

Association of Breast Cancer Stem Cells Identified by Aldehyde Dehydrogenase 1 Expression with Resistance to Sequential Paclitaxel and Epirubicin-Based Chemotherapy for Breast Cancers

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Abstract Purpose: Breast cancer stem cells have been shown to be associated with resistance to chemotherapy *in vitro*, but their clinical significance remains to be clarified. The aim of this study was to investigate whether cancer stem cells were clinically significant for resistance to chemotherapy in human breast cancers.

Experimental Design: Primary breast cancer patients ($n = 108$) treated with neoadjuvant chemotherapy consisting of sequential paclitaxel and epirubicin-based chemotherapy were included in the study. Breast cancer stem cells were identified by immunohistochemical staining of CD44/CD24 and aldehyde dehydrogenase 1 (ALDH1) in tumor tissues obtained before and after neoadjuvant chemotherapy. CD44⁺/CD24⁻ tumor cells or ALDH1-positive tumor cells were considered stem cells.

Results: Thirty (27.8%) patients achieved pathologic complete response (pCR). ALDH1-positive tumors were significantly associated with a low pCR rate (9.5% versus 32.2%; $P = 0.037$), but there was no significant association between CD44⁺/CD24⁻ tumor cell proportions and pCR rates. Changes in the proportion of CD44⁺/CD24⁻ or ALDH1-positive tumor cells before and after neoadjuvant chemotherapy were studied in 78 patients who did not achieve pCR. The proportion of ALDH1-positive tumor cells increased significantly ($P < 0.001$) after neoadjuvant chemotherapy, but that of CD44⁺/CD24⁻ tumor cells did not.

Conclusions: Our findings suggest that breast cancer stem cells identified as ALDH1-positive, but not CD44⁺/CD24⁻, play a significant role in resistance to chemotherapy. ALDH1-positive thus seems to be a more significantly predictive marker than CD44⁺/CD24⁻ for the identification of breast cancer stem cells in terms of resistance to chemotherapy.

Cancer stem cells are defined as rare tumor cells that are capable of self-renewal and give rise to multipotent progenitor cells, which ultimately differentiate into all cell types within the tumor (1–4). The cancer stem cell population is believed to be

small, accounting for only 0.1% to 1% of all tumor cells. Cancer stem cells were first documented in acute myeloid leukemia by taking advantage of cell sorting technology using various surface markers (5). Later studies of solid tumors, including breast tumors, brain tumors, lung tumors, and colon tumors, have indicated the presence of cancer stem cells in these tumors as well (6–9). With respect to breast cancer, Al-Hajj et al. were the first to distinguish tumorigenic cancer cells (stem cells) from nontumorigenic ones by using cell surface markers CD44 and CD24 (6). They showed that as few as 100 tumor cells with CD44⁺/CD24⁻ phenotype were able to produce tumors in immunodeficient mice, whereas tumor cells with other CD44/CD24 phenotypes were unable or rarely able to produce tumors even when as many as 10^5 to 10^6 tumor cells were inoculated into such mice. Furthermore, Abraham et al. conducted immunohistochemical studies of CD44⁺/CD24⁻ tumor cells in human breast tumors and showed that breast tumors containing a high proportion of CD44⁺/CD24⁻ cells were associated with distant metastases (10).

Recently, Ginestier et al. showed that aldehyde dehydrogenase 1 (ALDH1) is a better marker of breast cancer stem cells based on the finding that fewer ALDH1-positive than

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Translational Relevance

To realize the personalized chemotherapy for breast cancer patients, it is very important to develop a predictor of response to chemotherapy. Several parameters, including estrogen receptor, progesterone receptor, HER-2, Ki-67, and topoisomerase 2A, have been reported to be associated with pathologic complete response rates after sequential taxane and anthracycline-based chemotherapy, but they are not enough, and more accurate predictors need to be developed. In the present study, we have evaluated the clinical value of aldehyde dehydrogenase 1 (ALDH1)-positive cancer stem cells determined by immunohistochemistry in the prediction of response to the chemotherapy, because cancer stem cells are thought to be inherently chemoresistant and thus to have a potential to be used as a predictor of resistance. Actually, we have been able to show herein that ALDH1-positive cancer stem cells serve as a significant and independent predictor of resistance to the chemotherapy. Our present observation seems to be clinically important, because it is expected that response to sequential taxane and anthracycline-based chemotherapy can be estimated more accurately by adding ALDH1 to other conventional parameters.

CD44⁺/CD24⁻ tumor cells are required to produce tumors in immunodeficient mice (11). In addition, they have been able to show that immunohistochemically identified ALDH1 expression is associated with poor prognosis in human breast cancers. ALDH1 in cancer stem cells may be a significant enzyme in stem cell differentiation that regulates the conversion of retinoic acid to oxidizing retinol (12). The results of Abraham et al. (10) and Ginestier et al. (11) seem to point to the existence of breast cancer stem cells and their association with a biologically aggressive phenotype. Another important characteristic of cancer stem cells is that they usually express high levels of ATP-binding cassette transporters and thus are thought to be resistant to various chemotherapeutic agents effluxed by ATP-binding cassette transporters (13, 14). In fact, several *in vitro* studies have shown that cancer stem cells are resistant to paclitaxel, doxorubicin, 5-fluorouracil, and platinum (15–18). The implication that breast tumors may contain stem cells, which are supposedly resistant to chemotherapy, can be of major clinical importance for a better understanding of the mechanism of acquisition of drug resistance. Almost all breast tumors, although initially may respond to a given chemotherapy, ultimately become resistant to the chemotherapy. It is generally thought that tumor regrowth during chemotherapy results from clonal selection of tumor cells, which acquire their resistant properties due to various genetic/epigenetic mechanisms during the treatment (2). In the case of stem cells, however, it is considered that chemotherapy-resistant stem cells have been already present before chemotherapy and that tumor regrowth is attributable to the preferential proliferation of these stem cells. Taking all these findings into account leads to the speculation that breast tumors with a high proportion of stem cells may be associated

with resistance to chemotherapy and that the proportion of stem cells may increase after chemotherapy because they are resistant to chemotherapy. In the study presented here, we investigated the validity of these speculations in a neoadjuvant chemotherapy setting in human breast cancers. We employed the two methods for the identification of breast cancer stem cells, CD44/CD24 and ALDH1, to compare their clinical utility for the prediction of resistance to chemotherapy.

Materials and Methods

Patients and breast tumor tissues. The subjects recruited for this study comprised 108 primary invasive breast cancer patients (mean age, 50.8 years; range, 26–72 years) with a tumor >3 cm in diameter or with cytologically confirmed axillary lymph node involvement who were treated with neoadjuvant chemotherapy at Osaka University Hospital between June 2003 and April 2007 (4 stage IV patients with small distant metastases were included in these subjects). Tumor specimens were obtained before neoadjuvant chemotherapy by means of vacuum-assisted core needle biopsy. All patients were treated with 12 cycles of paclitaxel (80 mg/m²/wk) followed by 4 cycles of 5-fluorouracil 500 mg/m², epirubicin 75 mg/m², and cyclophosphamide 500 mg/m² every 3 weeks. Breast conserving surgery or mastectomy was conducted 3 to 4 weeks after the last treatment. Tumor specimens (surgical specimens) were also obtained at surgery. Informed consent was obtained from each patient.

It was possible that different sampling methods of tissue specimens might bias against the immunohistochemical results. Thus, we conducted a study to compare the immunohistochemical results of CD44/CD24 and ALDH1 between the vacuum-assisted core needle biopsy specimens obtained before surgery and the surgical specimens obtained at surgery in 40 primary invasive breast cancer patients [stage I (*n* = 24), stage II (*n* = 15), and stage III (*n* = 1)] who had not been treated with neoadjuvant chemotherapy. Concordance of CD44⁺/CD24⁻ tumor cell proportions (%) as well as ALDH1 status between vacuum-assisted core needle biopsy and surgical specimens was excellent, indicating that difference in sampling methods of tissue specimens was unlikely to bias against our results (Supplementary Fig. S1).

Antibodies. (a) CD24 [clone Ab-1 (SN3), monoclonal, IgG isotype, 1:100; Neomarkers], (b) Tyramide Signal Amplification Fluorescence System (1:50; Perkin-Elmer), (c) biotin-conjugated CD44 (clone 156-3C11, monoclonal, IgG isotype, 1:100; Neomarkers), (d) ALDH1 (monoclonal, IgG isotype, 1:100; BD Biosciences), (e) CD68 (clone PG-M1, monoclonal, IgG isotype, 1:100; DAKO Japan), (f) Ki-67 (clone MIB-1, monoclonal, IgG isotype, 1:100; DAKO Japan), (g) topoisomerase 2A (TOP2A; clone Ki-S1, monoclonal, IgG isotype, 1:100; DAKO Japan), (h) biotin Labeling Kit-NH₂ (Dojindo Molecular Technologies), and (i) Tyramide Signal Amplification Biotin System (1:50, Perkin-Elmer).

Double-fluorescence immunohistochemical identification of CD44⁺/CD24⁻ tumor cells. Antigen retrieval of tumor tissue paraffin sections (3 μm) was accomplished by microwaving in Target Retrieval Solution (pH 6.0; DAKO Japan). The sections were first incubated with anti-CD24 antibody (a) and then anti-mouse secondary antibody conjugated with peroxidase (1:100; The Jackson Laboratory) and subsequently visualized with a FITC-Tyramide Signal Amplification reaction (b; ref. 19). Next, the paraffin sections were incubated with anti-biotin-conjugated CD44 antibody (c) and subsequently visualized by means of anti-biotin secondary antibody conjugated with Cy3 (1:100; The Jackson Laboratory) and then counterstained with Hoechst (Invitrogen).

Fluorescent immunostaining of CD44 and CD24 was analyzed with a Zeiss LSM510 confocal microscope. The percentage of CD44⁺/CD24⁻ tumor cells (stained red) was determined with the aid of WinROOF imaging software (Mitani; ref. 20). CD44⁺/CD24⁻ tumor cells were selected by subtracting CD24⁺ tumor cells from CD44⁺ tumor cells. The number of CD44⁺/CD24⁻ tumor cells and the other tumor cells in the invasive

component was counted with visual check [three high-power ($\times 400$) fields]. Finally, the percentage of CD44⁺/CD24⁻ tumor cells per total tumor cells was calculated in each tumor. Threshold values used for the analysis of CD44 (Cy3) and CD24 (FITC) images were 33.3% (85 on the 0-255 grayscale) and 20.0% (51 on the 0-255 grayscale), respectively.

Immunohistochemical staining of ALDH1, CD68, Ki-67, and TOP2A. The expression of ALDH1, CD68, Ki-67, and TOP2A was immunohistochemically evaluated with the avidin-biotin-peroxidase method using anti-ALDH1 antibody (*d*), anti-CD68 antibody (*e*), anti-Ki-67 antibody (*f*), and anti-TOP2A antibody (*g*), respectively, according to the previously described method (21, 22). Antigen retrieval was accomplished by heating at 98°C for 40 min for ALDH1, CD68, and Ki-67 and for 1 h for TOP2A. The cutoff value for Ki-67 and TOP2A was 20%.

For differentiation of ALDH1-positive tumor cells from ALDH1-positive macrophages, double immunohistochemical staining of ALDH1 and CD68 (a marker for macrophages) were carried out in some tumors. In brief, paraffin sections (3 μ m) were incubated with anti-ALDH1 antibody (*d*) and subsequent conjugation of anti-mouse secondary antibody with alkaline phosphatase. Then, the sections were incubated with anti-CD68 antibody (*e*), treated with Biotin Labeling Kit-NH₂ (*h*), and incubated with anti-biotin secondary antibody conjugated with peroxidase using Tyramide Signal Amplification Biotin System (*i*; ref. 23). Finally, the sections were incubated with fuchsin (DAKO Japan) and 3,3'-diaminobenzidine (Merck). Incubation with primary antibodies were done at 4°C for overnight and that with secondary antibodies were done at room temperature for 1 h.

Histologic grade, estrogen receptor, progesterone receptor, and HER-2. The histologic grade was determined with the Scarff-Bloom-Richardson grading system (24). Estrogen receptor (ER) and progesterone receptor (PR) were defined as positive, when $\geq 10\%$ of the tumor cells were immunohistochemically stained positive (ER: clone 6F11; PR: clone 16; Ventana Japan and SRL). HER-2 was determined by fluorescence *in situ* hybridization using PathVysion HER-2 DNA Probe kits (SRL). When a tumor contained more than two genes per cell, it was considered HER-2 positive.

Assessment of pathologic response. Pathologic response of breast cancers to neoadjuvant chemotherapy was assessed for all patients. Multiple slides prepared from the primary tumors were examined for evaluation of chemotherapeutic effect according to the criteria specified

in the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 (25). In this study, pathologic complete response (pCR) was defined as the absence of residual invasive components regardless of the presence or absence of ductal carcinoma *in situ* components.

Colony formation assay. Colony formation assay of breast tumor cells was carried out to investigate the relationship of CD44⁺/CD24⁻ or ALDH1 positive with colony formation ability in 27 primary invasive breast cancers [stage 1 ($n = 2$), stage 2 ($n = 23$), and stage 3 ($n = 2$)] who had not been treated with neoadjuvant chemotherapy using the collagen gel droplet-embedded culture-drug sensitivity test kits (Nitta Gelatin; refs. 26–28). ALDH1-positive tumors showed a significantly ($P = 0.046$) higher colony formation than ALDH1-negative tumors, although there was no significant difference in colony formation between CD44⁺/CD24⁻ high and low tumors (Supplementary Fig. S2).

Statistical analyses. The SPSS software package version 12.1 was used for all statistical analyses. Association of the immunohistochemical results of CD44/CD24 and ALDH1 with the various clinicopathologic parameters were evaluated by the Mann-Whitney *U* test or χ^2 test. Changes in the immunohistochemical results of CD44/CD24 and ALDH1 before and after neoadjuvant chemotherapy were assessed by the Wilcoxon signed-rank test and χ^2 test, respectively. The relationship between pCR rates and various parameters was evaluated using a logistic regression method. Statistical significance was assumed for $P < 0.05$.

Results

Double-fluorescence immunohistochemical staining of CD44 and CD24. We analyzed CD44⁺/CD24⁻ tumor cells in human breast cancer tissues by the double-fluorescence immunohistochemical staining method. Representative results are shown in Fig. 1A (CD44), Fig. 1B (CD24), and Fig. 1C (CD44/CD24). CD44⁺ tumor cells and CD24⁺ tumor cells were selected by WinROOF imaging software (Fig. 1D and E, respectively), and CD44⁺/CD24⁻ tumor cells (Fig. 1F) were determined by subtracting CD24⁺ cells (Fig. 1E) from CD44⁺ cells (Fig. 1D). Finally, CD44⁺/CD24⁻ tumor cell proportion (%) was calculated in each tumor.

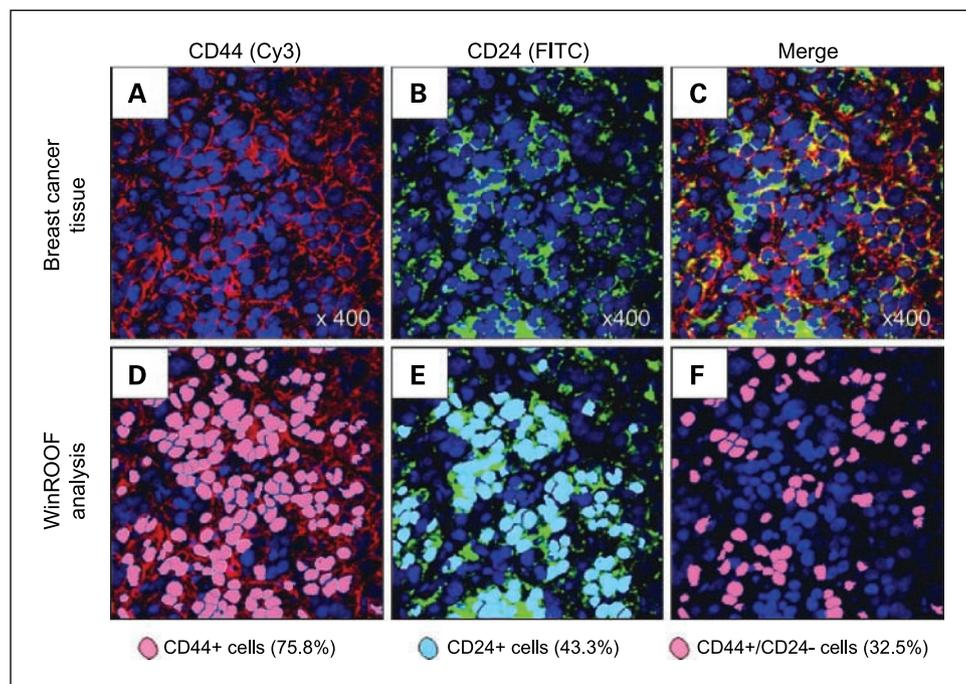
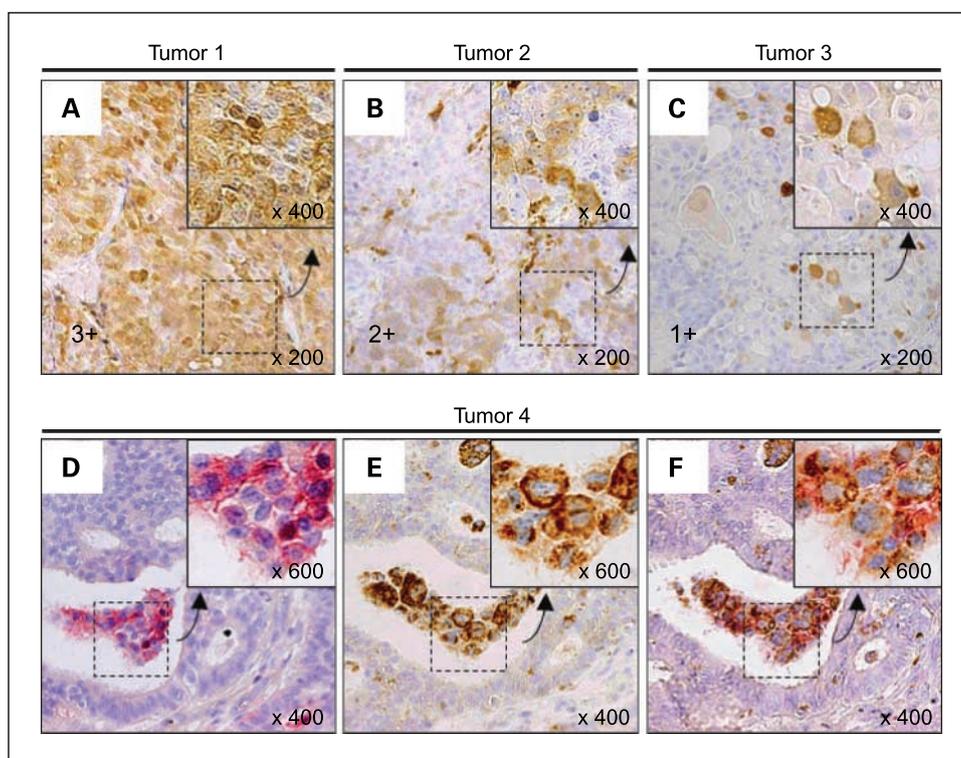


Fig. 1. Double-fluorescence immunohistochemical identification of CD44⁺/CD24⁻ tumor cells. Breast cancer tissue were subjected to double-fluorescence immunohistochemical determination of CD44⁺/CD24⁻ tumor cells. CD44⁺ tumor cells were stained red (A) and CD24⁺ tumor cells were stained green (B). Pictures A and B were merged into picture C. For the calculation of CD44⁺/CD24⁻ tumor cell proportions in breast cancer tissues, CD44⁺ tumor cells and CD24⁺ tumor cells were selected by WinROOF imaging software. CD44⁺ tumor cells were shown in pink (D), accounting for 75.8% of all tumor cells, and CD24⁺ tumor cells were shown in light blue (E), accounting for 43.3% of all tumor cells. Proportion (32.5%) of CD44⁺/CD24⁻ tumor cells (F) in all tumor cells was then determined by subtracting CD24⁺ cells (E) from CD44⁺ cells (D).

Fig. 2. Immunohistochemical identification of ALDH1-positive tumor cells. Representative results of immunostaining of ALDH1 in breast cancer tissues: (A) 3+ in tumor 1, (B) 2+ in tumor 2, and (C) 1+ in tumor 3. In tumor 4, besides ALDH1 immunostaining (fuchsin: red; D), CD68 immunostaining (3,3'-diaminobenzidine: brown; E) as well as ALDH1 and CD68 double immunostaining (F) were done in the adjacent sections.



Immunohistochemical staining of ALDH1. Representative results of immunohistochemical staining of ALDH1 in human breast cancer tissues were shown in Fig. 2. By using the criteria described by the report of Ginestier et al. (11), immunohistochemical staining of ALDH1 was classified into 3+ ($\geq 50\%$ positive tumor cells), 2+ ($< 50\%$, $\geq 10\%$), 1+ ($< 10\%$, $\geq 5\%$), and negative ($< 5\%$) groups. For the subsequent analysis, tumors showing 1+, 2+, and 3+ expression of ALDH1 were considered to be ALDH1 positive.

Because macrophages were positive for ALDH1 and morphologically similar to tumor cells, special attention was paid not to misinterpret macrophages as tumor cells positive for ALDH1. For this reason, in some tumors where differentiation between ALDH1-positive tumor cells and ALDH1-positive macrophages was difficult, immunostaining of CD68 as well as double staining of ALDH1 (fuchsin: red) and CD68 (3,3'-diaminobenzidine: brown) was done for the adjacent sections. Results for a representative tumor were shown in Fig. 2. In tumor 4 in Fig. 2, tumor-like cells in the ductal lumen were positive for ALDH1 (Fig. 2D) but also for CD68 (Fig. 2E), and double staining confirmed that these tumor-like cells were positive for both ALDH1 and CD68 (Fig. 2F), indicating that they were actually ALDH1-positive macrophages.

Relationship of CD44⁺/CD24⁻ or ALDH1 positive with clinicopathologic features of breast tumors as well as response to neoadjuvant chemotherapy. As shown in Table 1, there was no significant association of CD44⁺/CD24⁻ tumor cell proportions or of ALDH1-positive tumors with various clinicopathologic features such as menopausal status, tumor size, histologic grade, ER, PR, or HER-2. There was also no significant association between CD44⁺/CD24⁻ tumor cell proportions and ALDH1 status (Fig. 3B).

The pCR was achieved by 30 (27.8%) of the 108 patients treated with neoadjuvant chemotherapy. ALDH1-positive tumors were significantly associated with low pCR rates ($P = 0.037$; Fig. 4B), but there was no significant association between CD44⁺/CD24⁻ tumor cell proportions and pCR rates (Fig. 4A). Changes in the proportions of CD44⁺/CD24⁻ tumor cells or in grading of ALDH1-positive tumor cells before and after neoadjuvant chemotherapy were examined in 78 patients who did not achieve pCR (Fig. 4C and D). No significant changes were observed in the proportion of CD44⁺/CD24⁻ tumor cells before and after neoadjuvant chemotherapy (Fig. 4C). On the other hand, the grade of ALDH1-positive tumor cells after neoadjuvant chemotherapy significantly ($P < 0.001$) increased (Fig. 4D) in 9 patients (3 from 0 to 1+, 2 from 1+ to 2+, 2 from 1+ to 3+, and 2 from 2+ to 3+). A representative case where ALDH1 expression increased from 1+ before neoadjuvant chemotherapy (Fig. 4D, a) to 2+ after neoadjuvant chemotherapy (Fig. 4D, b) was shown.

Relationship between various biological factors and pCR rates. Association of various biological factors such as histologic grade, ER, PR, HER-2, Ki-67, TOP2A, CD44⁺/CD24⁻, and ALDH1 with pCR rates was also studied (Table 2). Univariate analysis showed a significant association of ER, PR, HER-2, Ki-67, TOP2A, and ALDH1 with pCR rates, and multivariate analysis showed a significant association of ER, Ki-67, and ALDH1 with pCR rates.

Discussion

Recent progress in cancer stem cell research has led to a better understanding about the mechanism of resistance to

chemotherapy as well as to the development of more effective chemotherapeutic regimens and new antitumor agents (29). Although their number is very small, cancer stem cells are thought to be inherently drug resistant, so that their eradication is essential for long-term success in cancer treatment (30, 31). An association between cancer stem cells and drug resistance in breast cancer cell lines has been shown *in vitro* (15–17), but such an association has not been shown yet clinically in human breast cancers. In the current study, we therefore investigated whether stem cells are associated with drug resistance in breast cancer patients treated with neoadjuvant chemotherapy.

Al-Hajj et al. have shown that CD44⁺/CD24⁻ tumor cells were highly tumorigenic in immunodeficient mice and that cancer stem cells in this population appeared to be enriched (6). It therefore seemed to be of considerable interest to identify the clinicopathologic characteristics of breast cancers with a high proportion of CD44⁺/CD24⁻ tumor cells. We studied breast cancer tissues with the double-fluorescence immu-

nohistochemistry and found that CD44⁺/CD24⁻ tumor cell proportions were not significantly associated with the conventional clinicopathologic features such as menopausal status, tumor size, lymph node status, histologic grade, ER, PR, or HER-2, which was also consistent with previously reported results (10).

Because cancer stem cells are thought to be inherently resistant to chemotherapy, CD44⁺/CD24⁻ high tumors can be expected to show a greater resistance to neoadjuvant chemotherapy than CD44⁺/CD24⁻ low tumors. Our study, however, has shown that there is no significant association between CD44⁺/CD24⁻ tumor cell proportions and pCR rates. Besides, CD44⁺/CD24⁻ tumor cell proportions have not shown a significant increase after neoadjuvant chemotherapy, although chemotherapy-resistant stem cells are expected to increase. These results seem to suggest that stem cells identified by immunohistochemistry of CD44⁺/CD24⁻ may not play an important role in the resistance to chemotherapy in human breast cancer. However,

Table 1. Relationship of CD44⁺/CD24⁻ tumor cell proportions (%) or ALDH1-positive tumors with clinicopathologic parameters

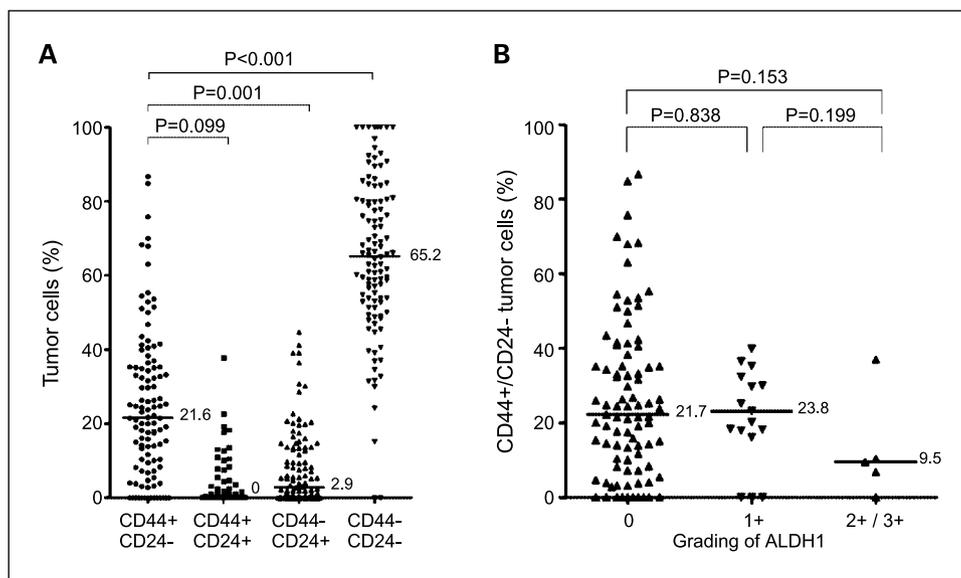
	n	CD44 ⁺ /CD24 ⁻ cell population (%)		ALDH1		P [†]
		Median (IQR)*	P [‡]	Positive, n (%)	Negative, n (%)	
All breast carcinomas	108			21 (19)	87 (81)	
Histologic type			0.020			0.360
Invasive lobular cancer	11	10.4 (0.0-17.3)		1 (9)	10 (91)	
Invasive ductal cancer	97	23.8 (10.4-35.3)		20 (21)	77 (79)	
Histologic grade						0.151
1	11	24.5 (12.4-38.1)		0 (0)	11 (100)	
2	76	22.4 (10.3-35.3)	0.878	15 (20)	61 (80)	
3	21	18.4 (8.2-29.7)	0.292	6 (29)	15 (71)	
Tumor size (cm)						0.145
T ₁	6	23.8 (4.0-26.8)		1 (17)	5 (83)	
T ₂	59	25.3 (10.3-42.0)	0.496	7 (12)	52 (88)	
T ₃	30	18.1 (4.7-24.8)	0.470	9 (30)	21 (70)	
T ₄	13	23.8 (10.1-35.3)	1.000	4 (31)	9 (69)	
Lymph node metastasis			0.276			0.124
N (-)	30	24.5 (14.5-35.2)		3 (10)	27 (90)	
N (+)	78	20.5 (7.2-35.3)		18 (23)	60 (77)	
ER			0.120			0.184
-	38	25.2 (16.1-36.5)		10 (26)	28 (74)	
+	70	19.7 (4.7-34.9)		11 (16)	59 (84)	
PR			0.610			0.797
-	59	21.7 (12.8-35.2)		12 (20)	47 (80)	
+	49	21.1 (7.2-35.2)		9 (18)	40 (82)	
HER-2			0.253			0.548
-	82	23.8 (9.5-36.5)		17 (21)	65 (79)	
+	26	19.2 (8.4-25.1)		4 (15)	22 (85)	
Ki-67			0.295			0.148
<20%	62	22.4 (10.3-35.3)		9 (15)	53 (85)	
≥20%	46	22.4 (10.3-35.3)		12 (26)	34 (74)	
TOP2A			0.037			0.029
<20%	59	21.1 (4.4-30.7)		7 (12)	52 (88)	
≥20%	49	25.3 (14.0-39.9)		14 (29)	35 (71)	
Stage						0.090
II	59	26.4 (14.8-42.0)		7 (12)	52 (88)	
III	45	17.7 (4.7-24.8)	0.002	13 (29)	32 (71)	
IV	4	9.6 (0.0-29.8)	0.142	1 (25)	3 (75)	

*IQR, interquartile range (25%, 75%).

[†]Mann-Whitney U test.

[‡]χ² test.

Fig. 3. Proportions of CD44⁺/CD24⁺ tumor cells in each tumor and their relationship with ALDH1 status. Breast cancers ($n = 108$) were classified into the four categories: CD44⁺/CD24⁻, CD44⁺/CD24⁺, CD44⁻/CD24⁺, and CD44⁻/CD24⁻. Proportions (%) of tumor cells in each category were plotted for each tumor (A). Breast cancers ($n = 108$) were graded by ALDH1 staining (0, 1+, 2+, and 3+), and their relationship with CD44⁺/CD24⁺ tumor cell proportions (%) was shown (B). *P*, Mann-Whitney *U* test. Bars, median.



Li et al. recently reported that neoadjuvant chemotherapy increased the proportions of CD44⁺/CD24⁻ tumor cells identified by flow cytometry (32). The reason for this discrepancy has been currently unknown, but it might be, at least in part, explained by the difference in the method for determination of CD44⁺/CD24⁻ tumor cells, that is, immunohistochemical double staining versus flow cytometry as well as the difference in the regimens and duration of neoadjuvant chemotherapy (paclitaxel followed by 5-fluorouracil 500 mg/m², epirubicin 75 mg/m², and cyclophosphamide 500 mg/m² every 3 weeks for 24 weeks versus docetaxel or doxorubicin/cyclophosphamide for 12 weeks).

Very recently, it was reported by Ginestier et al. that ALDH1 could function as a better marker of breast cancer stem cells than CD44⁺/CD24⁻ (11). We therefore also tried to clarify the clinicopathologic characteristics of ALDH1-positive breast tumors but found that ALDH1 expression was not significantly associated with any conventional clinicopathologic features. On the other hand, a significant association was found between ALDH1-positive breast tumors and resistance to neoadjuvant chemotherapy, because pCR rates were significantly lower in ALDH1-positive tumors (9.5%) than ALDH1-negative tumors (32.2%). In addition, a significant increase in the proportion of ALDH1-positive tumor cells was observed after neoadjuvant chemotherapy. These results seem to indicate that ALDH1-positive tumor cells play a significant role in resistance to chemotherapy. Because Ginestier et al. have reported that ALDH1 tumor cells are more tumorigenic than CD44⁺/CD24⁻ tumor cells (11), breast cancer stem cells are thought to be richer in ALDH1-positive tumor cells than in CD44⁺/CD24⁻ tumor cells. Consistently, we have also been able to show that ALDH1 positive, but not CD44⁺/CD24⁻, is significantly associated with colony formation in the collagen gel.

It has been reported that the subset of ALDH1-positive and CD44⁺/CD24⁻ tumor cells contain the highest proportion of breast cancer stem cells (11); thus, this subset is speculated to be most resistant to chemotherapy. However, our present study has shown that pCR rates in the ALDH1-positive and CD44⁺/CD24⁻ high subset (20%, 2 of 10) are not lowest among all the subsets, that is, the ALDH1-positive and CD44⁺/CD24⁻ low

subset (0%, 0 of 11), the ALDH1-negative and CD44⁺/CD24⁻ high subset (34.1%, 15 of 44), and the ALDH1-negative and CD44⁺/CD24⁻ low subset (30.2%, 13 of 43). Addition of CD44/CD24 status to ALDH1 status seems not to improve the prediction of response to chemotherapy. These findings taken together lead us to consider that ALDH1-positive tumor cells are likely to serve as a better marker for breast cancer stem cells than CD44⁺/CD24⁻ tumor cells at least for the prediction of resistance to chemotherapy. We speculate that ALDH1-positive tumors are resistant to chemotherapy, because such tumors contain a higher proportion of cancer stem cells. It is also possible, however, that ALDH1-positive tumor cells, irrespective of whether they are cancer stem cells or not, might be involved in resistance to chemotherapy, because ALDH1 itself has been shown to play a significant role in the resistance to chemotherapy in hematopoietic cells (33). Development of a highly specific marker for breast cancer stem cells, as well as further clarification of a role of ALDH1 in resistance to chemotherapy in breast cancers, is needed to elucidate a genuine role of breast cancer stem cells in resistance to chemotherapy.

Several biological factors, including ER, PR, HER-2, Ki-67, and TOP2A, have been reported to be associated with pCR rates after sequential taxane and anthracycline-based chemotherapy (34–38). In our study, we were able to obtain results consistent with previously reported ones in that high pCR rates were associated with negative ER, negative PR, positive HER-2, high Ki-67, and high TOP2A. Interestingly, multivariate analysis including these factors as well as ALDH1 has shown that three factors, ER, Ki-67, and ALDH1, are significant and mutually independent predictors of response to chemotherapy. We therefore believe that response to sequential paclitaxel and epirubicin-based chemotherapy can be estimated more accurately by adding ALDH1 to ER and Ki-67. The clinical significance of identification of these three markers for the prediction of response to sequential taxane and anthracycline-based chemotherapy therefore seems to deserve further investigation.

In conclusion, we were able to show that ALDH1 positive, but not CD44⁺/CD24⁻, was significantly associated with

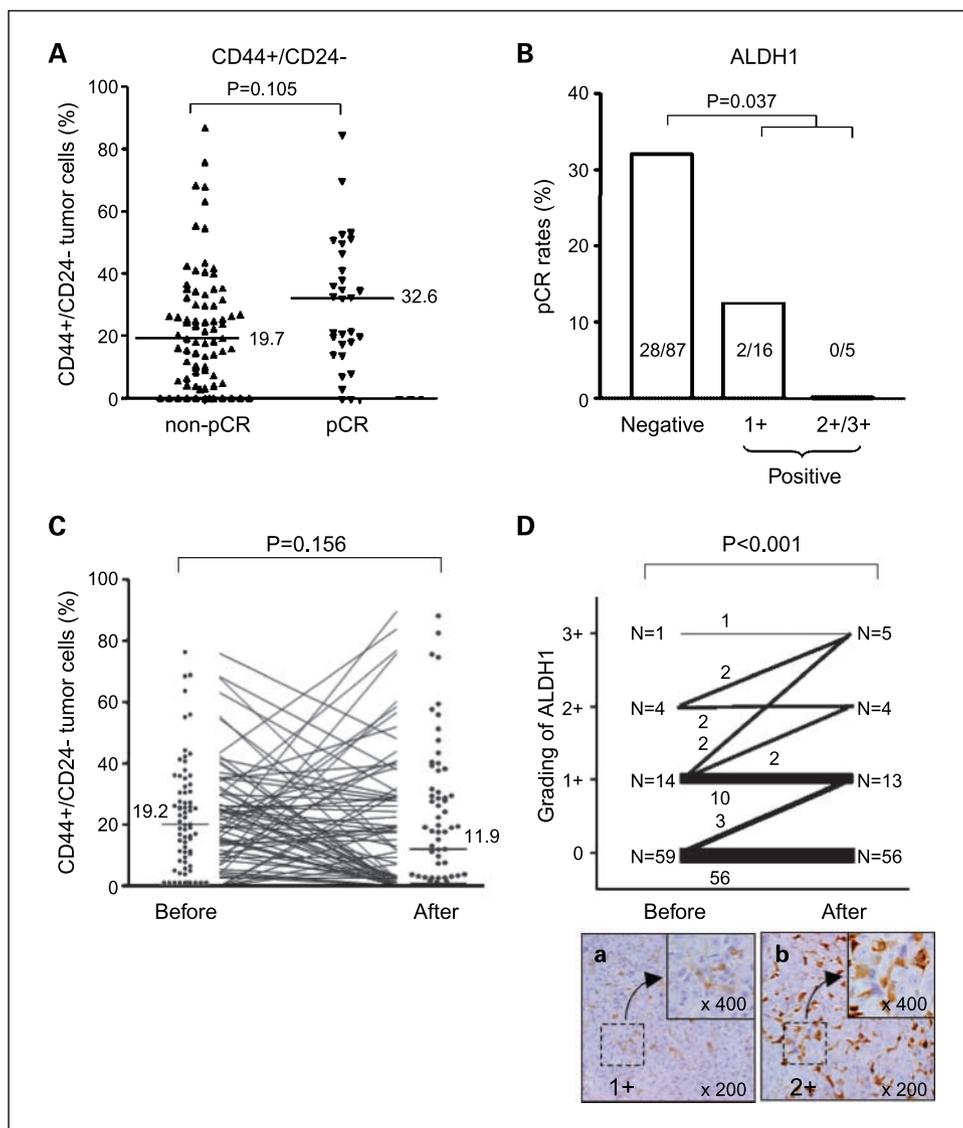


Fig. 4. Relationship of CD44⁺/CD24⁻ or ALDH1 positive with response to neoadjuvant chemotherapy. CD44⁺/CD24⁻ tumor cell proportions (%) were compared between tumors with pCR and non-pCR (A). *P*, Mann-Whitney *U* test. Bars, median. pCR rates were compared between ALDH1-positive and ALDH1-negative tumors (B). *P*, χ^2 test. Proportions of CD44⁺/CD24⁻ tumor cells (C) and ALDH1-positive tumor cells (D) were compared before and after neoadjuvant chemotherapy in 78 tumors not achieving pCR. *P*, Wilcoxon signed-rank test. Representative results of immunohistochemical staining of ALDH1 before (D, a) and after (D, b) neoadjuvant chemotherapy in the same patient, indicating up-regulation from ALDH1 1+ to 2+.

resistance to sequential paclitaxel and epirubicin-based chemotherapy and that the expression of ALDH1 increased after neoadjuvant chemotherapy, indicating that breast cancer stem cells identified by ALDH1 actually played a signif-

icant role in resistance to chemotherapy. This means that ALDH1 positive seems to be a better marker than CD44⁺/CD24⁻ for the identification of breast cancer stem cells at least for the prediction of resistance to chemotherapy. However,

Table 2. Univariate and multivariate analyses of various predictors of pCR

	pCR rate (%)	Univariate analysis		Multivariate analysis	
		Odds ratio	<i>P</i>	Odds ratio	<i>P</i>
Histologic grade (3/1, 2)	38.1/25.3	1.818	0.244		
ER (-/+)	50.0/15.7	5.362	<0.001	5.987	0.022
PR (-/+)	37.3/16.3	3.047	0.018	0.567	0.477
HER-2 (+/-)	46.1/22.0	3.048	0.019	2.479	0.114
Ki-67 ($\geq 20\%$ vs $< 20\%$)	45.7/14.5	4.944	<0.001	3.522	0.042
TOP2A ($\geq 20\%$ vs $< 20\%$)	38.8/18.6	2.763	0.022	1.329	0.638
ALDH1 (-/+)	32.2/9.5	4.508	0.037	8.584	0.011
CD44 ⁺ /CD24 ⁻ (high/low)*	31.5/24.1	1.449	0.391		

*CD44⁺/CD24⁻ high and low tumors were determined using a median value (21.6%) as the cutoff value.

our observation needs to be confirmed by a future study including a larger number of patients and different chemotherapeutic regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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