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/m^2/wk) followed by 4 cycles of 5-fluorouracil 500 mg/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 500 mg/m^2 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-NH_2 (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 (×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 μm) 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-NH_2 (*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 fucshin (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 ≥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.

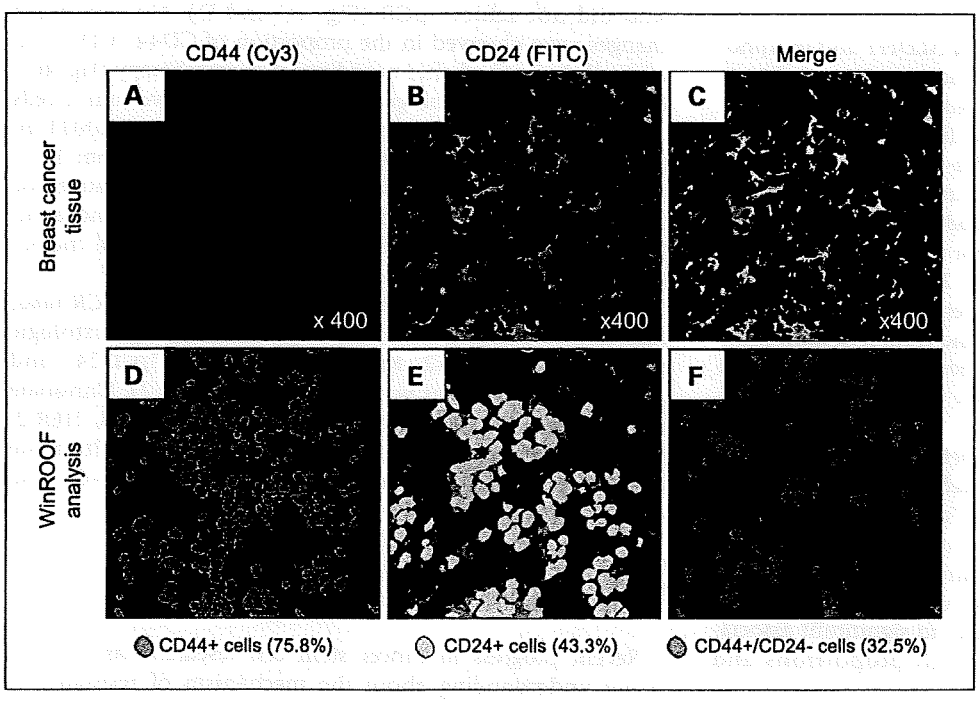

Fig. 1. Double-fluorescence immunohistochemical identification of $CD44^+/CD24^-$ tumor cells. Breast cancer tissue were subjected to double-fluorescence immunohistochemical determination of $CD44^+/CD24^-$ tumor cells. $CD44^+$ tumor cells were stained red (*A*) and $CD24^+$ tumor cells were stained 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*).

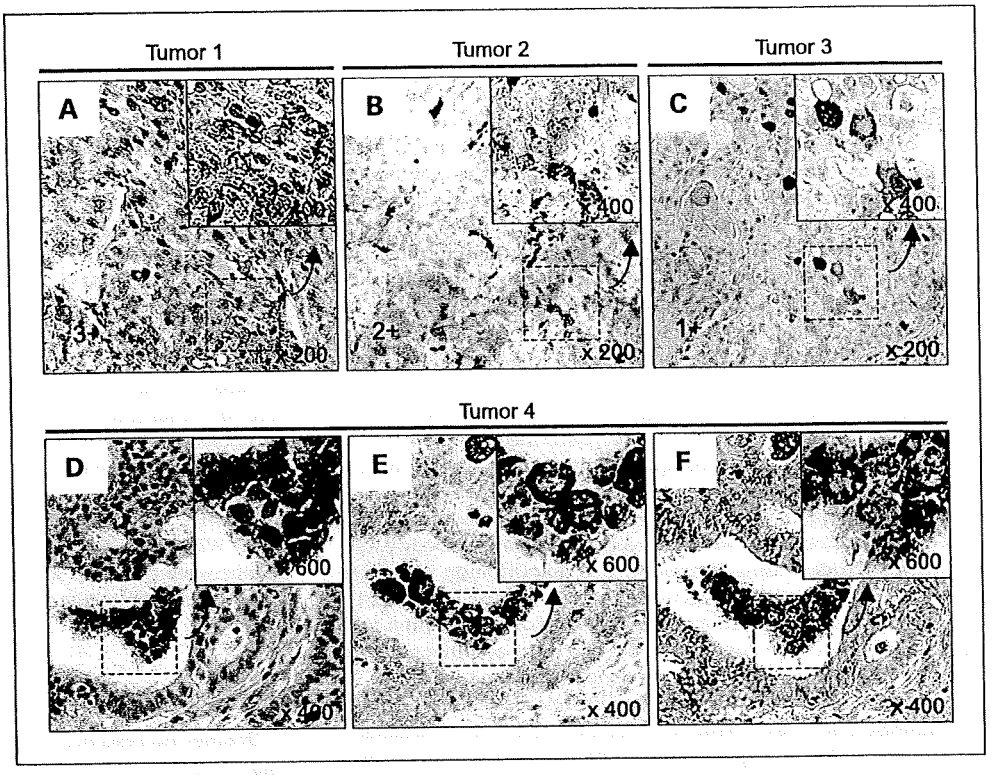

Fig. 2. Immunohistochemical identification of ALDH1-positive tumor cells. Representative results of immunostaining of ALDH1 in breast cancer tissues: (*A*) 3+ in tumor 1, (*B*) 2+ in tumor 2, and (*C*) 1+ in tumor 3. In tumor 4, besides ALDH1 immunostaining (fuchsin: *red*; *D*), CD68 immunostaining (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+ (≥50% positive tumor cells), 2+ (<50%, ≥10%), 1+ (<10%, ≥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 immunohistochemistry 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		
		Median (IQR)*	$P^\dagger$	Positive, *n* (%)	Negative, *n* (%)	$P^\ddagger$
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.
‡χ^2 test.

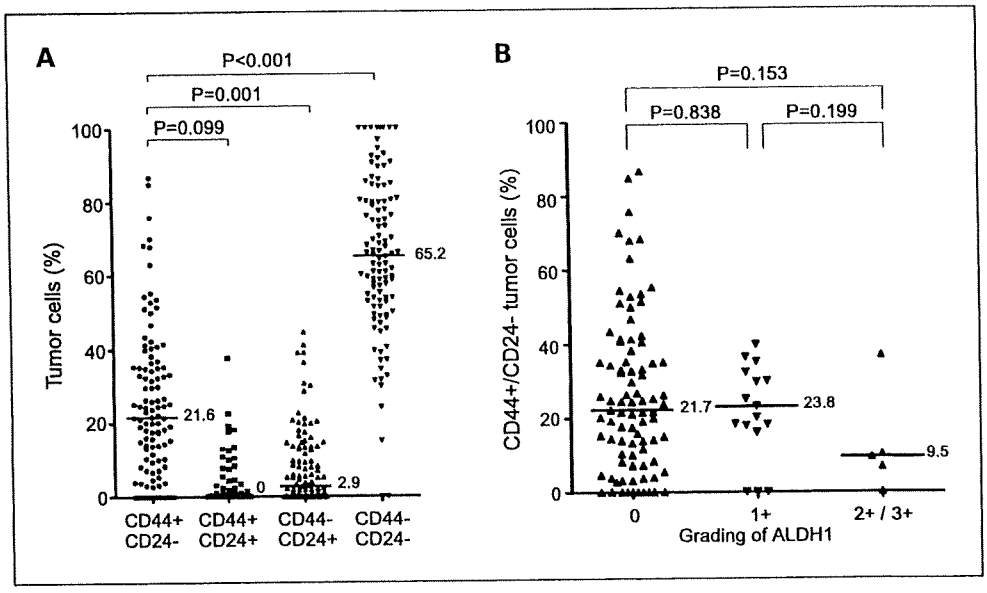

Fig. 3. Proportions of $CD44^+/CD24^+$ tumor cells in each tumor and their relationship with ALDH1 status. Breast cancers ($n = 108$) were classified into the four categories: $CD44^+/CD24^-$, $CD44^+/CD24^+$, $CD44^-/CD24^+$, and $CD44^-/CD24^-$. Proportions (%) of tumor cells in each category were 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/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 500 mg/m^2 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

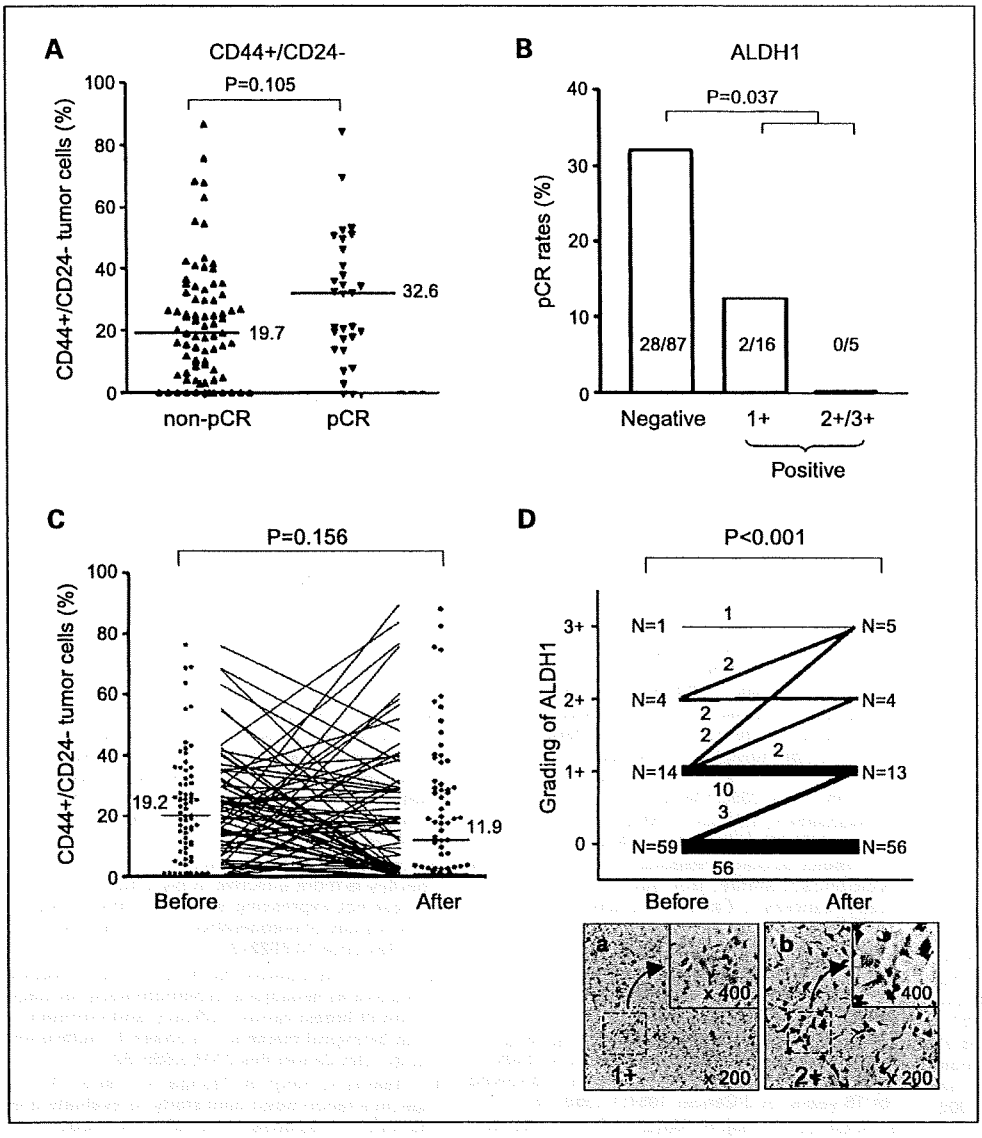

Fig. 4. Relationship of $CD44^+/CD24^-$ or ALDH1 positive with response to neoadjuvant chemotherapy. $CD44^+/CD24^-$ tumor cell proportions (%) were compared between tumors with pCR and non-pCR (*A*). *P*, Mann-Whitney *U* test. *Bars*, median. pCR rates were compared between ALDH1-positive and ALDH1-negative 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 significant 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	*P*	Odds ratio	*P*
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 (≥20% vs <20%)	45.7/14.5	4.944	<0.001	3.522	0.042
TOP2A (≥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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Genetic Polymorphisms of CYP2D6*10 and CYP2C19*2,*3 Are not Associated With Prognosis, Endometrial Thickness, or Bone Mineral Density in Japanese Breast Cancer Patients Treated With Adjuvant Tamoxifen

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BACKGROUND: The authors investigated the impact of the genetic polymorphisms cytochrome P450 (CYP) family 2, subfamily D, polypeptide 6, allele *10 (CYP2D6*10) and CYP family 2, subfamily C, polypeptide 19, allele *2,*3 (CYP2C19*2,*3) on disease recurrence in patients with breast cancer who received adjuvant tamoxifen and evaluated the impact of those polymorphisms on endometrial thickness, bone mineral density (BMD), and serum total cholesterol levels. **METHODS:** Patients with primary breast cancer (n = 173) who had hormone receptor-positive tumors and who also received adjuvant tamoxifen were included in the current study. Genetic polymorphisms of CYP2D6*10 and CYP2C19*2,*3 were analyzed. **RESULTS:** Recurrence-free survival (RFS) rates did not differ significantly between patients with the CYP2D6 *10/*10 genotype (n = 40) and patients with the CYP2D6 wild-type (wt)/wt or wt/*10 genotype (n = 133) or between patients with the CYP2C19 *2/*2, *2/*3, or *3/*3 genotypes (n = 41) and patients with the CYP2C19 wt/wt, wt/*2, or wt/*3 genotype (n = 132). Multivariate analysis indicated that, even after adjustment for well established prognostic factors, these CYP2D6 or CYP2C19 genotypes were not associated significantly with the RFS rate. Moreover, these genotypes did not affect endometrial thickness, BMD, or total cholesterol levels 1 year after the start of tamoxifen treatment. **CONCLUSIONS:** Neither the CYP2D6 *10/*10 genotype nor the CYP2C19 genotype is likely to have a clinically significant impact on prognosis, endometrial thickness, BMD, or total cholesterol levels in Japanese patients with breast cancer who are treated with adjuvant tamoxifen. **Cancer 2009;115:952–61. © 2009 American Cancer Society.**

KEY WORDS: breast cancer, cytochrome P450 family 2, subfamily D, polypeptide 6, cytochrome P450 family 2, subfamily C, polypeptide 19, tamoxifen.

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Tamoxifen has been used widely as a standard treatment in metastatic and adjuvant settings for estrogen receptor (ER)-positive and/or progesterone receptor (PgR)-positive breast cancers.[1,2] Tamoxifen as such has a low affinity for ER but is metabolized to active forms, such as 4-hydroxytamoxifen (4OH-TAM) and endoxifen, which have approximately 100 times higher affinity for ER than tamoxifen.[3] This metabolic activation of tamoxifen is mediated mainly by cytochrome P450 (CYP) family 2, subfamily D, polypeptide 6 (CYP2D6) and by CYP family 3, subfamily A, polypeptide 4/5 (CYP3A4/5). CYP2D6 is a highly polymorphic gene, and it has been established that several of its genetic variants, such as the CYP2D6*3, CYP2D6*4, and CYP2D6*5 alleles, do not carry any enzymatic activity.[4,5] Furthermore, a recent study demonstrated that patients with breast cancer who carry 2 of these null alleles have reduced levels of 4OH-TAM and endoxifen[6] compared with patients who have the CYP2D6 wt/wt genotype.

It is noteworthy that, in another recent study, patients with breast cancer who had the CYP2D6 *4/*4 genotype had worse clinical outcomes than patients who had other genotypes, probably because of the reduced activation of tamoxifen.[7] This finding may have important clinical implications for Western countries, because the CYP2D6 *4/*4 genotype occurs in 5% to 10% of Caucasians. Conversely, this genotype is very rare among Asians (<1%); however, the CYP2D6 *10/*10 genotype, which carries reduced enzyme activity (about 10% of the activity of the wild allele homozygote),[8] is relatively common and is observed in 15% to 20% of Japanese.[5] Very recently, it was demonstrated that progression of metastatic breast cancer occurs significantly sooner in patients who have the CYP2D6 *10/*10 genotype when they are treated with tamoxifen than in patients who have the CYP2D6 wt/wt or wt/*10 genotype[9] and that the prognosis for breast cancer patients who are treated with adjuvant tamoxifen is worse for those who have the CYP2D6 *10/*10 genotype than for those who have the CYP2D6 wt/wt or wt/*10 genotype.[10,11] These results appear to suggest that a reduced metabolism of tamoxifen in patients with the CYP2D6 *10/*10 genotype is important clinically in affecting the response to tamoxifen.

In addition to these findings for the CYP2D6 polymorphism, a recent study indicated that patients with the CYP2C19 *17 allele, which has higher activity than the wt allele, had a better prognosis than patients without it, probably because of the enhanced metabolism of tamoxifen into 4OH-TAM and endoxifen.[12] Although the occurrence of CYP2C19 *17 allele carriers is very rare in Japanese (<1% in Japanese but about 10% in Caucasians),[8] the occurrence of the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype (neither the *2 allele nor the *3 allele possesses enzyme activity) is relatively high, accounting for approximately 20% in Japanese.[13] It has been speculated that tamoxifen activation is reduced in patients who have these genotypes, resulting in high recurrence rates when they are treated with adjuvant tamoxifen, as reported for the CYP2D6 *4/*4 and CYP2D6 *10/*10 genotypes; whereas, to our knowledge, no studies have been reported on the relation between the CYP2C19 *2/*2, *2/*3, or *3/*3 genotype and recurrence rates.

For the report, we studied the impact not only of the CYP2D6 *10/*10 genotype but also of the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype on recurrence rates in patients with breast cancer who were treated with adjuvant tamoxifen. In addition, we studied the impact of these genetic polymorphisms on endometrial thickness, bone mineral density (BMD), and serum cholesterol levels, because these variables also can be expected to influence the effects of tamoxifen on these target organs.

MATERIALS AND METHODS

Patients

Serial patients with primary breast cancer (n = 173), who had hormone receptor-positive (ER-positive and/or PgR-positive) tumors, underwent mastectomy or breast-conserving surgery between October 1998 and December 2004 and were treated with adjuvant tamoxifen (20 mg daily), were included in this study. The median follow-up was 56 months (range, 8-109 months), and the median duration of adjuvant tamoxifen treatment was 52 months (range, 9-60 months). Characteristics of these patients are shown in Table 1. Seventy-three patients received tamoxifen alone, and 100 patients received tamoxifen, chemotherapy, and/or goserelin. Chemotherapy comprised either 6 cycles of combined oral cyclophosphamide (100 mg daily

Table 1. Characteristics of Patients Included in This Study

Characteristic	All Patients, n=173	CYP2D6 Wt/Wt or Wt/*10, n=133	CYP2D6 *10/*10, n=40	CYP2D6 P	CYP2C19 EM, n=132	CYP2C19 PM, n=41	CYP2C19 P
Median age (range), y	47 (22-73)	47 (22-72)	46 (27-73)	.47	47 (27-73)	47 (22-69)	.64
Menopausal status				**.59**			**.51**
Premenopausal	135	105	30		104	31	
Postmenopausal	38	28	10		28	10	
Tumor size, cm				**.81**			**.75**
≤2	98	76	22		75	23	
>2	75	57	18		57	18	
Lymph node status				**.82**			**.72**
Positive	50	39	11		36	14	
Negative	123	94	29		96	27	
ER status				**.47**			**.94**
Positive	157	123	34		120	37	
Negative	16	10	6		12	4	
PgR status				**.94**			**.52**
Positive	148	114	34		115	33	
Negative	25	19	6		17	8	
HER-2 status				**.44**			**.71**
Positive	13	9	4		11	2	
Negative	119	96	23		89	30	
Unknown	41	28	13		32	9	
Histologic grade				**.84**			**.54**
1	49	38	11		37	12	
2	119	91	28		90	29	
3	5	4	1		5	0	
Adjuvant treatment				**.88**			**.95**
TAM	73	56	17		57	16	
TAM and others†	100	77	23		75	25	

CYP2D6 indicates cytochrome P450 family 2, subfamily D, polypeptide 6; CYP2C19, cytochrome P450 family 2, subfamily C, polypeptide 19; Wt, wild-type allele; *10, allele *10; EM, extensive metabolizers; PM, poor metabolizers; ER, estrogen receptor; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; TAM, tamoxifen.
† Chemotherapy and/or goserelin.

on Days 1-14), intravenous methotrexate (40 mg/m^2 on Days 1 and 8), and intravenous 5-fluouroracil (5-FU) (600 mg/m^2 on Days 1 and 8; n = 8 patients); or 4 cycles of intravenous cyclophosphamide (600 mg/m^2 on Day 1) and intravenous epirubicin (60 mg/m^2 on Day 1; n = 32 patients); or others (n = 2 patients). Goserelin (3.75 mg every 4 weeks; n = 58 patients) was administered for 2 years. The patients who received paroxetine (a selective serotonin reuptake inhibitor [SSRI]) concomitantly with tamoxifen were excluded, because SSRIs are potent inhibitors of CYP2D6.[14,15] Informed consent was obtained from all patients.

Genotype Analysis

In brief, DNA was extracted from peripheral whole blood mononuclear cells and was subjected to TaqMan single-nucleotide polymorphism (SNP) Genotyping Assays (Applied Biosystems, Foster City, Calif) for identification of the CYP2D6 *10 allele. Polymerase chain reaction (PCR) was performed on the ABI prism 7900HT (Applied Biosystems) at 95°C for 10 minutes followed by 50 cycles at 92°C for 15 seconds and at 60°C for 90 seconds; and the fluorescent signal was detected by the ABI prism 7900HT (Applied Biosystems). The CYP2C19 *2

and *3 alleles were identified by using TaqMan SNP Genotyping Assays (Applied Biosystems). PCR was performed on the ABI prism 7900HT (Applied Biosystems) at 95°C for 5 minutes followed by 50 cycles at 92°C for 15 seconds and at 60°C for 90 seconds; and the fluorescent signal was detected by the ABI prism 7900HT (Applied Biosystems). Because the CYP2C19 *2 and *3 alleles do not have enzyme activity, patients with the CYP2C19 *2/*2, *2/*3, or *3/*3 genotypes were designated as poor metabolizers (PMs), and patients with the CYP2C19 wt/wt, wt/*2, or wt/*3 genotypes were designated as extensive metabolizers (EMs).[8,13,14]

Estrogen Receptor, Progesterone Receptor, and Human Epidermal Growth Factor Receptor 2

ER and PgR levels were defined as positive by immunohistochemistry (IHC) when ≥10% of tumor cells stained positive for these receptors (ER, clone 6F11; PgR, clone 16; Ventana Japan K.K. and SRL Inc., Tokyo, Japan). Human epidermal growth factor receptor 2 (HER-2) levels were determined by fluorescence in situ hybridization (FISH) using PathVysion Her-2 DNA Probe kits (SRL Inc.) or by IHC using the DAKO system scale (DAKO Diagnostics, Tokyo, Japan). When FISH indicated that a tumor contained >2 genes per cell or IHC indicated 3+ HER-2 staining, the tumor was considered HER-2-positive.

Recurrence-free Survival

To calculate RFS rates, distant recurrences, locoregional recurrences, ipsilateral in-breast recurrences, and contralateral breast cancers were included. RFS rates were estimated with the Kaplan-Meier method, and statistical significance was assessed with the log-rank test. Cox proportional hazard analyses (unadjusted and adjusted) also were performed.

Bone Mineral Density, Endometrial Thickness, and Total Cholesterol Levels

The influence of tamoxifen on BMD, endometrial thickness, and total cholesterol was studied in postmenopausal patients according to genotype (CYP2D6*10 or CYP2C19*2,*3). BMD of the lumbar spine (lumbar segments 2-4 [L2-L4]) was measured by using a dual-energy x-ray absorptiometer (Lunar DPX; GE Medical Systems, Tokyo, Japan) in 20 patients, and endometrial thickness was measured by transvaginal ultrasonography (SONOVISTA MSC; Siemens AG, Beyer, Munich, Germany) in 21 patients at baseline and 1 year after the start of tamoxifen. Total serum cholesterol levels also were measured by using the cholesterol oxidase method in 30 patients at baseline and 1 year after the start of tamoxifen. Changes from baseline in lumbar spine BMD, endometrial thickness, and cholesterol levels were assessed with the *t* test for paired data.

Statistical Analysis

Statview software (version 5.0 for Windows; SAS Institute Inc., Cary, NC) was used for statistical analyses. A *P* value <.05 was considered statistically significant.

RESULTS

Frequencies of CYP2D6*10 or CYP2C19*2,*3 Genotype and Relation With Clinicopathologic Features of Breast Tumors

TaqMan SNP genotyping assays were used to identify CYP2D6 and CYP2C19 genotypes in 173 patients. The frequency of the CYP2D6 *10/*10 genotype was 23.1%, and the frequency of the CYP2D6 wt/wt or wt/*10 genotype was 76.9%. The frequency of the CYP2C19 *2/*3, *2/*2, or *3/*3 genotype (ie, PMs) was 23.7%; and the frequency of the CYP2C19 wt/wt, wt/*2, or wt/*3 genotype (ie, EMs) was 76.3% (Table 2). None of these genotypes showed any significant association with various clinicopathologic parameters, including menopausal status, tumor size, lymph node status, ER, PgR, HER-2, histologic grade, and type of adjuvant therapy (Table 1).

Recurrence-free Survival Rates by CYP2D6*10 Genotype

RFS rates were not significantly different between patients with the CYP2D6 *10/*10 genotype (median follow-up, 63 months; range, 23-96 months; n = 40) and those with the wt/wt or wt/*10 genotype (median follow-up, 54 months; range, 8-109 months; n = 133; log-rank test;

$P = .98$) (Fig. 1a). When the analysis was limited to 73 patients who received adjuvant tamoxifen alone, again, there was no significant difference in RFS rates between these groups (log-rank test; $P = .57$) (Fig. 1b). Multivariate analysis indicated that, even after adjustment for well established prognostic factors such as tumor size, lymph node status, histologic grade, ER, and PgR, there still was no significant difference in RFS rates between patients with the CYP2D6 *10/*10 genotype and those with the CYP2D6 wt/wt or wt/*10 genotype (Table 3).

Table 2. Genotype Frequency of the Cytochrome P450 (CYP) 2D6*10 and CYP2C19*2,*3 Polymorphisms

Genotype	No. of Patients, n=173	%
CYP2D6		
Wt/Wt	74	42.8
Wt/*10	59	34.1
*10/*10	40	23.1
CYP2C19		
*1/*1	53	30.6
*1/*2	56	32.4
*1/*3	23	13.3
*2/*3	17	9.8
*2/*2	20	11.6
*3/*3	4	2.3

CYP2D6 indicates cytochrome P450 family 2, subfamily D, polypeptide 6; Wt, wild-type allele; *10, allele *10; CYP2C19, cytochrome P450 family 2, subfamily C, polypeptide 19.

*Recurrence-free Survival Rates by CYP2C19*2,*3 Genotype*

RFS rates did not differ significantly for the CYP2C19 PM genotypes (*2/*2, *2/*3, or *3/*3; median follow-up, 63 months; range, 36-109 months) and the EM genotypes (wt/wt, wt/*2, or wt/*3; median follow-up, 54 months; range, 8-109 months; log-rank test; $P = .19$) (Fig. 2a). When the analysis was limited to 73 patients who received adjuvant tamoxifen alone, again, there was no significant difference in RFS rates between these 2 groups (log-rank test; $P = .47$) (Fig. 2b). Multivariate analysis indicated that, after adjustment for the same prognostic factors described above, again, there was no significant difference in RFS rates between the CYP2C19 EM and PM genotypes (Table 3).

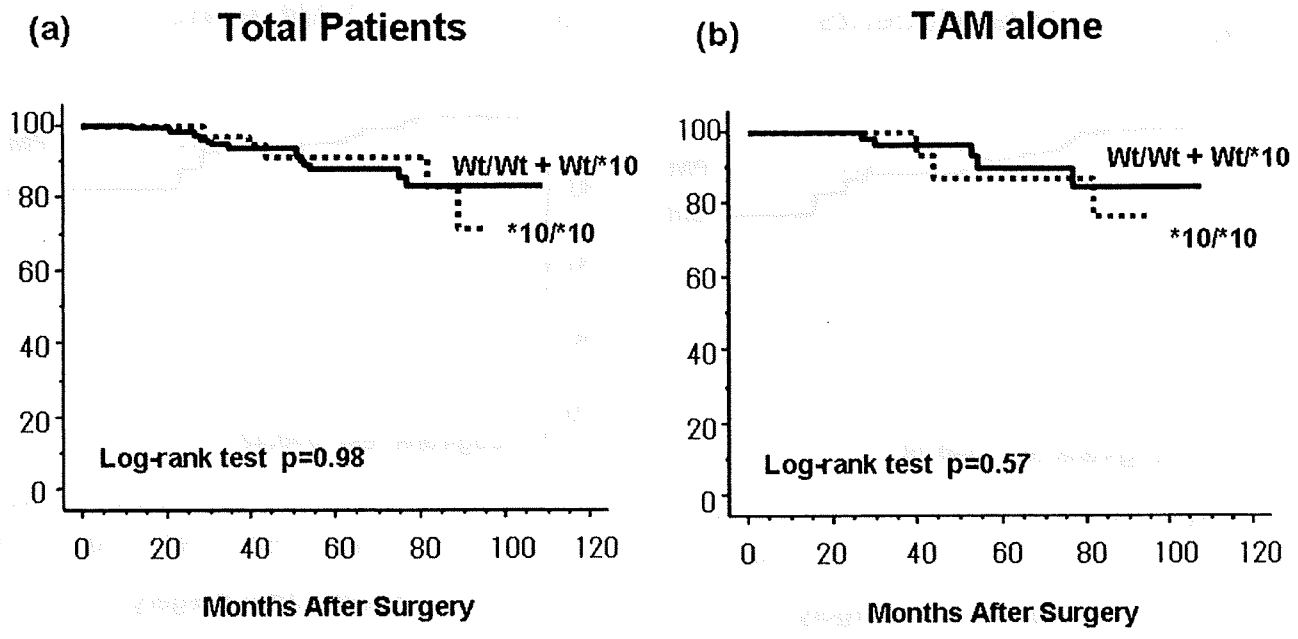

FIGURE 1. The prognosis of patients who received adjuvant tamoxifen according to cytochrome P450 (CYP) family 2, subfamily D, polypeptide 6, allele *10 (CYP2D6*10) genotype. Recurrence-free survival rates were calculated with the Kaplan-Meier method according to CYP2D6 genotype, ie, the CYP2D6 *10/*10 genotype and the CYP2D6 wild-type (wt)/wt or wt/*10 genotype, for all patients who received adjuvant tamoxifen (a) and for patients who received adjuvant tamoxifen alone (b).

*Influence of Tamoxifen on Endometrial Thickness, Bone Mineral Density, and Cholesterol Levels According to CYP2D6*10 or CYP2C19*2,*3 Genotype*

Because it is known that tamoxifen affects endometrial thickness, BMD, and serum total cholesterol, we also studied the impact of CYP2D6 and CYP2C19 genetic polymorphisms on these effects in postmenopausal patients. Changes from baseline in BMD (L2-L4), total cholesterol levels, and endometrial thickness 1 year after the start of tamoxifen are shown in Figure 3 for CYP2D6 and in Figure 4 for CYP2C19. A significant increase ($P < .05$) in BMD and a significant decrease ($P < .05$) in total cholesterol levels were observed regardless of CYP2D6 genotype after 1 year of treatment with tamoxifen; however, there was no significant difference in the extent of changes in BMD and total cholesterol levels between patients with the CYP2D6 *10/*10 genotype and those with the CYP2D6 wt/wt or wt/*10 genotype. Endometrial thickness increased significantly ($P < .05$) after a 1-year treatment with tamoxifen regardless of CYP2D6 genotype; however, there was no such significant difference in endometrial thickness between patients with the CYP2D6 *10/*10 genotype and those with the CYP2D6 wt/wt or wt/*10 genotype. Similar to the results obtained for the CYP2D6 genotype, there was no significant difference between CYP2C19 PM patients and CYP2C19EM patients in the extent of changes in BMD, total cholesterol levels, or endometrial thickness 1 year after the start of tamoxifen treatment.

Table 3. Univariate and Multivariate Analyses of the Cytochrome P450 (CYP) 2D6*10 and CYP2C19*2,*3 Polymorphisms on Recurrence-free Survival Rates

Variable	CYP2D6	CYP2C19
Univariate analysis		
HR	0.94	0.45
95% CI	0.34-2.60	0.13-1.55
P	.95	.2
Multivariate analysis†		
HR	0.6†	0.37†
95% CI	0.18-1.92	0.08-1.76
P	.39	.21

CYP2D6 indicates cytochrome P450 family 2, subfamily D, polypeptide 6; CYP2C19, cytochrome P450 family 2, subfamily C, polypeptide 19; HR, hazard ratio; CI, confidence interval.
† Adjusted for tumor size, lymph node status, histologic grade, progesterone receptor status, human epidermal growth factor receptor 2 status, and adjuvant therapy.

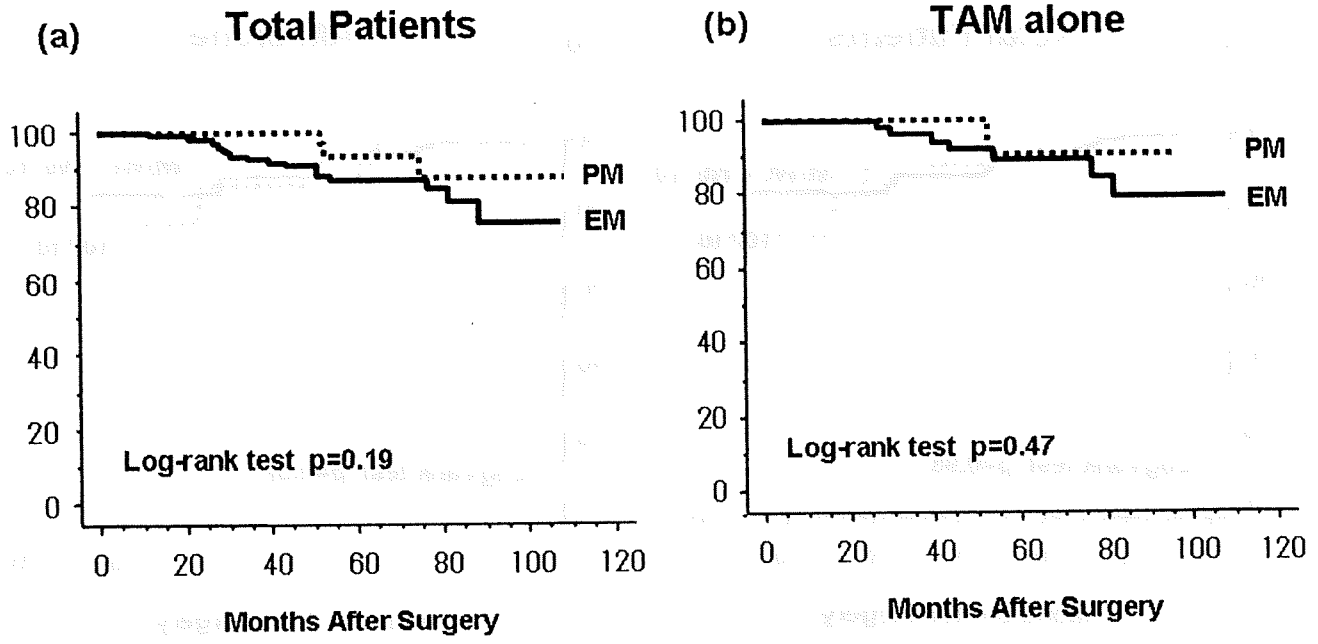

FIGURE 2. The prognosis of patients who received adjuvant tamoxifen (TAM) according to cytochrome P450 (CYP) family 2, subfamily C, polypeptide 19, allele *2,*3 (CYP2C19*2,*3) genotype. Recurrence-free survival rates were calculated with the Kaplan-Meier method according to CYP2C19 genotype, ie, poor metabolizers (PM) or extensive metabolizers (EM), for all patients who received adjuvant tamoxifen (a) and for patients who received adjuvant tamoxifen alone (b).

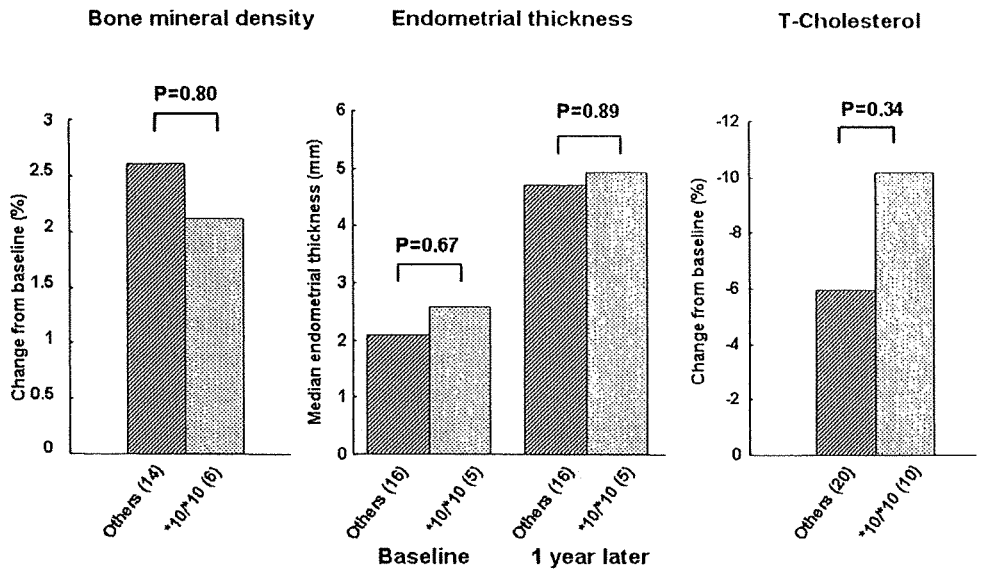

FIGURE 3. The influence of tamoxifen on bone mineral density, endometrial thickness, and total cholesterol (T-Cholesterol) levels by cytochrome P450 (CYP) family 2, subfamily D, polypeptide 6, allele *10 (CYP2D6*10) genotype in patients who received adjuvant tamoxifen. Changes in the percentages of bone mineral density, endometrial thickness, and total cholesterol levels after 1 year of tamoxifen treatment are shown according to CYP2D6 genotype, ie, the CYP2D6 *10/*10 genotype and the CYP2D6 wild-type (wt)/wt or wt/*10 genotype. Numbers in parentheses indicate the numbers of patients examined.

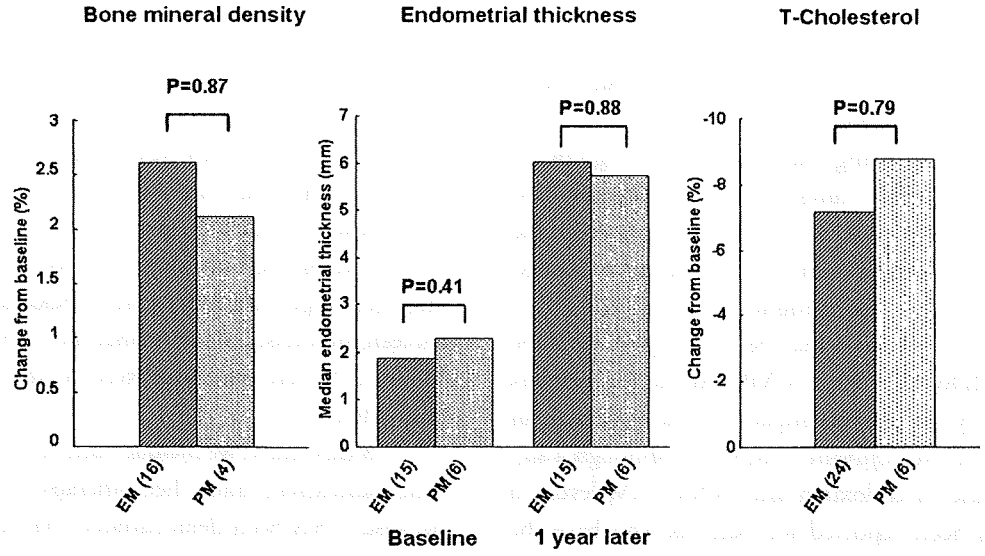

FIGURE 4. The influence of tamoxifen on bone mineral density, endometrial thickness, and total cholesterol (T-Cholesterol) levels in patients who received adjuvant tamoxifen according to cytochrome P450 (CYP) family 2, subfamily C, polypeptide 19, allele *2,*3 (CYP2C19*2,*3) genotype. Changes in the percentages of bone mineral density, endometrial thickness, and total cholesterol levels after 1 year of tamoxifen treatment are shown according to CYP2C19 genotype, ie, patients were designated as either poor metabolizers (PM) or extensive metabolizers (EM). Numbers in parentheses indicate the numbers of patients examined.

DISCUSSION

Because tamoxifen is used widely as 1 of the standard treatments in the metastatic and adjuvant settings, evidence of the suspected impact of CYP2D6*10 polymorphisms on the efficacy of tamoxifen would be of major consequence for clinical practice, especially in the case of Asian patients with breast cancer, because it is believed that these patients possess CYP2D6 *10/*10 homozygotes

Table 4. Meta-Analysis of the Prognostic Impact of Cytochrome P450 (CYP) 2D6*10/*10 Polymorphism

Study	No. of Patients	HR	95% CI
Kiyotani 2008[10]	58	10.04	1.17-86.27
Xu 2008[11]	152	4.7	1.1-20.0
Current study	173	0.6	0.18-1.92
Total	383	1.86	0.80-4.32

HR indicates hazard ratio; CI, confidence interval.

at a relatively high frequency (approximately 20%). However, unlike 2 previous reports, which claimed to demonstrate that the CYP2D6 *10/*10 genotype is associated with a poor prognosis, we did not observe any significant difference in RFS rates between the CYP2D6 *10/*10 genotype and the CYP2D6 wt/wt or wt/*10 genotype. The confidence intervals (CI) cited in those 2 previous reports revealed a wide range; ie, the hazard ratio was 10.04 (95% CI, 1.17-86.27) in the study by Kiyotani et al[10] and 4.7 (95% CI, 1.1-20.0) in the study by Xu et al.[11] Therefore, we used the method described by Parmar et al[16] to conduct an exploratory meta-analysis that included the 2 previous reports[10,11] and the current study. Our meta-analysis produced a hazard ratio of 1.86 (95%CI, 0.80-4.32) (Table 4), indicating that there was no significant association between RFS rates and CYP2D6 genotypes. In addition, it recently was reported that patients with the CYP2D6 *4/*4 genotype (null activity) are not associated not necessarily with a poor prognosis but, in fact, with a better prognosis.[17] Thus, it may be too early to conclude that the CYP2D6 *10/*10 or CYP2D6 *4/*4 genotypes have a clinically significant impact on the prognosis of patients who receive adjuvant tamoxifen. Although a significant decrease in endoxifen and 4OH-TAM levels in the blood has been reported for patients who have the CYP2D6 *10/*10 or CYP2D6 *4/*4 genotype compared with patients who have the CYP2D6 wt/wt genotype,[6,9,11] these tamoxifen metabolites still may be effective at such low levels, or the involvement of tamoxifen itself in the growth inhibition of breast tumors may be much greater than imagined.

It is known that tamoxifen has estrogenic effects on the endometrium, bone, and liver (total cholesterol levels), and these effects are thought to be mediated by its metabolites, endoxifen and 4OH-TAM. For this reason, we studied the impact of the CYP2D6 *10/*10 genotype on the effect of tamoxifen on these organs. Because normal organs are expected to respond to tamoxifen more homogeneously than tumors with their inherently heterogeneous response, the impact of the CYP2D6 *10/*10 genotype on these normal organs, if any, would be more evident than that on tumors. Consistent with the findings of previously reported studies,[18-20] tamoxifen treatment for 1 year in our study resulted in a significant increase in endometrial thickness and BMD as well as in a significant decrease in the total cholesterol levels. However, the extent of such an increase or decrease in any of these parameters was not significantly different between the CYP2D6 *10/*10 genotype and the CYP2D6 wt/wt or wt/*10 genotype. These results indicate that the CYP2D6 *10/*10 genotype has essentially no impact on the effects of tamoxifen in these target organs, which is consistent with our finding that the CYP2D6 *10/*10 genotype has no impact on the effect of tamoxifen on recurrence.

In the current study, we also investigated the association of CYP2C19 variant alleles *2 and *3 with the prognosis of patients who received adjuvant tamoxifen. Because the CYP2C19 *2 and *3 alleles possess no activity, patients with the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype can be expected to have lower levels of endoxifen and 4OH-TAM, which would lead to higher recurrence rates when these patients are treated with adjuvant tamoxifen. However, such an association was not observed in our study. In addition, there was no association between these genotypes and endometrial thickness, BMD, or total cholesterol levels, indicating that the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype is unlikely to influence the effect of tamoxifen.

In this study, we include patients who received adjuvant tamoxifen, and chemotherapy, and/or goserelin, because it has been demonstrated that tamoxifen significantly improves RFS rates even in the presence of these concomitant treatments[21,22] and because CYP2D6 and CYP2C19 essentially are not involved in the metabolism of epirubicin, methotrexate, or 5-FU, which were included in the chemotherapy. It is known that cyclophosphamide is activated by CYP2C19; thus, it has been speculated that cyclophosphamide, like tamoxifen, may be less effective for patients with the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype. However, the prognosis for patients with any of these genetic polymorphisms was

similar to that for the patients with the CYP2C19 wt/wt, wt/*2, or wt*3 genotype, indicating that any of the CYP2C19 polymorphisms are unlikely to have an impact on the effects of either tamoxifen or cyclophosphamide.

In conclusion, in the current study, we were able to demonstrated that neither the CYP2D6 *10/*10 genotype nor the CYP2C19 *2/*2, *2/*3, or *3/*3 genotype was associated with a poor prognosis for breast cancer patients who were treated with adjuvant tamoxifen. Moreover, we demonstrated that these genetic polymorphisms are not associated with endometrial thickness, BMD, or total cholesterol levels. When taken together, these results suggest that the effects of tamoxifen are unlikely to be influenced by these genetic polymorphisms. At least, it may be too early to conclude that either the CYP2D6 *10/*10 genotype or the CYP2C19 *2/*2, *2/*3, or *3/*3 genotype has a clinically significant impact on disease recurrence in patients who receive adjuvant tamoxifen. However, our current findings need to be verified by future studies that include larger numbers of patients.

Conflict of Interest Disclosure

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan.

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ORIGINAL PAPER

Incidence of joint symptoms and bone fractures in Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole

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Abstracts

Purpose Incidence of joint symptoms and bone fractures as well as changes in bone mineral density (BMD) in Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole were investigated to determine whether there is an ethnic difference from Caucasian patients in the incidence of these adverse events of anastrozole.

Methods Adjuvant anastrozole was used to treat 348 postmenopausal breast cancer patients for a median period of 22 months. Adverse events of anastrozole including joint symptoms, loss of BMD, and bone fracture were investigated by means of chart review.

Results Joint symptoms developed in 96 (27.5%) patients. Age (younger than 65) and prior chemotherapy was strongly associated with an increased risk of joint symptoms. Annual fracture incidence was 0.86 and 0.85% and lumbar BMD decreased by 1.3 and 2.8% at 1 and 2 years, respectively. In comparison, the ATAC trial reported corresponding figures of 2.0 and 2.7 and of 2.2 and 4.0%.

Conclusion Incidence and risk factors of joint symptoms are similar for Japanese and Caucasian patients, but the former tend to show a smaller decrease in BMD and a lower incidence of bone fractures, probably due to ethnic difference in the hormonal milieu.

Keywords Breast cancer · Anastrozole · Bone mineral density · Fracture · Joint symptoms

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Introduction

For more than a decade, 5-year treatment with tamoxifen has been the gold standard for hormonal therapy for postmenopausal patients with hormone receptor positive breast cancer. However, the ATAC trial has clearly shown that anastrozole, a potent third-generation aromatase inhibitor, is superior to tamoxifen in terms of improved disease-free survival (Baum et al. 2002; Howell et al. 2005). As a result, anastrozole is now accepted as a standard treatment for postmenopausal patients with hormone receptor positive breast cancer.

Besides anastorozole's superior efficacy it seems to cause fewer adverse events because the incidence of tamoxifen-related serious adverse events such as endometrial cancer, thrombophlebitis, and ischemic cerebrovascular disease, etc. is significantly lower for patients receiving anastrozole rather than tamoxifen (Baum et al. 2002; Howell et al. 2005; Buzdar et al. 2006). It has been reported, however, that patients being treated with anastrozole show a higher incidence of joint symptoms (joint pain and stiffness), loss of bone mineral density (BMD) and bone fractures (Howell et al. 2005; Buzdar et al. 2006). These reports are based on clinical trials involving Caucasian breast cancer patients. Since BMD and bone fracture incidence are different for Japanese and Caucasian postmenopausal woman (Ito et al. 1997) and since hormonal milieus of these two ethnicities also differ, i.e., serum estradiol levels are about in Caucasian postmenopausal women twice as high as in their Japanese counterparts (Shimizu et al. 1990), we considered it quite possible that the adverse effect of anastrozole on bone might also be different.

We published a preliminary report on the influence of anastrozole on BMD in Japanese postmenopausal breast cancer patients, and suggested that the negative impact of

anastrozole on bone might be milder in Japanese than Caucasian women, based on the observation that BMD loss 1 year after anastrozole treatment is 1.2% for Japanese patients but reportedly 2.2% for Caucasian patients (Yoneda et al. 2006). In the study presented here, we investigated the actual incidence of joint symptoms and bone fractures for Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole also studied changes in BMD after 2 years of this treatment.

Materials and methods

Patients

Between April 2002 and April 2007, 348 hormone receptor [estrogen receptor (ER) and/or progesterone receptor (PR)]-positive postmenopausal breast cancer patients were treated at our hospital with adjuvant anastrozole (1 mg/day) for a median period of 22 months ranging from 1 to 60 months. Adverse events of anastrozole on these patients, that is, joint symptoms, loss of BMD, and bone fracture, were investigated by means of chart review. Characteristics of the patients analyzed in this study are shown in Table 1. We also recorded the anastrozole-related joint symptoms (≥grade 1 according to Common Terminology Criteria for Adverse Events version 3.0) and fractures at any site.

Of the 348 patients, 122 had their lumbar spine (L2-4) BMD measured by means of dual-energy X-ray absorptiometry before the start of anastrozole treatment. Patients were categorized into three groups: normal bone mineral density [young adult mean (YAM ≥ 80%) ($n = 85$), osteopenia (80% > YAM ≥ 70%) ($n = 21$), or osteoporosis (YAM < 70%) ($n = 16$)]. Patients who were not osteopenic and those whose BMD was not measured before the start of anastrozole were not treated with preventive medication such as bisphosphonates, vitamin D, or calcium. However, patients who were osteopenic at the start of anastrozole and those who developed osteoporosis during anastrozole treatment received one or a combination of these medications.

Statistical analysis

The incidence of anastrozole-related joint symptoms was compared among various subgroups with the χ^2 test. Cumulative incidence of bone fractures was calculated with the Kaplan–Meier method. Changes from baseline in lumbar spinal BMD 1 and 2 years after the start of anastrozole treatment were assessed with the paired t test. Stat View software (Version 5.0 for Windows, SAS Institute Inc., Cary, NC, USA) was for statistical analysis. A P value less than 0.05 was considered to indicate statistical significance.

Table 1 Patient characteristics

	No. of patients
Demographics	
Age (years)	62 (8.2)[a]
Weight (kg)	56 (8.6)
Height (cm)	155 (4.7)
Body mass index (kg/m^2)	23 (3.2)
Stage	
I	140 (40.0%)
II	174 (49.7%)
III	24 (6.9%)
Hormone-receptor status	
ER positive	344 (98.2%)
PR positive	259 (74.0%)
Adjuvant chemotherapy	
None	226 (64.9%)
Epirubicin[b]	47 (13.4%)
Taxane[c]	7 (2.0%)
Epirubicin → Taxane[d]	63 (18%)
CMF	5 (1.5%)

[a] SD

[b] Epirubicin-containing regimens (epirubicin 75 mg/m^2 or 100 mg/m^2, 4–6 cycles, q3w)

[c] Taxane includes paclitaxel (80 mg/m^2, 12 cycles, q1w) or docetaxel (60 mg/m^2, 4 cycles, q3w)

[d] Epirubicin-containing regimens followed by paclitaxel or docetaxel

Results

Joint symptoms

Of the 348 patients, 96 (27.5%) developed joint symptoms (Table 2). The median time until development of joint symptoms was 3 months, ranging from 1 to 20 months. Of these 96 patients, 79 (82%) showed spontaneous resolution of joint symptoms with a median time until resolution of 3 months (range 1–18 months) even though anastrozole was continued without any medication for the symptoms. The symptoms of the remaining 17 (18%) patients deteriorated, so that anastrozole was discontinued and replaced with exemestane in, for two, tamoxifen or toremifene for 12, and no further treatment for three patients.

Risk factors for joint symptoms are displayed in Table 3. Age <65, but not BMI, was strongly associated with a higher risk of joint symptoms ($P = 0.0004$). Presence of adjuvant chemotherapy was also strongly correlated with an increased risk of joint symptoms regardless of the regimen, since the incidence of joint symptoms was 21.2% for the patients not treated with chemotherapy, 44.2%