved by docetaxel or paclitaxel monotherapy are too low[28] to be used as meaningful marker, so that we used clinical response instead. It has been reported that clinical response is also associated with a favorable patient prognosis.[29] We therefore believe that clinical response, especially if assessed accurately by MRI, can be a reliable marker of response to chemotherapy. Another limitation of the present study is a small number of patients analyzed. So, definitive conclusions are unlikely to be drawn from this study. Our findings need to be confirmed by a future study including a larger number of patients.

Conclusion

In the present study, we have suggested, though preliminary due to a small number of patients, that GSTP1 protein expression, but not GSTP1 methylation status, can serve as a marker for resistance to both docetaxel and paclitaxel in primary breast cancers. Since GSTP1 protein expression can be determined by immunohistochemistry, it seems to have a potential to be applied to a routine clinical test after relevant clinical studies to confirm the clinical significance of GSTP1.

Conflict of interest

This manuscript does not have any potential conflicts.

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Prognostic Significance of CD55 Expression in Breast Cancer

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Abstract

Purpose: Our recent study revealed that CD55-high population in breast cancer cell line was resistant to apoptosis and formed colonies *in vitro* more efficiently than CD55-low population. The present study was conducted to examine whether CD55-high population in breast cancer cell line possesses higher tumorigenic potential *in vivo* and presence of CD55-high cells in breast cancer affects clinicopathologic behavior of patients.

Experimental Design: CD55-high and CD55-low population was sorted from breast cancer cell line, injected into immunodeficient mice, and the resultant tumor volume was measured. CD55 expression was immunohistochemically examined in clinical samples from 74 cases with breast cancers, and cases with >1% of tumor cells showing high level of CD55 expression were categorized as CD55 high.

Results: The xenotransplanted tumor volume derived from CD55-high population was significantly larger than that from CD55-low population. Fifty (67.6%) of 74 cases of breast cancer were CD55-high. A significant correlation was observed between CD55-high character and relapse rate ($P < 0.001$). Univariate analysis showed that tumor size ($P = 0.005$) and CD55 expression ($P = 0.005$) were unfavorable prognostic factors. Multivariate analysis revealed that the tumor size ($P = 0.013$) and CD55 expression ($P = 0.011$) were independent prognostic factors.

Conclusions: CD55 play an important role in tumorigenesis of breast cancer, and presence of small population of cells with strong CD55 expression would be sufficient to predict poor prognosis of patients.

Neoplasia is a proliferation of a single clone of cell, which becomes to comprise heterogeneous cell populations along with progression of proliferation because of additional genetic abnormalities (1). In such condition, the capability to sustain tumor formation and growth is restricted to a small population of cells with high tumorigenic ability, called cancer stem cells or tumor-initiating cells (2, 3). These tumor-initiating cells have been identified in various kinds of malignancies (4–13). The heterogeneous properties of monoclonally proliferating cells could be shown even in cell lines derived from cancers. MCF7 breast cancer cell line contains two distinct populations, which are stained with Hoechst 33342 dye in a different manner; one expelling dye and the other stained with dye strongly (14, 15). The former is called side population (SP) and the latter non-SP. SP cells give rise to both SP and non-SP cells, and are tumorigenic in mice (14). Through characterization of SP cells in several cell lines, Patrawala et al. (15) reported that the SP cells are more tumorigenic than non-SP cells. These findings indicate that SP cells contain higher proportion of cancer stem cells than non-SP cells.

CD55 is a glycosylphosphatidylinositol-anchored protein, which regulates complement activation pathway (16, 17). As in the case of many glycosylphosphatidylinositol-anchored proteins, CD55 is either bound to the cell membrane or released from the membrane into the microenvironment (18–20). CD55 binds to C3 convertases generated from both the classic and alternative complement pathways, prevents C3b deposition, thus inhibits the formation of membrane attack complex (16, 17). Physiologically, CD55 is expressed in cells exposing to the complement system and plays a role in protection of cells against complement attack. Complement attack is a powerful innate mechanism in protection of the host against pathogens, including cancer (21).

To escape from the complement attack, cells of several cancer cell lines are known to express CD55. The expression levels of CD55 are various among monoclonal cell lines. Staining of HT29 colon cancer cell line with anti-CD55 antibody showed a broad spectrum of expression levels among cancer cells (19). This broad spectrum is also observed in MCF7 breast cancer cell line (22). Very recently, we found that CD55-high population in the MCF7 cells is resistant to apoptotic stimuli and forms colonies *in vitro* more efficiently than CD55-low population (22). CD55 expression level is higher in SP cells than in non-SP of MCF7 cells. These findings suggest that the high expression of CD55 may be a character of breast cancer stem cells. In the

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present study, the significance of CD55-high population was studied in the clinical samples of breast cancer.

CD55 is detected in clinical samples from various kinds of malignant tumors (23–26). CD55 expression level is higher in prostatic carcinoma than in normal prostate epithelium (23). The staining intensity of CD55 in both differentiated and undifferentiated gastric adenocarcinomas is significantly higher than that in normal gastric epithelium (24). In bulky lymphoma, the expression level of CD55 is positively correlated with tumor size and resistance to rituximab treatment (25). The knocked-down expression of CD55 reduces tumorigenicity of prostatic adenocarcinoma cell line in severe combined immunodeficient mice (Scid; ref. 27). These findings suggest that CD55 plays an important role in tumorigenicity of cancer cells.

Whether the overexpression of CD55 is an unfavorable factor for cure of cancer or not was studied. In colorectal carcinoma, the overexpression of CD55 is an independent factor for poor prognosis (28). However, a contradictory result is obtained in breast cancers; a loss of CD55 is associated with the aggressiveness of breast cancers (29). We examined CD55 expression immunohistochemically in 74 cases with breast cancers: strong expression of CD55 was found in a quite small population of tumor cells in two thirds of cases. Such cases might be judged as CD55-low in the previous report. In the present study, attention was paid on the population expressing CD55 at a high level. Cases showing the high level of CD55 expression in >1% of tumor cells were considered as CD55-high. With this criteria, the recurrence rate in CD55-high cases was significantly higher than that in CD55-low cases. These findings suggested that the presence of CD55-high population was a poor prognostic marker in breast cancers, even if the frequency was quite low.

Table 1. Summary of characteristics of 74 breast cancer patients

	Number of patients
Menopausal status	
Premenopausal	31
Postmenopausal	43
Histologic type	
Papillotubular	26
Solid-tubular	26
Scirrhous	18
Others	4
Histologic grade	
I+II	59
III	13
Estrogen receptor status	
Positive	43
Negative	28
Progesterone receptor status	
Positive	31
Negative	40
Tumor size (cm)	
<2	8
>2, ≤5	61
>5	5
Lymph node metastasis	
Positive	45
Negative	29
Stage	
IIA	32
IIB	28
III	14

Materials and Methods

Cells and flow cytometry. The cell line MCF7 from human breast adenocarcinoma was purchased from the American Type Culture Collection, and were cultured in DMEM (Sigma) supplemented with 10% FCS (Nippon Bio-supp. Center). MCF7 cells were sorted as CD55-high population and CD55-low population as described previously (22).

Mice and xenograft transplantation. Six- to 8-week-old female nonobese diabetic (NOD)/Scid mice were purchased from Charles River Laboratories Japan and kept under specific pathogen-free conditions. Before xenotransplantation, the mice were deeply anesthetized. All animal experiments were done according to the guideline of Osaka University Animal Center and approved by the institutional review board of committee of animal experiments (No. 753). For xenograft transplantation, sorted MCF7 cells (8×10^4) were suspended in 0.2 mL of Matrigel (BD Biosciences), and were injected s. c. in the mammary fat pad of NOD/Scid mice. CD55-high and CD55-low population was injected into the fat pad of left side and right side of the same animal, respectively. In some experiments, a whole MCF7 cells (8×10^4) were injected, and tumor volume was estimated using the formula: $(\text{width})^2 \times (\text{length})/2$ according to the report by Meyer-Siegler et al. (30).

Patients and tumor tissues. There were 74 female patients who underwent breast-conserving surgery or mastectomy for invasive breast cancers with stage II and stage III during the period from 1998 to 2001 at Osaka University Hospital: they were selected for the current study. The character of these 74 patients was summarized in Table 1. The age of patients ranged from 35 to 82 y (median age, 55 y). Neither chemotherapy nor hormonal therapy before surgery were used in these patients. Histologic specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology of Osaka University Hospital at room temperature, and were sectioned at 4-μm thickness at the time of staining with H&E, and immunoperoxidase procedure. After surgery, 25 patients were treated with tamoxifen (20 mg/d; $n = 16$), tamoxifen + goserelin ($n = 6$), and Toremifen ($n = 3$). As an adjuvant chemotherapy, 6 cycles of CMF (100 mg/d cyclophosphamide p.o. days 1-14 + 40 mg/m^2 methotrexate i.v. days 1 and 8 + 600 mg/m^2 5-fluorouracil i.v. days 1 and 8) were used in 8 patients, 4 cycles of EC (60 mg/m^2 epirubicin i.v. day 1 + 600 cyclophosphamide mg/m^2 i.v. day 1) in 3 patients, and Docetaxel in 4 patients. Thirty patients were treated with combined chemotherapy [CMF ($n = 5$), EC ($n = 10$), or other chemotherapies ($n = 9$)] and hormone therapy [tamoxifen ($n = 18$), tamoxifen + goserelin ($n = 2$), and Toremifen ($n = 5$)]. Four patients did not receive any adjuvant therapy. The median follow-up period ranged from 17 to 100 mo (median, 83 mo). Histologic types of tumors were papillotubular carcinoma (26 cases), solid-tubular carcinoma (26 cases), scirrhous carcinoma (18 cases), and others (4 cases; 2 invasive lobular carcinoma and 2 squamous cell carcinoma). The actual overall and disease-free 7-year survival rate was 86% and 58%, respectively. The present study was done in accordance with the author's institutional guidelines and was approved by institutional review boards. Informed consent was obtained from each patient.

Immunohistochemistry. Immunoperoxidase procedure (avidin-biotin-complex method) was done on the paraffin-embedded sections. After antigen retrieval with Pascal pressurized heating chamber (DAKO A/S), the sections were incubated with anti-CD55 antibody (clone 133-30; Abcam Ltd), and a tyramide signal amplification system (CSAII kit; Dako) was applied. As the negative control, staining was carried out in the absence of primary antibody. Staining of endothels and mast cells for CD55 was used as internal positive control. Intensity of the staining in each tumor cell was categorized as none, weak, and strong. At least 10,000 tumor cells were evaluated. When >1% of the tumor

cells strongly expressed CD55, the tumor was judged as CD55-high. The evaluation for CD55 staining was done independently by two pathologists (J. I. and E. M); the results were compared and discussed for patients with discrepant findings. The xenotransplanted tumors were routinely processed for paraffin embedding, and sections were immunohistochemically stained with anti-CD55, anti-Bcl2 (clone 124; Dako), and anti-Ki67 (clone MIB-1; Dako antibodies). For detection of the signals for Bcl2 and Ki67, the Envision system (Dako) was used. The microvessels in xenotransplanted tumors were stained with anti-CD34 antibody (Hycult Biotechnology), and their number per high-power field (200-fold) was counted.

Reverse transcription-PCR analysis. Total RNAs from MCF7 cells of CD55-high and CD55-low populations were extracted with RNeasy kit (Qiagen) with DNase I treatment and subjected to reverse transcription by Superscript III (Invitrogen). One microliter of the reverse-transcribed product was added to 25 μL of PCR mixture containing 1.25 U of Taq DNA polymerase (Roche Diagnostics GmbH) and 25 pmol of each of the primers. The sequence of primers was as follows: 5′-GCCAGCACACAAGAAAGCCAGTTC and 5′-CCTCCGAGAAGGCAGCCAGATT for EphA8 gene, 5′-GCAGGTGAACGTGAACGACTATCT and 5′-AACTCGTAGCCCAGAGAGAAGGCGCT for ephrinA3 gene, and 5′-GCCGAGCGGGAAATCGTGCG and 5′-ACGATGGAGGGGCCGGACTC for actin gene.

Statistical analysis. Statistical analysis for the tumor volume in xenograft transplantation was carried out using Student's *t* tests. The values were shown as the mean ± SE. The relationship between CD55 expression level and clinicopathologic variables was analyzed with χ^2 test or Mann-Whitney's *U* test. The relapse-free survival curves were calculated by the Kaplan-Meier method, and the log-rank test was used for evaluating differences. The independent prognostic factors were analyzed with the Cox proportional hazard model. Statical significance was defined as having a *P* value of <0.05. The sample size calculation showed that the expected difference of 50% in disease-free survival between CD55-high (95% survival rate) and CD55-low populations (45% survival rate) can be detected on a significant level of a *P* value of <0.05 (two-sided test) with 90% power and an α level of 0.05 if 18 patients are included in each of the two groups (31). Some more patients were recruited into the present study because a certain imbalance between both groups was expected.

Results

We previously reported that the MCF7 breast cancer cell line expressing CD55 at a high level forms colonies *in vitro* more efficiently than that expressing CD55 at a low level. To examine the role of CD55 for tumorigenicity *in vivo*, CD55-high and CD55-low populations of MCF7 cells were injected into NOD/Scid mice. At 2 weeks after injection, tumor mass was found only in the injected site of CD55-high cells (Fig. 1A). Although the small mass was detected in the injected site of CD55-low cells at 4 weeks, the size of mass was significantly larger in the injected site of CD55-high cells throughout the observed period (Fig. 1A and B).

Microscopically, both tumor cells derived from CD55-high population and CD55-low population formed a glandular structure (Fig. 2A and B). When compared with the tumors derived from CD55-low population (Fig. 2B), the tumors derived from CD55-high population contained a lot of collagen fibers and vasculatures (Fig. 2A). Immunohistochemistry revealed the concomitance of both CD55-high and CD55-low cells in the tumors derived from CD55-high population (Fig. 2C). In contrast, CD55-high cells were never detected in the tumor derived from CD55-low population (Fig. 2D). Bcl-2 expression was not detected immunohistochemically in both the tumors derived from CD55-high or CD55-low population. Ki-67 labeling index in tumor from CD55-high population was comparable with that in tumor from CD55-low population (Fig. 2E and F). Injection of a whole MCF7 cells into NOD/Scid mice generate tumors with sizes intermediate between tumors from CD55-high and CD55-low population (Fig. 2G).

The number of microvessels in tumors derived from CD55-high population was compared with that from CD55-low population by staining endothelium with anti-CD34 antibody. The number of microvessels in the tumors from CD55-high population was higher than that from CD55-low population (Fig. 2H). High expression of Eph and ephrin is reported to be related to high microvessel density (32), and then the expression level of Eph and ephrin was examined. Expression levels of EphA8 and ephrinA3 were higher in MCF7 cells in CD55-high population than those in CD55-low population (Fig. 2I).

Immunohistochemical detection of CD55 expression in 74 breast cancer tissues was carried out. The intensity of the staining was evaluated at least in 10,000 tumor cells, and shown as none, weak, and strong. When >1% of the tumor cells showed the strong expression, this tumor was judged as CD55-high. Representative results were shown in Fig. 3. Strong positivity of CD55 staining was detected in the cytoplasm of tumor cells (Fig. 3A and C). Fifty of 74 cases (67.6%) was categorized as CD55-high and the remaining as CD55-low.

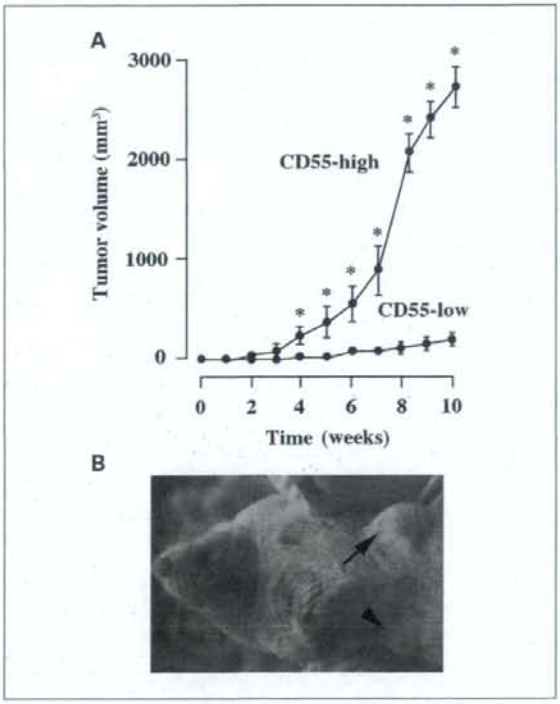

Fig. 1. *A*, growth of CD55-high and CD55-low population of MCF-7 breast cancer cell line after injection into the mammary fat pad of NOD/Scid mice. Tumor size was measured every week, and the volume was calculated by the formula: $(\text{width})^2 \times (\text{length})/2$. Points, mean of three mice; bars, SE. In some cases, the SE is too small to be shown by bars. *, $P < 0.05$ by Student's *t* test versus the value of tumor derived from CD55-low population. *B*, tumors derived from CD55-high (*arrow*) and CD55-low (*arrowhead*) population.

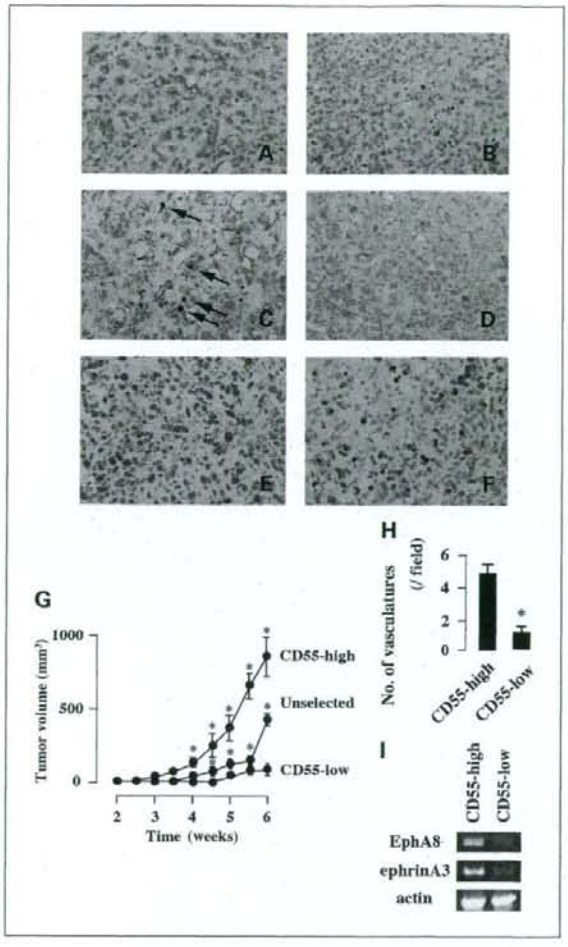

Fig. 2. Microscopic appearance of the tumor derived from CD55-high population (*A*) and CD55-low population (*B*). Staining of the tumor derived from CD55-high (*C* and *E*) and from CD55-low (*D* and *F*) population with anti-CD55 antibody (*C* and *D*; *arrows*, positive cells) and with anti – Ki-67 antibody (*E* and *F*). Magnification, ×200. *G*, growth of CD55-high, CD55-low, and a whole population of MCF-7 breast cancer cell line after injection into the mammary fat pad of NOD/Scid mice. Points, mean of three mice; bars, SE. In some cases, the SE is too small to be shown by bars. *, $P < 0.05$ by Student's *t* test; the value of tumor derived from CD55-high or unselected population versus the value of tumor derived from CD55-low population. *H*, number of vasculatures in tumors derived from CD55-high and CD55-low populations. *, $P < 0.01$ by Student's *t* test. *I*, reverse transcription-PCR. Expression level of EphA8 and ephrinA3 was compared in MCF7 cells of CD55-high and CD55-low population.

Correlation of CD55-high expression with the clinicopathologic features was evaluated. A statistically significant correlation was observed between CD55-high character and relapse rate ($P < 0.001$). Whereas other variables such as menopausal status, histologic type, tumor size, histologic grade, occurrence of lymph node metastasis, estrogen receptor status, and progesterone receptor status did not show a statistically significant correlation with CD55 expression (Table 2). There was a statistically significant difference in relapse-free survival rates ($P < 0.001$) and overall survival rates ($P = 0.024$) between patients with CD55-high and CD55-low tumors (Fig. 4). Proportions of patients treated with adjuvant hormonal therapy, chemotherapy, or chemohormonotherapy were similar between patients with CD55-high and CD55-low tumors (Table 2). Univariate analysis showed that tumor size and CD55 expression were significant prognostic factors for prognosis (Table 3). The multivariate analysis revealed that the tumor size and CD55 expression were independent prognostic factors (Table 3).

Discussion

Our recent study on the breast cancer cell line revealed that the CD55-high population was more resistant to apoptotic stimuli and formed colonies *in vitro* more efficiently than CD55-low population (22). This previous findings on *in vitro* colony formation might suggest the high tumorigenic potential of CD55-high population *in vivo*. In the present study, the CD55-high population of breast cancer cell line was transplanted into the NOD/Scid mice, and the size of tumor from this population was compared with that from CD55-low population. As expected, the size of tumors derived from CD55-high population was significantly larger than that derived from CD55-low population. This was consistent with the report by Loberg et al. (27), in which the knocked-down expression of CD55 in prostatic cancer cell line reduces the tumorigenic potential when transplanted into Scid mice. These findings suggest that CD55 play an important role in tumorigenesis.

When stained with anti-CD55 antibody, CD55-high cells were detected in tumor derived from CD55-high population but not in tumor derived from CD55-low population. Cancer stem cells are known to self-renew but noncancer stem cells do not (8). In this respect, CD55-high population may contain the cancer stem cells.

CD55-low population formed tumors when transplanted into NOD/Scid mice, although the size was small. There might be two explanations for this: (*a*) a small population of CD55-high cells escaped from sorting and contaminated into CD55-low population, and (*b*) CD55-low cells themselves have a low potential of tumorigenicity. These two possibilities seemed to be plausible because the previous *in vitro* colony formation assay showed that a small number of colonies appeared from CD55-low cells, although their sizes was significantly smaller than the colonies derived from CD55-high cells.

The growth rate of tumors derived from CD55-high population was significantly high compared with that from CD55-low population. The growth rate of tumors derived from a whole MCF7 cells was intermediate between that from CD55-high and that from CD55-low cells. These findings indicated that the growth rate seemed to be parallel to the content of CD55-high population in transplanted cells, and that CD55-high population was significantly tumorigenic. Then, we analyzed why the tumors derived from CD55-high population showed a rapid growth. Our previous study showed that CD55-high population abundantly expressed Bcl-2 compared with CD55-low population (22). However, the expression of Bcl-2 was hardly detectable at immunohistochemistry in tumor cells derived from both CD55-high and CD55-low population. Number of apoptotic cells was comparable between these two tumors. From these findings, it is suggested that the antiapoptotic effect of Bcl-2 might not play a role in tumor

formation of MCF7 cells in NOD/Scid mice. The proliferation rate of Ki-67 labeling index was rather similar between tumors from CD55-high and CD55-low cells, suggesting similar proliferative activity among these cells. The inhibitory effect of CD55 on complement attack could not explain the high growth rate of tumors from CD55-high population because human CD55 is a poor inhibitor of mouse complement (33). Presence of a lot of collagen fibers and vasculatures in the tumors derived from CD55-high population compared with those from CD55-low population was distinctive. The rapid growth of tumors derived from CD55-high population may be attributable, at least in part, to an increase of mesenchymal components around the tumor cells. In fact, EphA8 and ephrinA3, which are vasculogenic factors in tumors, were expressed abundantly in CD55-high MCF7 cells.

Next, the relationship between presence of CD55-high population in the tumors and the clinicopathologic behavior was examined in 74 patients with breast cancers. When breast cancer tissues were stained with anti-CD55 antibody, a marked variation of signal intensity was observed. This variation was in consistent with the results of other studies on CD55 staining for colorectal tumors: flow cytometric analysis revealed the broad spectrum of CD55 expression level among tumor cells (28), and the immunohistochemistry revealed the proportion of CD55-expressing cells was various from case to case (26). The aim of our study is to investigate the relationship between presence of CD55-high population and the clinicopathologic behavior of tumors. The tumors containing >1% of tumor cells with strong CD55 expression were judged as CD55-high: 50 of 74 cases (67.6%) was CD55-high cases. Majorities of clinicopathologic features including histologic grade and type were not associated with CD55 expression level. This was in agreement with the previous reports on colon and prostatic carcinoma, in which CD55 expression level is not associated with tumor stage or differentiation status of tumor cells. Only the recurrence rate showed significant correlation with CD55 expression level in the present series. In the present study, grade and lymph node status were not significant variable in the

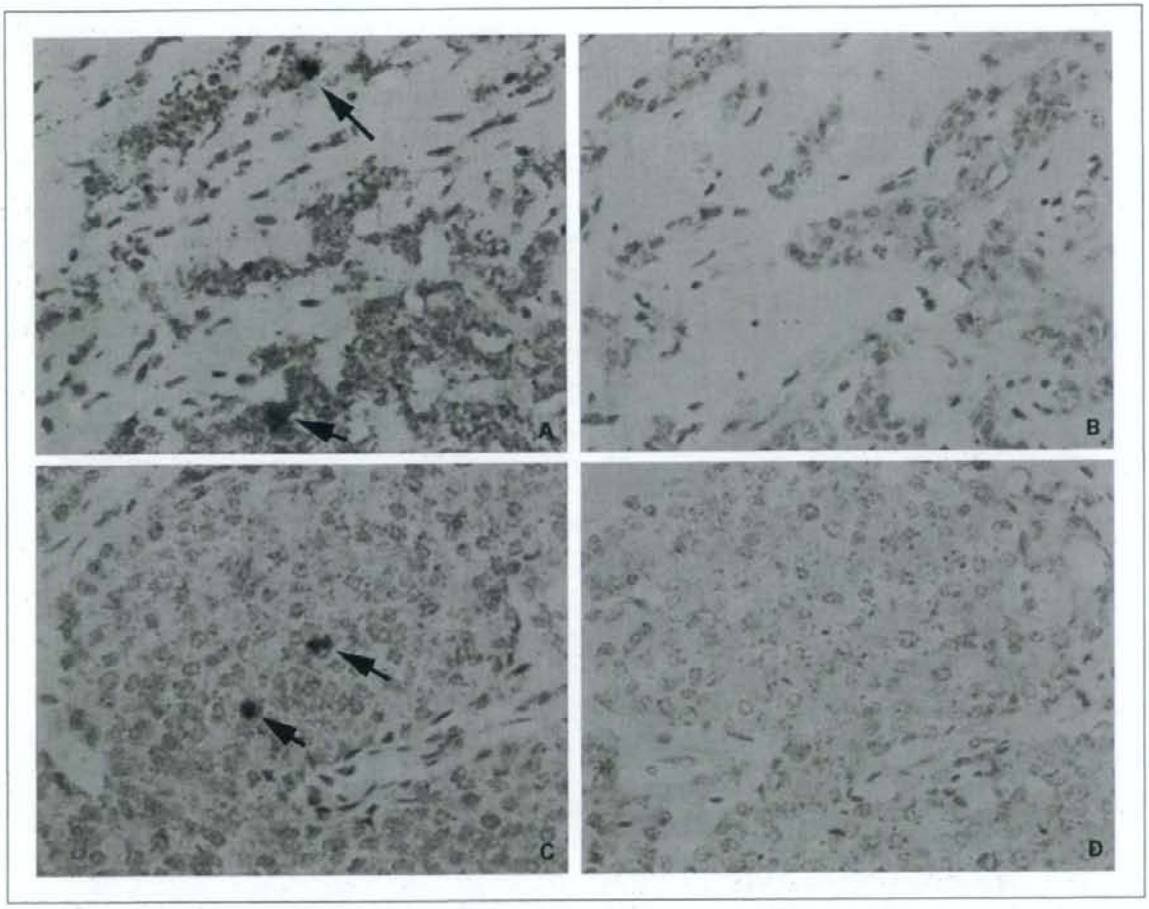

Fig. 3. Expression of CD55 in breast cancer samples as revealed by immunohistochemistry. *A*, weak signals for CD55 were detected in almost all tumor cells, and two tumor cells showed strong signal (*arrows*). *B*, negative control in which no anti-CD55 antibody was added to the sample of (*A*). *C*, no signal was detected in almost all tumor cells, but strong signal was detected in two tumor cells (*arrows*). *D*, negative control in which no anti-CD55 antibody was added to the sample of (*C*). Magnification, ×400.

Table 2. Correlation between CD55 expression and clinicopathologic variables

	CD55 expression		P
	Low	High	
Menopausal status			
Premenopausal	7	24	
Postmenopausal	17	26	0.120
Histologic type			
Papillotubular	6	20	
Solid-tubular	6	20	
Scirrhous	10	8	
Others	2	2	0.079
Tumor size (cm)			
<2	4	4	
>2, ≤5	20	41	
>5	0	5	0.083
Histologic grade			
I+II	22	39	
III	2	11	0.127
Lymph node metastasis			
Positive	17	28	
Negative	7	22	0.216
Stage			
IIA	11	21	
IIB	10	18	
III	3	11	0.600
Estrogen receptor status			
Positive	18	27	
Negative	6	23	0.078
Progesterone receptor status			
Positive	14	18	
Negative	10	32	0.070
Recurrence			
Positive	1	31	
Negative	23	19	<0.001
Adjuvant therapy			
None	2	2	
Hormonotherapy	11	14	
Chemotherapy	4	11	
Chemohormonotherapy	7	23	0.332

univariate analysis. This may be attributable to the patients' inclusion criteria; patients with stage II and III breast cancers were included in the present study. Multivariate analysis revealed the high expression of CD55 to be an independent prognostic factor for recurrence. Durrant et al. (28) reported that the high CD55 expression level was a poor prognostic factor for survival in colorectal cancer. The present study clearly showed that presence of cells showing strong CD55 expression is a sign for poor prognosis of patients with breast cancer.

Brandt et al. (34) reported that the subcellular localization of CD55 is changeable by status of cells. Because the migration ability of cancer cells through endothels could reflect an invasive potential of cancer, they selected the subclones with higher migration ability than the parental breast cancer cell line. CD55 is localized both in the cell membrane and cytoplasm of the invasive subclone, whereas CD55 is localized only in the cell membrane of original noninvasive cell line. In the present study, strong expression of CD55 was found in the cytoplasm of tumor cells. The present findings that CD55-high population had more strong tumorigenic potential than CD55-low population is consistent with the results of Brandt et al. (34).

The previous study on the breast cancer showed that negative expression of CD55 was correlated with aggressiveness of breast cancer (29). This conflicting results to the present study might be partly due to the difference in criteria for evaluation of CD55 expression. In the previous study, the grade of CD55 staining intensity in each tumor cell was multiplied with the proportion of CD55-positive cells among total tumor cells. The resultant value was used as the index for categorization of each case as CD55-high or CD55-low. The different criteria for CD55 high expression was used in the present study: cases with >1% of tumor cells showing strong CD55 expression were judged as CD55-high. Because only tumor cells with significantly strong CD55 staining were counted in the present study, the proportion of CD55-low cases was higher than the previous study. In any way, the present study showed the presence of cells with high CD55 expression could reflect tumorigenic potential of cases containing such cells.

Recently, heterogeneous characters of cells constituting tumor is well-known. It was reported that a small population of tumor cells affected the characters of tumor. For example, Naoi et al. (35) reported a significant association between connexin 26 expression of tumor cells and relapse rate of breast cancer when a very low cutoff value of 1% is used. They used various cutoff values at evaluation of the association between connexin 26 expression and prognosis, and found that a cutoff value of as low as 1% was meaningful at the separation of patients with poor prognosis from those with favorable

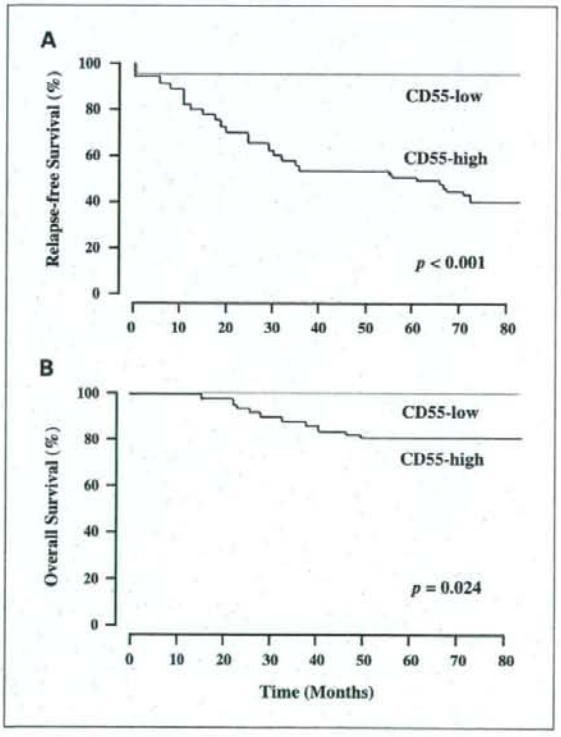

Fig. 4. Relapse-free survival (*A*) and overall survival (*B*) of patients with breast cancer who had CD55-high and CD55-low character. A significant difference was observed between two groups.