Detection of ER activity in MCF-7 and sublines

To quantify the ER activities of MCF-7–E10 cells and PAC-1–PAC-3 cells in terms of the intensity of GFP expression, we previously developed an automated image analysis system for GFP expression in collaboration with Olympus Life Science Company (Tokyo, Japan) [22]. Using this system, estrogen-induced ER activation was analyzed.

Statistical analysis

Statistical analyses were performed using the Stat Flex version software program (Artech Co., Ltd., Osaka, Japan). In comparisons among three or more groups, ANOVA, two-sample t tests, and Fisher's exact test were used to assess the statistical significance of differences. Data are expressed as means ± SD $P < 0.05$ was considered statistically significant.

Results

Clinical response to taxanes in NAC

First, we studied the relationship between ER expression and clinical response to taxanes in 190 breast cancer patients receiving NAC. As shown in Table 1, 6.8% of the 190 patients showed cCR (clinical complete response) by taxanes. ER-negative patients achieved a higher cCR rate than ER-positive patients (12.5 vs. 4.0%, $P = 0.028$), while no significant difference in cCR rate was observed for HER2 expression ($P = 0.498$). ER-negative patients also tended to have a higher cCR plus cPR (clinical partial response) rate than ER-positive patients (57.9 vs. 42.9%, $P = 0.051$). These results suggest ER-negative breast cancers to be more sensitive to taxanes than ER-positive cancers.

Table 1 Clinical complete response to taxanes in NAC-treated patients

Characteristic	cCR rate of patients	
	No./total (%)	
ER		
Positive ($n = 126$)	5/126 (4.0)	$P = 0.028$*
Negative ($n = 64$)	8/64 (12.5)	
HER2		
Positive ($n = 71$)	6/71 (8.5)	$P = 0.498$
Negative ($n = 119$)	7/119 (5.9)	
Total ($n = 190$)		

cCR clinical complete response

* Chi-square test

Evaluation by ad-ERE–GFP assay of ER status in NAC

In our previous studies, we produced our own focused microarray for estrogen-regulated genes [16], and found that ER-positive breast cancers did not always show high expression of a target gene [unpublished data]. This suggests some discrepancy between the expression levels of ER protein and its function as a transcription factor. Using the ad-ERE–GFP assay system to detect ERα transcriptional activity in breast cancer cells, we analyzed the significance of ERα in the clinical response to NAC.

First, as shown in Fig. 1a and b, the relationship between response to paclitaxel and ERα expression was evaluated by IHC and the ad-ERE–GFP method, respectively, in 31 clinical samples. The cCR and cPR (clinical partial response) were sensitive to paclitaxel and the cSD (clinical stable disease) and cPD (clinical progressive disease) were insensitive. When ERα status was determined by IHC, 15 of 24 ER-positive cases (62.5%) and 4 of 7 ER-negative cases (57.1%) were paclitaxel-insensitive, while when ERα status was assessed based on its function by the ad-ERE–GFP method, 10 of 13 cases with high ER activity (76.9%) and 9 of 18 cases with low ER activity (50.0%) were paclitaxel-insensitive. These results suggest that the ad-ERE–GFP method might be more useful for the selection of ER-functional and paclitaxel-insensitive cases than the IHC method.

Next, we analyzed the response to paclitaxel in the 24 cases considered to be ERα-positive based on IHC (Fig. 1c). Seven cases showed pCR (pathological complete response) and 17 cases did not (non-pCR). Twelve cases of 24 cases showed low ER activity, indicating that ERα in these cases could not function as a transcription factor. The rate of non-pCR cases in the high ER activity group was 83.3% (10 of 12 cases), which was much higher than that in the low ER activity group, at 58.3% (7 of 12 cases). Consistent with the results on clinical response in NAC, the cases for which ERα did function as a transcription factor hardly achieve pCR. To further clarify the role of ERα in the sensitivity to paclitaxel, we carried out the following experiments in vitro.

Knockdown of ERα of MCF-7 cells increased the sensitivity to paclitaxel

On the basis of the clinical results, we analyzed whether or not ERα expression directly affects the sensitivity of breast cancer cells to paclitaxel. Figure 2a shows that siRNA-treated (si-1, si-2) MCF-7 cells were more sensitive to paclitaxel than the parent and scramble si-RNA cells. Figure 2b shows the ERα knockdown in si-1 and si-2 cells. While the IC50 values for the parent MCF-7 and scramble cells were 6.7 and 5.1 nM, those for si-1 cells and si-2 cells were 2.8 and 3.6 nM, respectively (Fig. 2c). These results suggest that ERα knockdown rendered MCF-7 cells more sensitive to paclitaxel than parent MCF-7 cells.

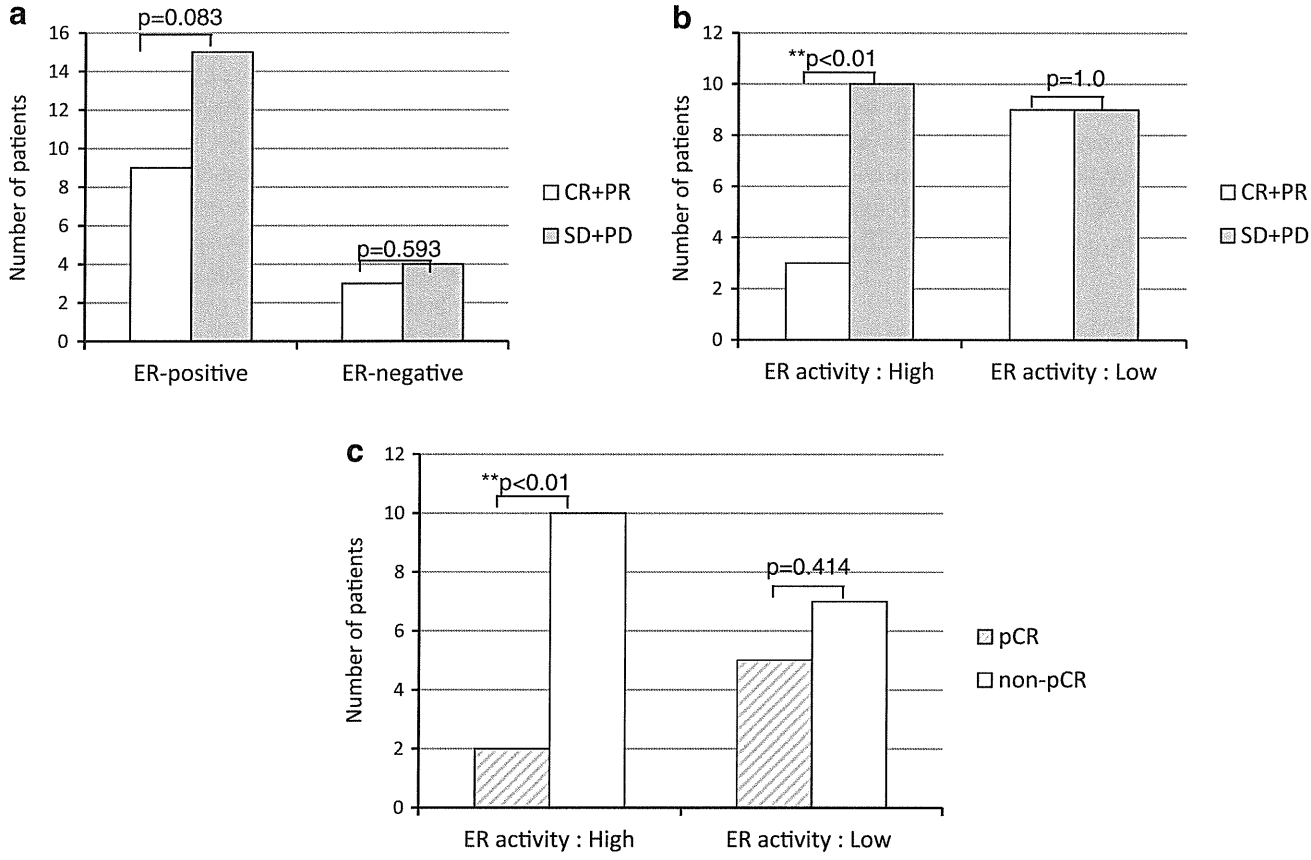

Fig. 1 The clinical evaluation and ER expression or activities in breast cancers in pre-NAC. **a** ERα status were evaluated by IHC method in 31 patients. **b** ERα activities were evaluated using adenovirus ERE–GFP system in 31 patients. **c** ER activities in pCR and non-pCR cases for 24 ER protein positive breast cancers

ERα expression decreased the sensitivity of ERα-negative SKBR3 cells to paclitaxel

Next, we examined the effect of ERα expression on the sensitivity of the SKBR3 cell line to paclitaxel. We generated SKBR3 cells expressing ERα, and compared the paclitaxel sensitivity of SK-ERpos cells with those of parent SKBR3 and SKBR3-vector control (SKBR3-cont) cells (Fig. 2d). Figure 2e shows ERα expression in SK-ERpos cells by real-time PCR. The IC50 values for the parent SKBR3 and SKBR3-cont cells were 3.6 and 3.1 nM, respectively, whereas that of SK-ERpos cells was >10 nM (Fig. 2f). These results suggest that ERα overexpression rendered SKBR3 cells more resistant to paclitaxel than parent SKBR3 cells.

SiRNA knockdown of ERα decreased deacetylation of α-tubulin

Paclitaxel promotes microtubule stabilization, which disrupts cellular processes, inhibits cell division, and ultimately induces apoptosis [23, 24]. On the other hand, estrogen promotes the deacetylation of tubulin through HDAC6, a downstream gene of ER, thereby increasing cell motilities via microtubule destabilization within the cells [18]. We examined the relationship between the presence of the ER and acetylation of α-tubulin.

Figure 3 shows results of the analysis for acetylation of α-tubulin in MCF-7 and ERα knockdown MCF-7 cells. We detected a significant increase in acetylated α-tubulin protein in ERα knockdown MCF-7 cells, suggesting that suppression of tubulin deacetylation by knockdown of ERα renders MCF-7 cells more sensitive to paclitaxel.

Upregulation of ERα function in paclitaxel-resistant MCF-7–E10 cells

To further evaluate the function of ERα in determining sensitivity to taxanes, we established paclitaxel-resistant MCF-7–E10 cells. MCF-7–E10 is an estrogen signal reporter cell line, which was derived from MCF-7 cell line via stable transfection of the ERE–GFP gene for the purpose of assessing ER activation [19, 20]. Paclitaxel-resistant MCF-7–E10 cells showed resistance to paclitaxel-

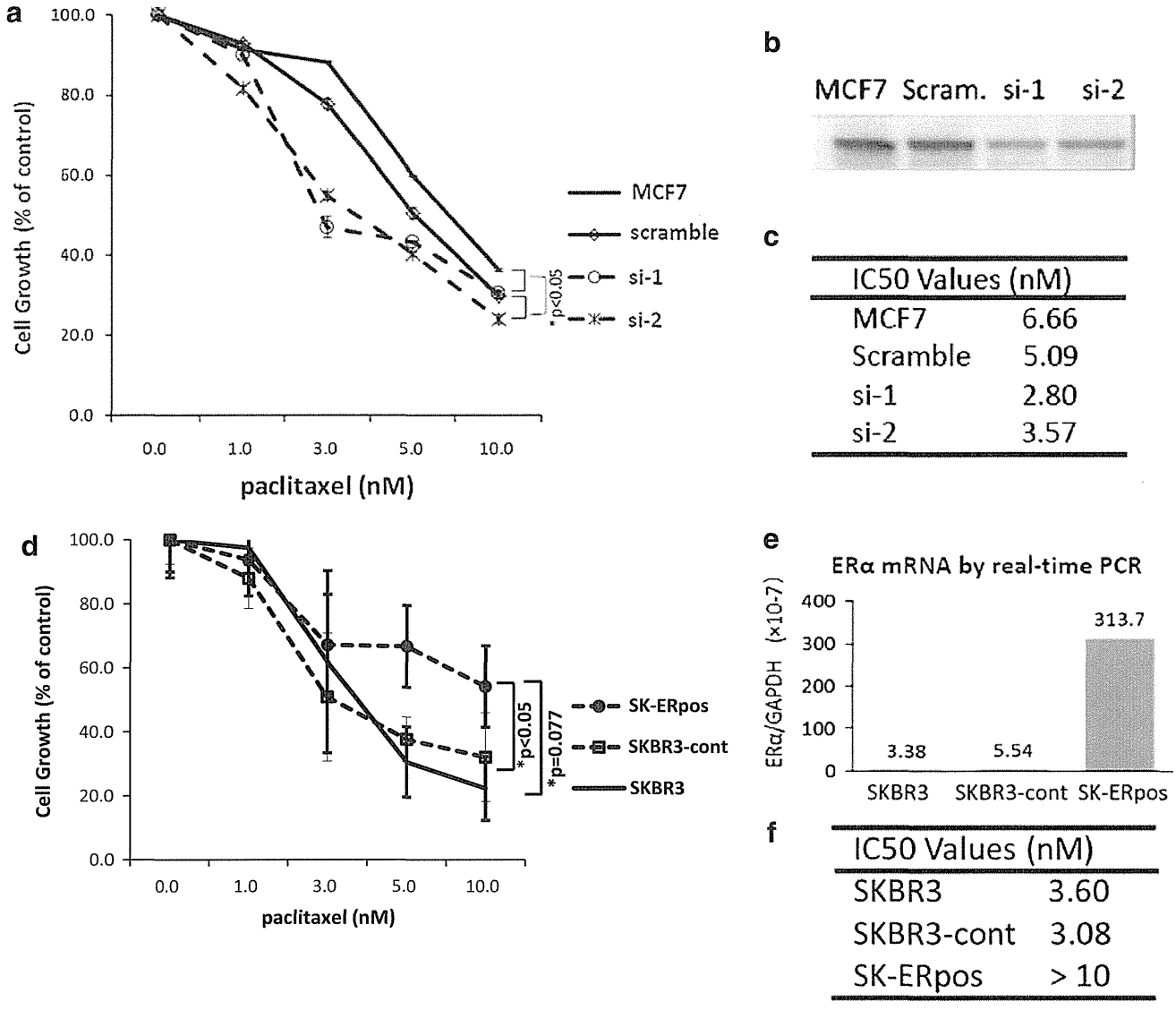

Fig. 2 Knockdown of ERα in MCF-7 cells increased sensitivity to paclitaxel and over-expression of ERα decreased sensitivity to paclitaxel in SKBR3 cell lines. **a** Growth inhibition by paclitaxel in scramble and ERα siRNA-treated MCF-7 cells (si-1 and si-2), and parent MCF-7 cells. Cells were treated with paclitaxel at the indicated concentrations for 3 days in the normal culture medium supplemented with 10% FCS, which had not been deprived of estrogen, and the cell number was counted. The data shown as a percentage of the control are means ± SD of duplicate determinations of two independent experiments. *Bars* indicate SD. $*P < 0.05$ for ERα knockdown in MCF-7 versus parent and scramble MCF-7 by ANOVA. **b** Knockdown of ERα in MCF-7–si-1 and MCF-7–si-2 cells. ERα protein levels were assessed by western blotting. **c** IC50 values of paclitaxel in **a**. Knockdown of ERα rendered MCF-7 cells more sensitive to paclitaxel. **d** Growth inhibition by paclitaxel in parent, control, and ERα over-expressing SKBR3 cell lines. Cells were treated with paclitaxel at the indicated concentrations for 3 days and the cell number was then counted. The data shown as a percentage of the control are the means ± SD of duplicate determinations. *Bars* indicate SD. **e** ERα mRNA levels determined by real-time RT-PCR in parent, control, and ERα over-expressing SKBR3 cell lines. ERα mRNA levels were normalized relative to GAPDH. **f** IC50 values of paclitaxel shown in **d**

induced growth inhibition compared with parent MCF-7–E10 cells (Fig. 4a).

Next, we measured estrogen-dependent ERα activity via GFP expression using our automated system [22, 25]. ERα activities in paclitaxel-resistant MCF-7–E10 cells were measured after culture with estrogen for 3 days. Estrogen-dependent ERα activity in paclitaxel-resistant MCF-7–E10 clones was higher than that in parent MCF-7–E10 cells (Fig. 4b).

We examined the relationship between the presence of the ER and acetylation of HDAC6. Figure 4c shows ERα mRNA expression to be increased in paclitaxel-resistant

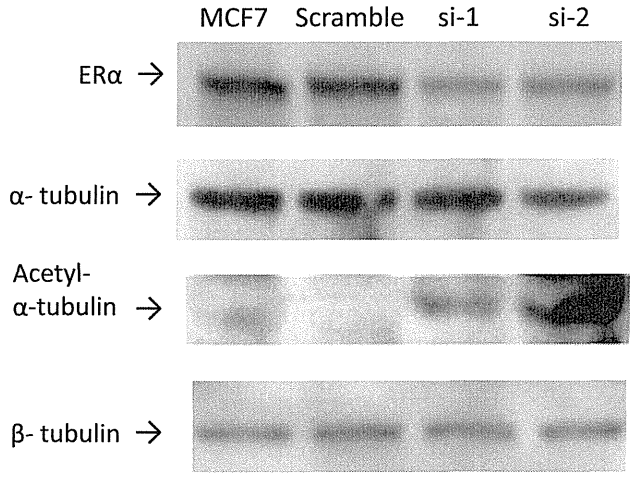

Fig. 3 SiRNA knockdown of ERα decreased deacetylation of α-tubulin. Western blotting of ERα, α-tubulin, and acetylated α-tubulin in scramble and ERα-siRNA-treated MCF-7 cells and parent cells. α-Tubulin was more acetylated in ERα-knockdown si-1 and si-2 MCF-7 cells

MCF-7–E10 cells. Figure 4d shows HDAC6 mRNA levels to be significantly increased in paclitaxel-resistant MCF-7–E10 cells. These results suggest that paclitaxel-resistant clones could be developed by increasing in HDAC6 expression via regulation of ERα expression.

Discussion

Patients with advanced breast cancer are at risk of tumor recurrence and death. Chemotherapy, especially NAC, has become the standard treatment for these patients to eradicate distant micrometastatic disease. In addition, NAC provides an ideal model for evaluating the role of biological markers such as the ER as prognostic factors. However, in the field of chemotherapy, there are few reports on predictive molecular markers for these patients. Therefore, we investigated the significance of ERα expression in the sensitivity of breast cancer to paclitaxel.

First, we compared clinical responses to taxanes in NAC between ER-positive and ER-negative cases. The results shown in Table 1 clearly demonstrate that ER-negative tumors are more sensitive to taxanes than ER-positive tumors. This result is consistent with previously reported observations for adjuvant chemotherapy.

Next, we analyzed the relationship between ERα expression and chemosensitivity to paclitaxel by comparing pCR, cCR, and cPR cases. We observed a discrepancy between the expression level of ER protein and ER transcription activity. This might be responsible for the difficulty choosing of the most appropriate chemotherapy. With the IHC method, 37.5 and 42.9% of ERα-positive and ERα-negative breast cancers, respectively, were sensitive to paclitaxel (Fig. 1a). With the ERE–GFP method, 23.0 and 50.0% of high and low ER activity breast cancers, respectively, were sensitive to paclitaxel (Fig. 1b), suggesting that ERα-nonfunctional breast cancers are sensitive to paclitaxel. The ERE–GFP method differs significantly from the IHC method in the selection of ERα-nonfunctional cases.

Among 31 cases, we next analyzed 24 ERα positive breast cancers using the IHC method as shown in Fig. 1c. Twelve cases (50.0%) showed high ER activity when evaluated by the ad-ERE–GFP method. The cases with low ER activity showed a higher pCR rate (41.7%) than those with high ER activity (16.7%). Our results clearly show that, like ERα-negative breast cancer, the cases for which ERα protein could be detected but did not function as a transcription factor were liable to achieve pCR. Our result suggests that the ad-ERE–GFP method might more accurately predict the efficacy of chemotherapy than the IHC method, although studies with more samples are needed.

To examine the relationship between the presence versus absence of ERα expression and sensitivity to paclitaxel, we used two breast cancer cell lines, ERα-positive and HER2-negative MCF-7 cells and ERα-negative and HER2-positive SKBR3 cells. For each cell line, we analyzed the effects of altering in ERα expression levels on sensitivity to paclitaxel, and found that ERα expression directly affects the sensitivity to paclitaxel regardless of HER2 expression (Fig. 2a–f).

Finally, we analyzed the function of ERα in paclitaxel-resistant MCF-7 clones established in this study (Fig. 4a). Estrogen-dependent ERα activity in these PAC-resistant MCF-7 clones was higher than that in parent MCF-7 cells (Fig. 4b). Increased ERα expression is one mechanism whereby they showed high ERα activity (Fig. 4c), and expression of HDAC6 (Fig. 4d), identified as a target gene of the estrogen signal in our previous study [16], was also increased in these cells. These results that development of resistance to paclitaxel is associated with increased ERα expression and its activity in MCF-7 cells. We inferred from these observations that ER-positive breast cancer subjected to long-term treatment with paclitaxel chemotherapy becomes more sensitive to anti-estrogen drugs. We intend to study the interactions of paclitaxel and anti-estrogen drugs in treating ER-positive breast cancer further.

Taxanes act by shifting the dynamic equilibrium between tubulin and microtubules to the direction of microtubule assembly [23, 26]. Tubulin is the major component of microtubules, which play a critical role in cell migration, cell morphology, cell–cell interactions, and tumor interactions. Hubbert et al. reported that HDAC6 has been shown to deacetylate tubulin, target of taxane [18], and Palasso et al. suggested HDAC6 to be an important regulator of cell

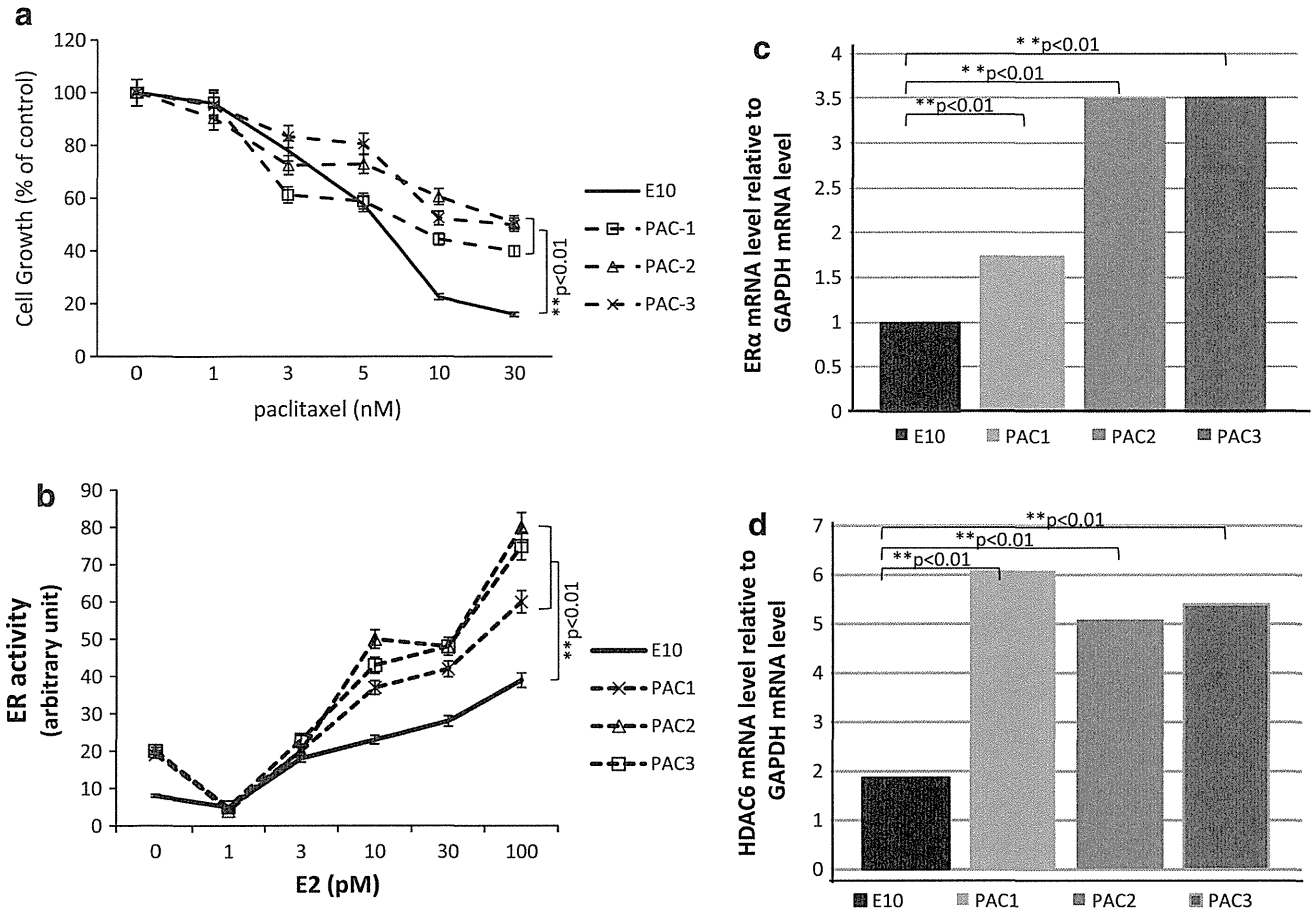

Fig. 4 Estrogen-dependent ER activities increased in paclitaxel-resistant MCF-7–E10 cells. **a** Resistance to paclitaxel-induced growth inhibition in PAC-1, PAC-2, and PAC-3–MCF-7–E10 cells. Cells were treated with paclitaxel at the indicated concentrations for 3 days and the cell number was then counted. The data are shown as a percentage of the means ± SD of duplicate determinations. *Bars* indicate SD. **b** Estrogen-dependent ER activities in PAC-1, PAC-2, and PAC-3–MCF-7–E10 cells. After 3 days of culture in estrogen-deficient medium, the cells were treated with estrogen at the indicated concentrations for 3 days. ER activities were evaluated by expression of GFP using the automated image analysis system. $*P < 0.01$ for ERα activity in PAC-1, PAC-2, and PAC-3–MCF-7–E10 versus that in parent cells, by ANOVA. **c** ERα mRNA expression was increased in paclitaxel-resistant MCF-7–E10 cells (PAC-1, PAC-2, and PAC-3 cells). $*P < 0.01$ for ERα activity in PAC-1, PAC-2, and PAC-3–MCF-7–E10 versus that in parent cells, by ANOVA. **d** HDAC6 mRNA expression was increased in paclitaxel-resistant MCF-7–E10 cells (PAC-1, PAC-2, and PAC-3 cells). $*P < 0.01$ for ERα activity in PAC-1, PAC-2, and PAC-3–MCF-7–E10 versus that in parent cells, by ANOVA

motility, especially in ER-positive breast cancer [26]. In addition, in our previous study, HDAC6 overexpression caused tubulin deacetylation and enhanced the motility of breast cancer cells, while the inhibition of HDAC6 activity reduced motility [27]. Figure 3 shows α-tubulin to be more acetylated in ERα-knockdown MCF-7 cells and that ERα expression induced deacetylation of α-tubulin. The expression of HDAC6 was decreased in ERα-knockdown MCF-7 cells and increased in ERα-overexpressing SKBR3 cells (data not shown). HDAC6 and ERα mRNA expressions were also increased in paclitaxel-resistant MCF-7–E10 cells as described above. These results led us to hypothesize that ERα is involved in the regulation of tubulin, one of the targets of paclitaxel via HDAC6 expression.

Figure 5 is a schematic diagram showing how our findings in the present and previous studies are linked to the pharmacological properties of paclitaxel. In breast cancers having functional ERα, estrogen-induced HDAC6 expression caused tubulin deacetylation, which decreased the efficacy of paclitaxel via destabilization of tubulin. In other words, estrogen signals directly influence the effects of paclitaxel, which targets tubulin formation. Inhibition of HDAC6 might increase sensitivity to paclitaxel in ER-positive breast cancer, and we aim to establish more effective agents by developing HDAC6-targeting therapy.

This is the first report, to our knowledge, to provide evidence that ERα directly regulates tumor sensitivity to paclitaxel, primary via estrogen-induced deacetylation of

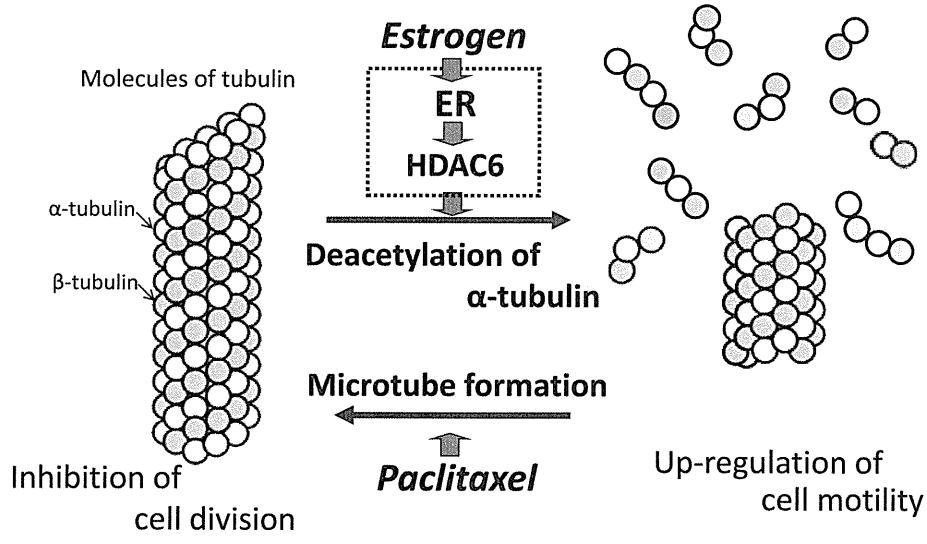

Fig. 5 Paclitaxel and estrogen show the opposite effects on tubulin assembly. Paclitaxel inhibits the proliferation of tumor cells via induction of microtubule formation, whereas estrogen induces deacetylation of α-tubulin via HDAC6. Our previous study has showed that deacetylation of α-tubulin causes up-regulation of cell motility [27]

tubulin involving HDAC6. These findings provide a new understanding of the mechanisms underlying the roles of the ER and chemotherapeutic agents such as paclitaxel, as well as insights into developing new molecular targets for breast cancer treatment.

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ORIGINAL ARTICLE

Aberrant expression of tumor suppressors CADM1 and 4.1B in invasive lesions of primary breast cancer

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Abstract
Background The tumor suppressor genes *CADM1/TSLC1* and *DAL-1/4.1B* are frequently inactivated by promoter methylation in non-small cell lung cancer. The proteins they encode, CADM1 and 4.1B, form a complex in human epithelial cells and are involved in cell–cell adhesion.
Methods Expression of CADM1 and 4.1B proteins was examined by immunohistochemistry in 67 primary breast cancer and adjacent noncancerous tissues. CADM1 and 4.1B messenger RNA (mRNA) was detected by reverse-transcription polymerase chain reaction (RT-PCR). The methylation status of the *CADM1* and *4.1B* promoters was determined quantitatively by bisulfite treatment followed by pyrosequencing.
Results CADM1 and 4.1B protein signals were detected along the cell membrane in normal mammary epithelia. By contrast, 47 (70%) and 49 (73%) of 67 primary breast cancers showed aberrant CADM1 and 4.1B staining, respectively. Aberrant CADM1 staining was more frequently observed in pT2 and pT3 tumors and for stages II and III ($P = 0.045$ and $P = 0.020$, respectively), while aberrant 4.1B staining was more often observed in tumors with lymph node metastasis, for pT2 and pT3 tumors, and for stages II and III ($P = 0.0058$, $P = 0.0098$, and $P = 0.0007$, respectively). Furthermore, aberrant CADM1 and 4.1B expression was preferentially observed in invasive relative to noninvasive lesions from the same specimen ($P = 0.036$ and $P = 0.0009$, respectively). Finally, hypermethylation of *CADM1* and *4.1B* genes was detected in 46% and 42% of primary breast cancers, respectively.
Conclusions Our findings suggest that aberrant CADM1 and 4.1B expression is involved in progression of breast cancer, especially in invasion into the stroma and metastasis.

Keywords CADM1 · 4.1B · Tumor suppressor protein · Breast cancer · Methylation

Introduction

Breast cancer is the most common malignancy in women, and its incidence has been increasing in recent years in Japan. Many clinical and pathological factors are routinely used to categorize patients with breast cancer in order to assess prognosis and determine the most appropriate therapy. Patient age, tumor size, lymph node metastasis, nuclear grade, lymphovascular invasion, expression status of hormone receptors including the estrogen receptor (ER) and progesterone receptor (PgR), and status of human epidermal growth factor receptor 2 (Her2) have been recognized as major prognostic factors. Although these factors are mostly useful, more effective indicators are required to determine prognosis precisely and to evaluate the risk of recurrence in patients with breast cancer. Recently,

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additional new techniques, such as gene expression profiling [1], have been developed to improve subclassification of breast cancer and to determine the most appropriate therapy as well. However, these mRNA-based diagnostic approaches have several disadvantages in practical use.

The tumor suppressor gene *CADM1* (*Cell adhesion molecule 1*)/*TSLC1* (*Tumor suppressor in lung cancer 1*) was identified on chromosome 11q23.2 by functional complementation of tumorigenicity of non-small cell lung cancer (NSCLC) cells [2–5]. *CADM1* encodes an immunoglobulin superfamily molecule that is involved in cell–cell adhesion in a variety of human epithelia, including those of the mammary gland [5]. Previous studies have revealed that *CADM1* is frequently inactivated in various cancers, such as lung [2, 3], prostate [6], liver, pancreas [4], and breast cancer [7], especially in those with invasion and metastasis to lymph nodes and distant organs. Goto et al. [15] reported that CADM1 expression was preferentially lost in invasive lesions relative to noninvasive lesions of lung adenocarcinoma. Hypermethylation of the *CADM1* promoter has been shown to be one of the main mechanisms to inactivate the gene in these cancers [2–4, 6–8].

The tumor suppressor gene *DAL-1/4.1B* (*differentially expressed in adenocarcinoma of the lung*) was shown to be located on chromosomal fragment 18p11.3 and was inactivated in lung, breast, and brain tumors [9, 10]. The protein it encodes, 4.1B, belongs to the protein 4.1 superfamily of scaffold proteins. Yageta et al. [10, 11] reported that CADM1 interacts with the actin filament through 4.1B at the cell–cell attachment site, where complex formation of CADM1 and 4.1B is dependent on the integrity of the actin cytoskeleton, and that CADM1 and 4.1B are responsible for stable adhesion between adjacent cells. It has also been reported that loss of 4.1B expression and methylation of the *4.1B* promoter are involved in development and progression of NSCLC, providing a possible indicator of poor prognosis [12]. Thus, the CADM1–4.1B cascade appears to be involved in cell–cell attachment, while functional loss of CADM1 and/or 4.1B would play a role in invasion and metastasis of tumor cells in advanced stages.

In breast cancer, promoter methylation of *CADM1* was found in 33% of surgically resected tumors using bisulfate sequencing analysis [7]. Furthermore, loss of 4.1B expression was observed in 83% of breast cancer cell lines using Western blotting [13]. Previous studies have also shown that *CADM1* and/or *4.1B* methylation correlates with tumor grade and hormone receptor status in breast cancer [14].

In the present study, we examined expression of CADM1 and 4.1B in 67 primary breast cancer specimens and the methylation status of *CADM1* and *4.1B* promoters in 6 breast cancer cell lines and 50 primary breast cancers. We then examined whether expression of CADM1 and 4.1B as well as their methylation status correlated with the clinicopathological factors of primary breast cancers in order to investigate the significance of CADM1 and 4.1B in breast tumorigenesis. Our results suggest that loss of CADM1 and 4.1B expression is involved in development and progression of breast cancer, especially in invasion and metastasis.

Materials and methods

Tissue samples and cell lines

A breast cancer cell line, MCF7, was obtained from the Human Science Research Resources Bank (Osaka, Japan). Four breast cancer cell lines (SK-BR-3, MDA-MB-361, MDA-MB-231, and BT474) were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-453, another breast cancer cell line, was obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were cultured according to the suppliers' recommendations. A series of 67 primary invasive breast cancers without systemic drug therapy before surgery were obtained from patients who underwent surgery at the Department of Breast and Endocrine Surgery of Juntendo University, Tokyo during the period 2006–2010. These included 65 invasive ductal carcinomas (8 papillotubular, 21 solid-tubular, and 36 scirrhous carcinomas) and 2 invasive lobular carcinomas. In 39 of 67 tumors, both invasive and noninvasive lesions were present within the same specimen. Informed consent was obtained from patients before surgery for specimens to be used for this research. As summarized in Table 1, the average age of the patients at diagnosis was 56.9 years, ranging from 32 to 82 years. Of 67 patients, 24 patients were under 50 years of age, while 43 patients were over 50 years of age at time of diagnosis. Twenty-one cases had lymph node metastasis. Seventeen cases had lymphovascular invasion. Fifty-six cases had positive ER and/or PgR hormone receptor expression. Ten cases had Her2 overexpression. Twenty-three, 37, and 7 cases were diagnosed as nuclear grade 1, 2, and 3, respectively, whereas 31, 33, and 3 cases were diagnosed as pT stage 1, 2, and 3, respectively. Twenty-three cases were categorized as pathological stage I, 30 cases as stage IIA, 10 cases as stage IIB, and 4 cases as stage III. Fifty of 67 tumors with diameter equal to or greater than 1.5 cm were obtained surgically, and tissue samples were frozen immediately and stored at −80°C. This study was approved by the ethics committees of Juntendo University and the Institute of Medical Science, The University of Tokyo. All samples were diagnosed and classified according to the World Health Organization (WHO) grading system and the General Rules for Clinical

Table 1 Clinicopathological characteristics of patients with primary breast cancer and expression of CADM1 and 4.1B

	n	CADM1 (%)	*P* value	4.1B (%)	*P* value
		Negative staining		Negative staining	
Total	67	47 (70)		49 (73)	
Age (years)					
<50	24	20 (80)	NS	19 (79)	NS
≥50	43	27 (63)		30 (70)	
Lymph node metastasis					
0	46	29 (63)	NS	29 (63)	
1–3	17	15 (88)		16 (94)	0.0058
≥4	4	3 (75)		4 (100)	
Lymphovascular invasion					
−	50	32 (64)	NS	33 (66)	NS
+	17	15 (88)		16 (94)	
Hormone receptor status					
+/+ or +/−	56	41 (73)	NS	40 (71)	NS
−/−	11	6 (55)		9 (82)	
Her2					
Negative	57	39 (68)	NS	41 (72)	NS
Positive	10	8 (80)		8 (80)	
NS					
1	23	15 (65)	NS	15 (65)	NS
2	37	26 (70)		29 (78)	
3	7	6 (86)		5 (71)	
pT stage[a]					
1	31	18 (58)		18 (58)	
2	33	27 (82)	0.045	28 (85)	0.0098
3	3	2 (67)		3 (100)	
Pathological stage[b]					
I	23	12 (52)		11 (48)	
II	40	32 (80)	0.020	34 (85)	0.0007
III	4	3 (75)		4 (100)	

P values calculated using the χ^2 test

NS not significant

[a] Size of tumor classified according to the tumor–node–metastasis (TNM) pathological classification

[b] According to the TNM pathological classification

and Pathological Recording of Breast Cancer established by the Japanese Breast Cancer Society [16].

Immunohistochemical (IHC) study

Antibody against CADM1 was generated by immunizing rabbits with the C-terminal peptide of CADM1 as described previously [17]. Antibody against 4.1B, sc-10046, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Formalin-fixed, paraffin-embedded tissue sections of 8 μm were examined. After deparaffinization and dehydration through graded alcohols and xylene, antigen unmasking was performed using Histofine pH 9 (NichireiI, Tokyo, Japan) in an autoclave for 20 min at 121°C, followed by cooling to room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 30 min. After rinsing and blocking with 5% normal donkey serum, the sections were incubated overnight at 4°C with primary antibodies, including anti-CADM1 (diluted at 1:500) and anti-4.1B (diluted at 1:300) and then washed and incubated for 1 h at room temperature with the secondary antibody [DAKO EnVision kit/HRP

(AEC); DAKO, Hamburg, Germany]. Diaminobenzidine [DAKO EnVision kit/HRP (DAB)] was used for detection. Finally, the sections were counterstained with hematoxylin.

The signals of CADM1 and 4.1B proteins were detected along the membrane in normal mammary epithelial cells. Cancer cells with membrane staining of CADM1 or 4.1B were defined as having membrane expression, while cells with no or greatly reduced signals were defined as having low expression. Cancer cells with cytoplasmic immunoreactivity of CADM1 and 4.1B but no membrane staining were defined as showing aberrant expression. We calculated the percentage of cancer cells with membrane expression in the entire area of invasive and noninvasive lesions and scored the tumors as 0 (0–10% cells with membrane expression), 1 (11–30%), 2 (31–60%), and 3 (61–100%). Finally, we defined tumors with scores 1, 2, and 3 as positive staining and tumors with score 0 as negative staining for CADM1 or 4.1B expression.

Reverse-transcription PCR

Genomic DNA and total cellular RNA were extracted from cell lines, frozen breast cancer specimens, and noncancerous breast tissues using an AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Expression of CADM1 and 4.1B mRNA in breast cancer cell lines was detected by reverse-transcription PCR (RT-PCR). An aliquot of total cellular RNA (1 μg) was reverse-transcribed using the Transcriptor first-strand complementary DNA (cDNA) synthesis kit (Roche, Switzerland). PCR was carried out using KOD FX (TOYOBO Life Science, Osaka, Japan). Primer sequences used for RT-PCR are shown in Supplementary Table 1. The expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was examined as an internal control to confirm RNA integrity.

Pyrosequencing analysis

For the methylation analysis, 600 ng genomic DNA was subjected to bisulfite conversion using a MethylCode bisulfite conversion kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The purified bisulfate-converted samples were eluted in a 10 μl volume and stored at −20°C. An aliquot of bisulfate-treated DNA (60 ng) was amplified by PCR with biotinylated primers and Platinum Taq DNA polymerase (Invitrogen). The sequences of all primers used for PCR as well as the reaction conditions are presented in Supplementary Table 1. The PCR products were purified using a Qiaquick PCR purification kit (QIAGEN), and single-strand DNA was prepared using Dynabeads M280 streptavidin (Invitrogen). Pyrosequencing was performed with single-strand DNA as a template, the Exo-Klenow fragment (Ambion, USA), and the single-strand binding protein (New England Biolabs, Ipswich, MA, USA) using a small DNA analyzer (Hitachi, Ltd., Central Research Laboratory, Tokyo, Japan). The methylation rate of the cytosine residue was quantified as (peak of the signals of methylated C)/(peak of methylated C + peak of unmethylated C) using the software provided for the Handylumi analyzer (Hitachi, Ltd.). The average methylation rates of 5 CpG sites that are located at −497, −480, −467, −440, and −433 bp from the first nucleotide at the translational start site of the *CADM1* gene and 8 CpG sites that are located at −158, −154, −152, −150, −139, −128, −117, and −115 bp from the first nucleotide in exon 1 of the *4.1B* gene were calculated as the methylation rate of each gene. For *CADM1*, methylation rates of more than 20%, between 5% and 20%, and less than 5% were defined as hypermethylation, partial methylation, and nonmethylation, respectively. For *4.1B*, methylation rates of more than 20%, between 10 and 20%, and less than 10% were defined as hypermethylation, partial methylation, and nonmethylation, respectively. Methylation rates of *CADM1* and *4.1B* were less than 5% and 10%, respectively.

Statistical analysis

All statistical analyses were carried out using Fisher's exact tests or the Pearson χ^2 test. Results were considered significant at P value <0.05.

Results

Loss of CADM1 and 4.1B protein expression in primary breast cancer

Expression of CADM1 and 4.1B proteins was examined by immunohistochemistry (IHC) in 67 primary breast cancers and 39 corresponding noncancerous breast tissues. Signals of CADM1 and 4.1B proteins were detected on the cell membrane at the cell–cell attachment sites in normal luminal epithelial cells but not in normal myoepithelial cells or interstitial cells (Fig. 1). On the other hand, 47 of 67 (70%) tumors showed negative staining with more than 90% of cancer cells presenting low or aberrant expression of CADM1 protein when examined by IHC (Fig. 2). Clinicopathological examination of the tumors showed that the incidence of negative CADM1 staining was significantly higher in tumors with diameter greater than 2 cm (pT2 and pT3; 29 of 36, 81%) than in those with diameter less than 2 cm (pT1; 18 of 31, 58%) ($P = 0.045$) (Table 1). Negative CADM1 staining was also observed at significantly higher incidence in tumors with pathological

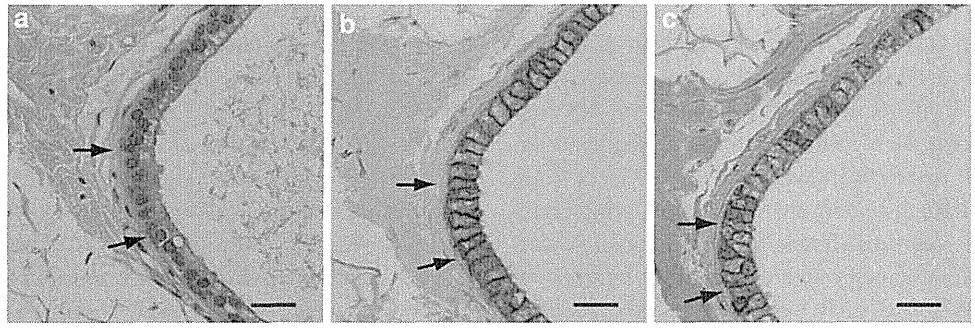

Fig. 1 Immunohistochemical analysis of CADM1 and 4.1B in normal mammary duct epithelial cells. **a** Hematoxylin and eosin (HE) staining. **b** and **c** Immunohistochemical staining of CADM1 protein by anti-CADM1 antibody (**b**) and anti-4.1B antibody (**c**). Normal membrane staining of CADM1 and 4.1B proteins is detected in mammary duct epithelia, whereas no staining is observed in normal myoepithelial cells, indicated by *arrows*. *Bars* 20 μm

stages II and III (35 of 44, 80%) than in those with stage I (12 of 23, 52%) ($P = 0.020$) (Table 1). IHC also revealed that expression of 4.1B protein was mostly lost or aberrant in at least 49 of 67 (73%) primary breast cancers. Negative staining of 4.1B protein was preferentially observed in tumors with lymph node metastasis (20 of 21, 95%) relative to those without lymph node metastasis (29 of 46, 63%) ($P = 0.0058$). Negative staining of 4.1B was also observed at significantly higher incidence in tumors with pT2 and pT3 (31 of 36, 86%) than in those with pT1 (18 of 31, 58%) ($P = 0.0098$) or in tumors with pathological stages II and III (38 of 44, 86%) than in those with stage I (11 of 23, 48%) ($P = 0.0007$).

Next, we combined the expression status of CADM1 and 4.1B and divided the 67 tumors into three groups: group 1, tumors with positive staining of both CADM1 and 4.1B; group 2, tumors with negative staining of either CADM1 or 4.1B; group 3, tumors with aberrant expression of both CADM1 and 4.1B (Table 2). Then, we combined groups 2 and 3 with loss of at least one of CADM1 or 4.1B protein. As summarized in Table 2, the numbers of tumors in groups 1, 2, and 3 were 7, 24, and 36, respectively (Table 2). Among the 24 tumors in group 2, 13 tumors expressed CADM1 but not 4.1B, whereas 11 tumors expressed 4.1B but not CADM1. Clinicopathological comparison demonstrated that the tumors from patients aged 50 years or older ($P = 0.037$) and the tumors with advanced pathological stages, II and III ($P = 0.029$), were more preferentially observed in group 2 and 3 tumors relative to group 1 tumors. In addition, no tumors with lymph node metastasis, lymphovascular invasion, nuclear grade 3, and pT stage 3 were found in group 1 tumors, although these scores were not statistically significant. On the other hand, the expression status of the hormone receptors ER or PgR, or that of Her2, and nuclear grade were not associated with the combined expression status of CADM1 and 4.1B. Moreover, no significant difference was observed between group 2 and group 3 tumors in terms of any clinicopathological characters.

Comparative analysis of CADM1 and 4.1B expression in invasive and noninvasive lesions from the same tumor

Thirty-nine out of 67 breast cancer tissues examined in this study were histologically heterogeneous and contained both invasive and noninvasive lesions within the same specimen (Fig. 3a, e). Thus, we next examined the expression status of CADM1 and 4.1B proteins in these lesions. As representatively shown in Fig. 3b, c, f, and g, normal membrane staining of CADM1 and 4.1B was detected in most of the noninvasive lesions. By contrast, CADM1 and 4.1B expression was low or aberrant in invasive lesions (Fig. 3b, d). As summarized in Table 3, 25 out of 39 tumors retained CADM1 expression in noninvasive lesions. Among them, 18 (72%) tumors showed low or aberrant expression of CADM1 in their invasive lesions. On the other hand, 14 of 39 tumors already showed low or aberrant CADM1 expression in noninvasive lesions, and all of them lacked normal CADM1 expression in their invasive lesions. Similarly, 12 out of 24 (50%) tumors expressing 4.1B in noninvasive lesions showed low or aberrant 4.1B expression in their invasive lesions, whereas all 15 tumors showing low or aberrant 4.1B expression in noninvasive lesions lacked normal 4.1B expression in invasive lesions as well.

Promoter methylation of the *CADM1* and *4.1B* genes in primary breast cancers and cell lines

We next examined CADM1 and 4.1B mRNA expression in human breast cancer cell lines by RT-PCR analysis. Of 6 cell lines examined, loss of CADM1 and 4.1B mRNA was observed in 3 and 4 cell lines, respectively (Fig. 4a). Two

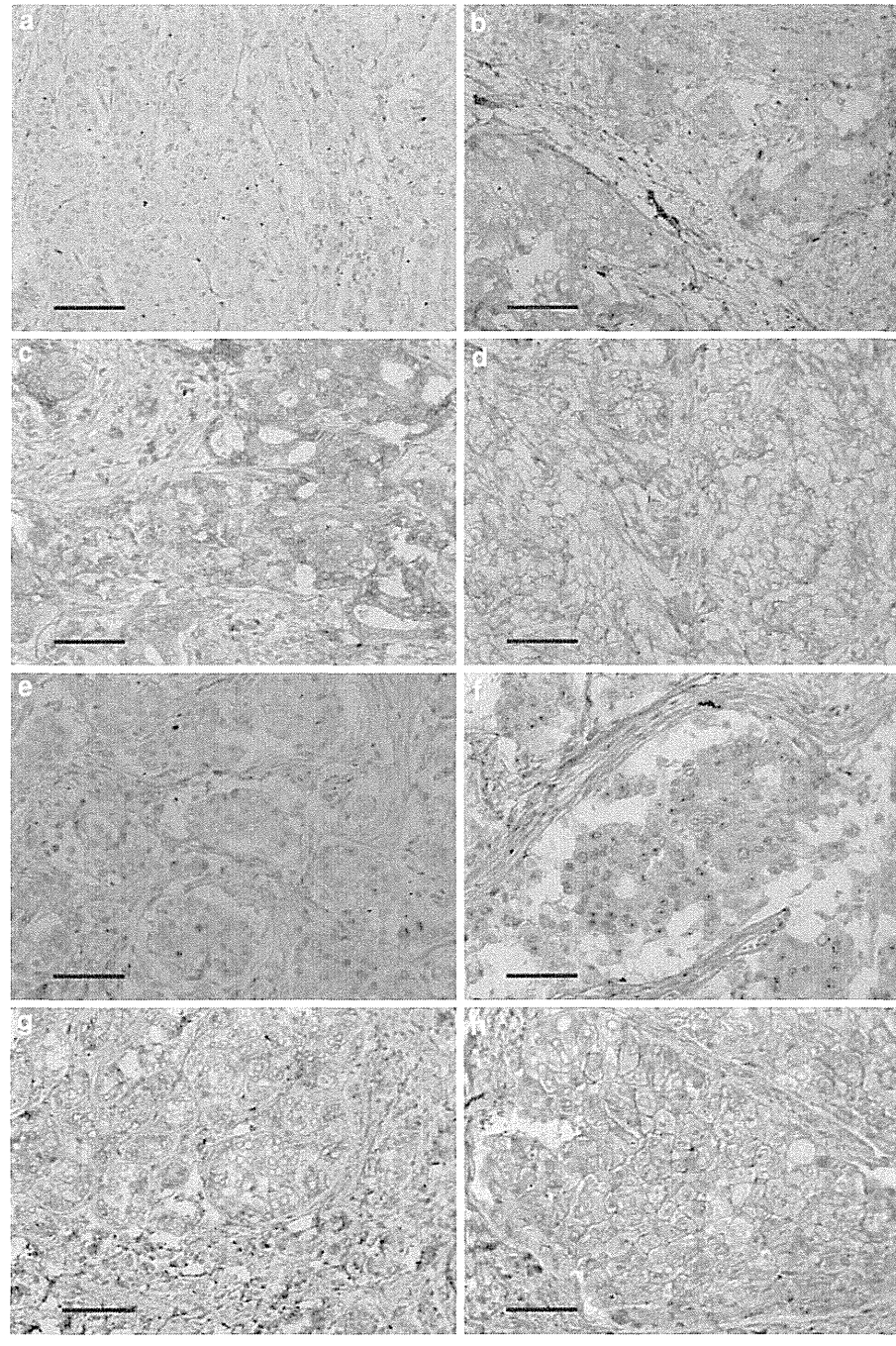

Fig. 2 Immunohistochemical analysis of CADM1 and 4.1B in invasive ductal carcinoma of the breast. Representative images of breast cancers expressing CADM1 (**a**–**d**) and 4.1B (**e**–**h**) are shown. The content of cancer cells with normal membrane staining of CADM1 or 4.1B in tumors with score 1 is 0–10% (**a**, **e**); score 2, 11–30% (**b**, **f**); score 3, 31–60% (**c**, **g**); and score 4, 61–100% (**d**, **h**)

cell lines, MDA-MB-361 and MDA-MB-453, showed loss of expression of both genes, while SK-BR-3 cells retained expression of both genes. Then, using pyrosequencing analysis, we performed quantitative analysis of DNA methylation at 5 and 8 CpG sites with the *CADM1* and *4.1B* gene promoter, respectively (Fig. 4b, c). Significant level of methylation of *CADM1* was detected in two cell lines, MDA-MB-453 and MDA-MB-231, showing loss of CADM1 mRNA (Fig. 4a, b). On the other hand, significant methylation of *4.1B* was observed in five cell lines: BT474, MCF7, MDA-MB-453, MDA-MB-361, and MDA-MB-231 (Fig. 4c). Among these, 4 cell lines, except for MDA-MB-231, lost 4.1B mRNA expression (Fig. 4a).

The methylation status of the *CADM1* and *4.1B* was subsequently analyzed in 50 primary breast cancers using pyrosequencing. *CADM1* and *4.1B* methylation was observed in 23 (46%) and 21 (42%) of 50 tumors, respectively (Table 4). Among them, most of the tumors (18 of 23 and 19 of 21) showed loss or greatly reduced expression of CADM1 and 4.1B proteins by IHC,

Table 2 Clinicopathological characteristics of patients with primary breast cancer and combined state of CADM1 and 4.1B expression

	n	Group 1 CADM1 (+) 4.1B (+)	Groups 2 + 3	*P* value	Group 2 CADM1 (−) or 4.1B (−)	Group 3 CADM1 (−) 4.1B (−)
	67	7 (10)	60 (90)		24 (36)	36 (54)
Age (years)						
<50	24	0	24 (100)		9 (38)	15 (62)
≥50	43	7 (16)	36 (84)	0.037	15 (35)	21 (49)
Lymph node metastasis						
0	46	7 (15)	39 (85)		20 (43)	19 (42)
1–3	17	0	17 (100)	NS	3 (18)	14 (82)
≥4	4	0	4 (100)		1 (25)	3 (75)
Lymphovascular invasion						
−	50	7 (14)	43 (86)		21 (42)	22 (44)
+	17	0	17 (100)	NS	3 (18)	14 (82)
Hormone receptor status						
+/+ or +/−	56	6 (11)	50 (89)		19 (34)	31 (55)
−/−	11	1 (10)	10 (90)	NS	5 (45)	5 (45)
Her2						
Negative	57	6 (11)	51 (89)		22 (39)	29 (50)
Positive	10	1 (10)	9 (90)	NS	2 (20)	7 (70)
Nuclear grade						
1	23	3 (13)	20 (87)		10 (43)	10 (43)
2	37	4 (11)	33 (89)	NS	11 (30)	22 (59)
3	7	0	7 (100)		3 (43)	4 (57)
pT stage[a]						
1	31	5 (16)	26 (84)		16 (52)	10 (32)
2	33	2 (6)	31 (94)	NS	7 (21)	24 (73)
3	3	0	3 (100)		1 (33)	2 (67)
Pathological stage[b]						
I	23	5 (22)	18 (78)		13 (56)	5 (22)
II	40	2 (5)	38 (95)	0.029	10 (25)	28 (70)
III	4	0	4 (100)		1 (25)	3 (75)

P values calculated using the χ^2 test

NS not significant

[a] Size of tumor was classified according to the TNM pathological classification

[b] According to the TNM pathological classification

respectively (Table 4). On the other hand, about a half of tumors with negative staining of CADM1 (19 of 37) or 4.1B (19 of 38) showed an unmethylated promoter in each gene.

Discussion

Expression of CADM1 and 4.1B proteins was investigated in 67 primary tumors by IHC analysis. Low or aberrant expression of CADM1 and 4.1B was observed in 70% and 73% of primary breast cancers, respectively (Table 1). Statistical analysis demonstrated that low or aberrant expression of CADM1 in tumors was significantly associated with advanced pT stages of pT2 and pT3 and advanced pathological stages of II and III (Table 1). On the other hand, low or aberrant expression of 4.1B in tumors was significantly associated with lymph node metastasis, advanced pT stages of pT2 and pT3, and advanced pathological stages of II and III (Table 1). In addition, when we combined the expression status of CADM1 and 4.1B, tumors lacking at least one of CADM1 or 4.1B expression tended to show more malignant pathological features than tumors expressing both CADM1 and 4.1B. These results suggest that dysfunction of the CADM1–4.1B cascade plays a role in progression of primary invasive breast

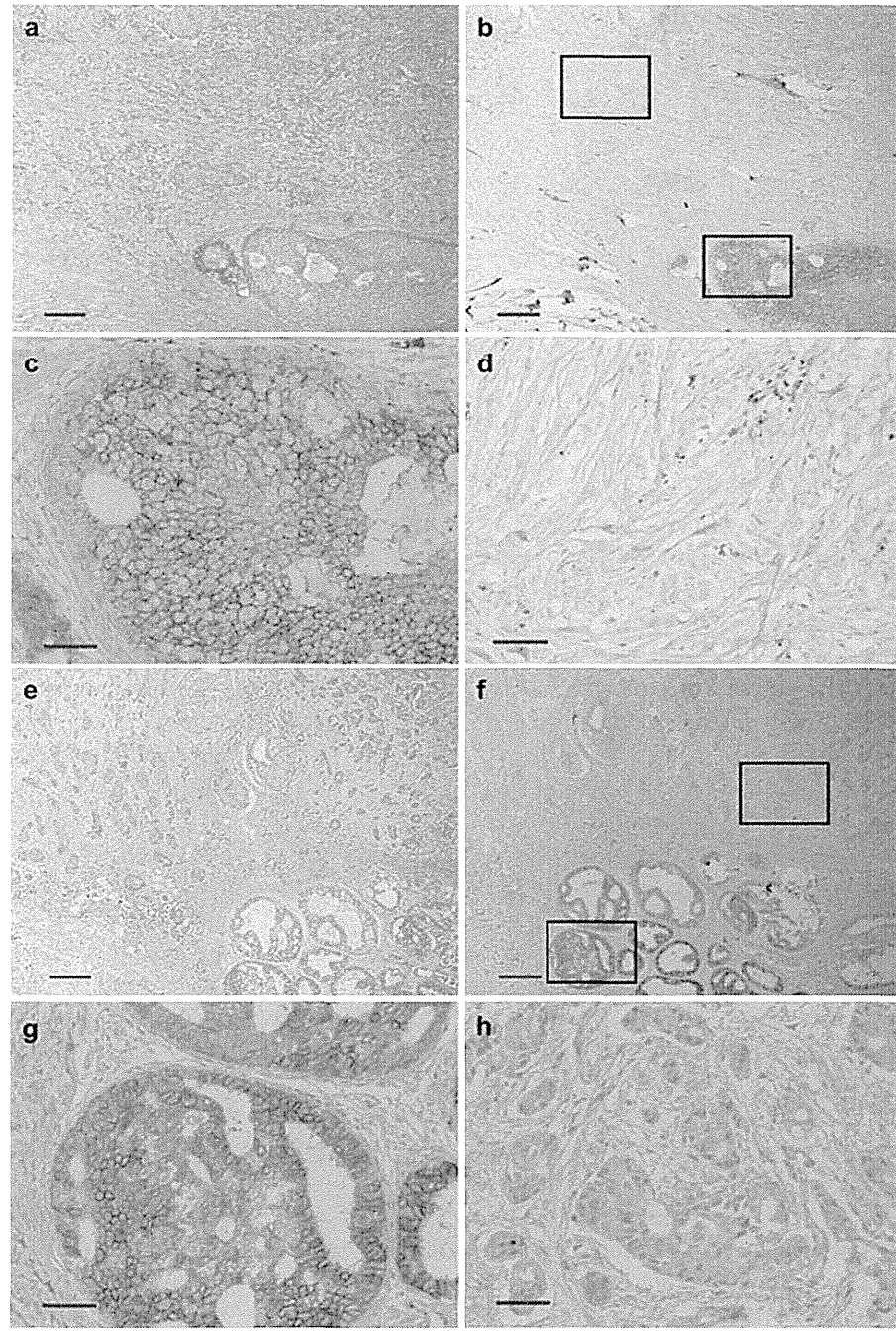

Fig. 3 Immunohistochemical analysis of CADM1 and 4.1B in invasive and noninvasive lesions of primary breast cancer. Representative images with HE staining (**a** and **e**) and stained with anti-CADM1 antibody (**b–d**) and with anti-4.1B antibody (**f–h**) are shown. Noninvasive lesions (**c** and **g**) and invasive lesions (**d** and **h**) are shown at high magnification from the same specimens (**b** and **f**). *Bars* 200 μm (**a**, **b**, **e**, and **f**) and 50 μm (**c**, **d**, **g**, and **h**)

cancer. On the other hand, no difference in clinicopathological features was detected between the tumors with low or aberrant expression of either CADM1 or 4.1B (group 2) versus both CADM1 and 4.1B (group 3). This could be consistent with previous findings that CADM1 and 4.1B act in the same cascade by interacting with each other [10]. In this connection, comparison of incidence in tumors with normal or aberrant expression of CADM1 and 4.1B proteins suggests that aberrant expression of CADM1 and 4.1B occurred independently.

Involvement of dysfunction of the CADM1–4.1B cascade in breast cancer invasion was also supported by comparative analysis of CADM1 and 4.1B expression in invasive and noninvasive lesions within the same tumors from 39 breast cancer patients (Fig. 3). As shown in Table 3, 72% (18 of 25) of tumors expressing CADM1 and 50% (12 of 24) of tumors expressing 4.1B in noninvasive lesions showed selective loss of normal expression of these proteins in their invasive lesions. Inversely, no tumor was observed that showed aberrant expression of CADM1 or

Table 3 Preferential loss of CADM1 and 4.1B expression in invasive lesions of primary breast cancer

Non-Invasive lesions	Invasive lesions: CADM1 (+)	Invasive lesions: CADM1 (-)	total	*P*-value
CADM1 (+)	7	18	25	
CADM1 (-)	0	14	14	0.036
total	7	32	39	

	4.1B (+)	4.1B (-)		
4.1B (+)	12	12	24	
4.1B (-)	0	15	15	0.0009
total	12	7	39	

P values calculated using the χ^2test

4.1B in noninvasive lesions but retained normal membrane expression of these proteins in invasive lesions. Preferential loss of CADM1 expression in invasive lesions has been also reported in lung adenocarcinoma [15]. These results appear to be consistent with previously reported experimental evidence that CADM1 suppresses epithelial–mesenchymal transition (EMT) and oncogenic signaling [18, 19].

It should be noted that we could identify a special group of tumors retaining expression of both CADM1 and 4.1B by combinatorial expression analysis of these two proteins (group 1 in Table 2). Although only 10% (7 of 67) of tumors were subclassified into this group, these 7 tumors showed neither lymph node metastasis nor lymphovascular invasion, suggesting that this group of tumors shows better prognosis. Inversely, all 21 tumors with lymph node metastasis or all 17 tumors with lymphovascular invasion showed low or aberrant expression of either or both of the CADM1 and 4.1B proteins. It is also important to note that aberrant expression of CADM1 or 4.1B was observed in significant portions of noninvasive lesions [14 of 39 (36%) and 15 of 39 (38%), respectively] (Table 3) as well as in tumors without lymph node metastasis, those without lymphovascular invasion, those with pT1, and those with pathological stage I [39/46 (85%), 43/50 (86%), 26/31 (84%), 18/23 (78%), respectively; Table 2]. Therefore, it is tempting to speculate that tumors with low or aberrant expression of either or both of the CADM1 and 4.1B proteins might have some potential for breast cancer recurrence even if they do not show lymph node metastasis or lymphovascular invasion at time of surgery. Further studies on the prognosis of these cases would be required to prove this hypothesis. It is also noteworthy that all 7 tumors with normal expression of CADM1 and 4.1B proteins were from patients who were more than 50 years old. Considering the possible distinct molecular pathways underlying tumorigenesis between premenopausal and postmenopausal breast cancer, dysfunction of the CADM1–4.1B cascade appears to be a prerequisite for premenopausal breast cancer.

In the present study, promoter methylation of the *CADM1* and *4.1B* genes was also examined to elucidate the molecular mechanism of gene inactivation, because hypermethylation of *CADM1* and *4.1B* and the resultant gene silencing have been reported in various cancers [2, 3, 6–8, 12, 20]. To analyze the methylation status of the gene promoter, we carried out pyrosequencing of the specific fragments within the gene promoter containing several CpG residues after bisulfate treatment. Since pyrosequencing can detect the content of methylcytosine and unmethylated cytosine, which are converted to cytosine and thymine by bisulfite, respectively, it provides a significant advantage in quantifying the level of methylation at the specified CpG sites over various other methods, including bisulfate sequencing and methylation-specific PCR (MSP). Heller et al. [14] used MSP to examine the methylation status of the *CADM1* and *4.1B* genes in breast cancers. Although MSP is a convenient method for assessing methylation status, evaluation of the results of MSP, especially those obtained from surgical specimens, can be difficult and sometimes even misleading because MSP is not quantitative; it only detects the methylation status of a single CpG site per primer and inevitably includes false-positive and/or false-negative results.

Using bisulfate treatment coupled with pyrosequencing, we found strong correlation of promoter methylation with loss of mRNA expression in both *CADM1* and *4.1B* genes in breast cancer cell lines (Fig. 4). We also used this technique to examine the methylation status in primary breast cancer and found that 46% and 42% of tumors showed promoter methylation of the *CADM1* and *4.1B* genes, respectively. It is interesting that most of the tumors with a methylated promoter of *CADM1* (17 of 23) and *4.1B* (19 of 21) showed low or aberrant expression of each

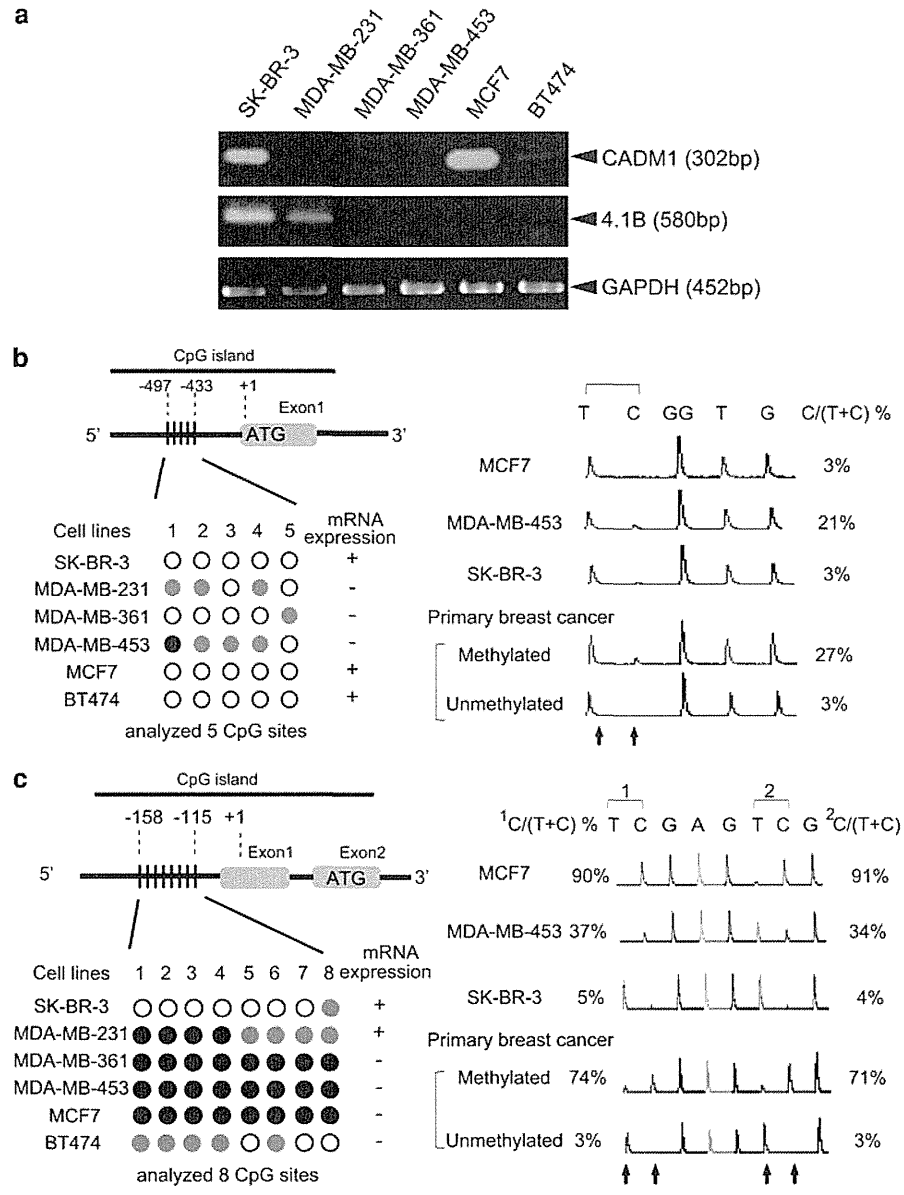

Fig. 4 mRNA expression and promoter methylation analyses of *CADM1* and *4.1B*. **a** RT-PCR analyses of CADM1 and 4.1B mRNA in six breast cancer cell lines. GAPDH serves as an internal control. **b**, **c** *left*, a schematic representation of the CpG island of the *CADM1* (**b**) and *4.1B* (**c**) genes and summary of methylation status. The *gray box* indicates exons. *Vertical bars* indicate CpG sites examined for methylation, while *black*, *gray*, and *white circles* represent hypermethylation, partial methylation, and unmethylation, respectively, as described in "Materials and methods." *Right*, representative results of bisulfate pyrosequencing of a cytosine residue(s) at −497 bp in the *CADM1* promoter (**b**) and at −158 and −154 bp in the *4.1B* promoter (**c**). *Arrows* indicate T and C residues converted by bisulfate from unmethylated C and methylated C residues, respectively

Table 4 Promoter methylation state and protein expression of CADM1 and 4.1B in primary breast cancer

	CADM1 methylation (%)			*4.1B* methylation (%)		
	Unmethylated	Methylated	Total	Unmethylated	Methylated	Total
Protein expression						
Positive	8	5	13	10	2	12
Negative	19	18	37	19	19	38
Total	27 (54)	23 (46)	50 (100)	29 (58)	21 (42)	50 (100)

protein, implying that promoter methylation would inactivate these genes in at least some breast cancers. However, approximately half of the tumors with low or aberrant expression of CADM1 or 4.1B showed no methylation of these genes. This observation may be attributable to the noncancerous cells or noninvasive cancer cells that are inevitably present in tumor tissues. Alternatively, some additional mechanisms other than promoter methylation, including loss of each chromosomal locus, transcriptional repressors, and microRNA (miRNA), may suppress the

expression of CADM1 or 4.1B. Since frequent loss of heterozygosity (LOH) at the chromosomal region, 11q23 and 18p11, on which *CADM1* and *4.1B* are located, has been reported in breast cancer [7, 21, 22], LOH would be one of the possible molecular mechanisms of the second hit to inactivate these genes. Further study will be necessary to clarify this issue.

In conclusion, the present study demonstrated strong correlation between low or aberrant expression of CADM1 and 4.1B proteins and local invasion, lymph node metastasis, and lymphovascular invasion in primary breast cancer. Expression status of CADM1 and 4.1B may serve as a novel significant biomarker predicting postoperative metastasis or recurrence of breast cancer when utilized in combination with other established diagnostic indicators.

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ORIGINAL ARTICLE

The role of chemoradiotherapy in patients with unresectable T4 breast tumors

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Abstract
Purpose Unresectable T4 tumors of the breast are usually treated with systemic therapies, while the role of local therapies remains debatable. This study aims to evaluate the effectiveness of chemoradiotherapy as a part of T4 breast cancer treatment, and to assess the role of local radiotherapies in patients with unresectable T4 breast tumors.

Materials/methods Between February 1998 and June 2010, 39 unresectable T4 breast tumors were treated with chemoradiotherapy at our institutes. Clinical stages included stage IIIB ($n = 15$), stage IIIC ($n = 3$), and stage IV ($n = 21$). Twenty-one cases had undergone previous systemic therapies, whereas the remaining 18 cases reported no history of previous treatment. Radiation doses of 59–66 Gy (median 60 Gy) were administered to the breast in addition to concurrent chemotherapies. Acute adverse effects were assessed on a weekly basis during treatment to 2 weeks after completion of treatment, and were scored by the Common Terminology Criteria for Adverse Events v3.0. Treatment response was assessed at 1 month after completion of chemoradiotherapy. Statistical analysis of survival was calculated using the Kaplan–Meier method.
Results Chemoradiotherapy was completed in all cases. Greater than grade 3 hematological toxicities were observed with regard to lymphocytes (33%), platelets (8%), neutrophils (3%), and hemoglobin (3%). Greater than grade 3 nonhematologic toxicities included chemoradiation dermatitis (23%) and pneumonitis (5%). Sixteen T4 tumors (41%) achieved complete response, whereas 23 (59%) achieved partial response. All patients were treated with chemotherapy and/or endocrine therapy following chemoradiotherapy. The median follow-up period was 20 months (range 3–96 months). Nineteen patients died because of progressive breast cancer. Infield recurrence or relapse was observed in 11 cases during the course of treatment, but only 3 cases were symptomatic. The 2-year overall local control rate was 73.6%, and the survival rate was 65.9%.
Conclusion Chemoradiotherapy represents a viable option for local treatment of unresectable T4 breast tumors.

Keywords Chemoradiotherapy · Advanced breast cancer

We reported a part of this study at the 18th Annual Meeting of the Japan Breast Cancer Society.

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Introduction

Advanced breast cancer requires multimodality treatment. Usually, patients with resectable primary tumors undergo chemotherapy, endocrine therapy, molecular targeting therapy supported by surgery, and postoperative radiotherapy [1]. However, the treatment strategy for unresectable T4 tumors is particularly problematic. In general, patients with these tumors are administered systemic therapies while waiting for appropriate indications to perform surgical treatment. In such inoperable situations, we believe that chemoradiotherapy, which plays a key role in locally advanced cancers such as those of head and neck, lung, esophagus, and uterine cervix, represents a feasible treatment option [2, 3].

We treated advanced breast cancer with chemoradiotherapy using taxanes, doxifluridine (5′DFUR), or capecitabine (CAP). If the treatment was not likely to extend survival time, local symptomatic control was considered beneficial in improving quality of life. As per our knowledge, only a few successful reports are available in relation to chemoradiotherapy for advanced breast tumors [4–6] Therefore, we report the results of our study on the effectiveness of chemoradiotherapy for unresectable T4 breast tumors and discuss the importance of this therapy for such advanced cases.

Materials and methods

Eligibility

Patients eligible for this study had histologically proven T4 breast tumors. Other criteria included bone marrow function tolerance with neutrophil count >1500/μl, aspartate transaminase/alanine transaminase levels <1.5× the upper normal limit (UNL), alkaline phosphatase levels <2.5× UNL, total bilirubin <UNL, life expectancy >3 months, and absence of infection or severe respiratory complications. Before commencement of treatment, all patients underwent uniform staging and preparatory procedures, including computed tomography (CT) scanning of the chest and abdomen, magnetic resonance imaging (MRI) of the head, and bone scintigraphy. Written informed consent was obtained from all patients after the treatment methods, expected results, and potential adverse effects were explained to them in detail.

Chemotherapy

The following describes the treatment regimens used by our research team since 1998. In 1998, docetaxel (DTX) was approved for treatment of breast cancer in Japan. From February 1998 to September 2001, we performed a clinical study of DTX combined with radiotherapy. On study initiation, DTX was administered biweekly at dose of 30 mg/m^2, with subsequent dose adjustments based on tolerance and bone marrow or liver functions. From February 2000, a weekly DTX schedule was introduced on the basis of data reporting reduced myelosuppression and lung toxicity. The weekly dose rate was set at 20 mg/m^2, with additional dose reductions in patients with reduced organ function. DTX was diluted in a 250-ml solution of 5% glucose and infused over 1 h before the patient underwent radiotherapy. Antihypersensitivity medications were administered prior to chemotherapy if the patient carried a history of allergic reactions.

From November 2002 to May 2005, a clinical study of concomitant paclitaxel (PTX) and 5′DFUR with radiotherapy was performed in order to establish more effective concurrent therapy. We initially preferred a combination of CAP + PTX, which reportedly gave good results; however, CAP was not approved at that time in Japan. Therefore, 5′DFUR was used in place of CAP. During ongoing radiotherapy, 5′DFUR was administered 5 days per week at dose of 600 mg/body, and PTX was administered twice every week at dose of 20–35 mg/m^2 with the same dilution procedure as described for DTX. Initially, dosage escalation of PTX started from 20 mg/m^2; however, after confirming tolerance in 3 patients, dosage was gradually increased by 5 mg/m^2 up to a maximum rate of 35 mg/m^2. After chemoradiotherapy, PTX was administered once in every 3 weeks at dose of 60 mg/m^2 followed by 1 week of rest. This was continued until onset of progressive disease (PD) was noted.

From June 2005 to the present, we have been using normal doses of CAP combined with radiotherapy. Monotherapy with normal doses of CAP can generally be used for breast cancer treatment because it is easily accepted by patients owing to oral administration and low likelihood of toxicities. CAP at dose of 2400 mg/body is administered over a period of 3 weeks followed by 1 week of rest. This course is administered along with and after radiotherapy, and is continued after radiotherapy until onset of PD is noted.

Radiotherapy

In principle, all patients, including those with distant metastases, were administered radical radiotherapy doses to the primary tumor for local control. Radiotherapy in fractions of 2 Gy was delivered 5 times per week to the whole breast and ipsilateral axillary regions with tangential 4-MV photon beams until a total dose of approximately 60 Gy was administered. In some patients, single anterior electron beams were additionally used to cover tumor