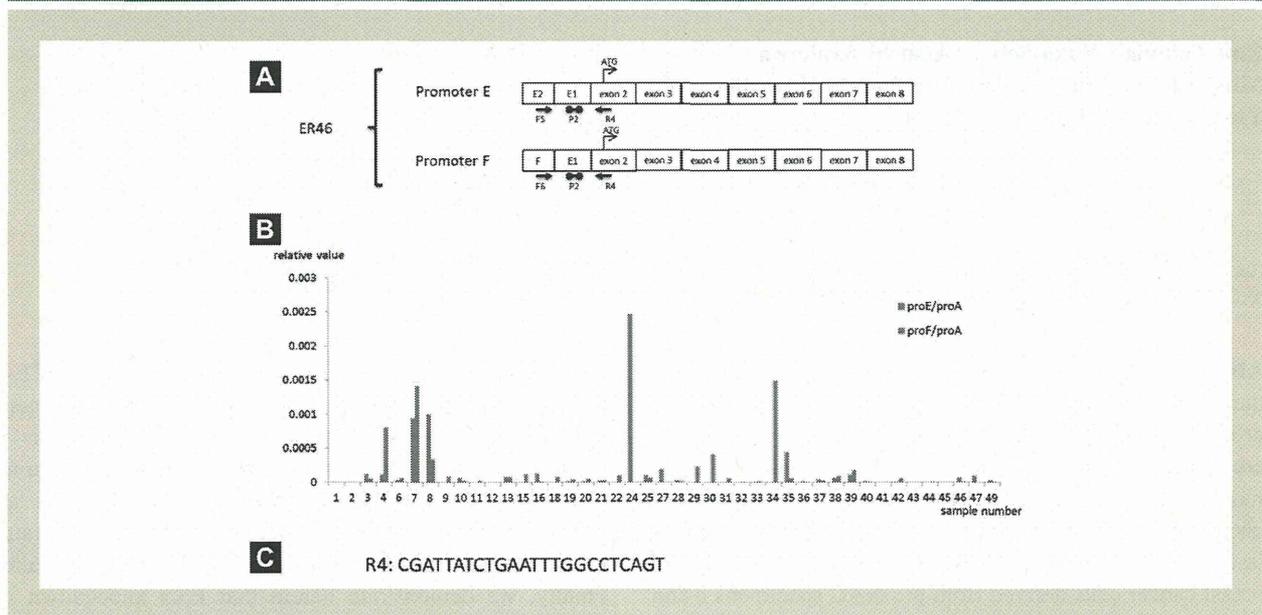


Variation in Use of ER- $\alpha$  Positive Breast Cancer

Supplemental Table 1 The Sequence of Primers and Probes Used in This Study

Primer List	Sequence (5'→3')
ER $\alpha$ Promoter A Forward Primer (F1)	CTGTGCTCTTTTCCAGGTG
ER $\alpha$ Promoter B Forward Primer (F2)	CAGCGACGACAAGTAAAGTG
ER $\alpha$ Promoter C Forward Primer (F3)	GTTCTTGATCCAGCAGGGTG
ER $\alpha$ Promoter D Forward Primer (F4)	CACCTGAGAGAGCCAGTG
ER $\alpha$ Promoter Common Reverse Primer (R1)	AGGGTCATGGTCATGGTC
ER $\alpha$ Promoter E Forward Primer (F5)	ACCAATCCTTTTGATTGTGAA
ER $\alpha$ Promoter F Forward Primer (F6)	GCATAAGAAGACAGTCTCTGAGTGA
ER $\alpha$ Promoter Common Reverse Primer (R2)	GGCAGAAGGCTCAGAAACC
ER $\alpha$ Promoter Common Probe (P1)	CCGGTTTCTGAGCCTTCTGCCC
ER $\alpha$ Promoter Common Probe (P2)	ACATTCTCCGGGACTGCGGTACCA
Total ER $\alpha$ Exon7 Forward Primer (F7)	CTCCACATCAGGCACAT
Total ER $\alpha$ Exon8 Reverse Primer (R3)	CTCCAGCAGCAGGTCATA

**Supplemental Figure 1** Analyses of ER Alpha Variants With This Promoter-Specific Method. (A) Exon Structure of the ER $\alpha$  Variant ER $\alpha$ 46. This Messenger RNA (mRNA) Originates From the Same Promoters, E and F, as Those of Normal ER $\alpha$  Gene. Two 5'-UTR Exons of This mRNA Were Directly Spliced to Exon 2, but not to Exon 1. (B) The Real-Time Polymerase Chain Reaction Analysis of ER $\alpha$ 46 Transcripts. The Amount of ER $\alpha$ 46 Transcripts Originating From Both Promoter E and F was Normalized to That of the Transcripts From Promoter A. The Horizontal Axis Indicates the Identification Number of Breast Cancer Samples and Vertical Axis Indicates the Levels of Transcripts Originating From Both Promoter E and F, Relative to Those Originating From Promoter A. (C) The Sequence of the Reverse Primer Designed for the Analysis of ER $\alpha$ 46



Abbreviations: proA = promoter A; proE = promoter E; proF promoter F.

## Estrogen receptor- $\alpha$ directly regulates sensitivity to paclitaxel in neoadjuvant chemotherapy for breast cancer

Emi Tokuda · Yuko Seino · Atsushi Arakawa ·  
Mitsue Saito · Fujio Kasumi · Shin-ichi Hayashi ·  
Yuri Yamaguchi

Received: 19 May 2011 / Accepted: 24 August 2011 / Published online: 11 September 2011  
© Springer Science+Business Media, LLC. 2011

**Abstract** Neoadjuvant chemotherapy (NAC) has become the standard treatment for advanced breast cancer. Several prognostic markers, including estrogen receptor- $\alpha$  (ER $\alpha$ ), are used to predict the response to NAC. However, the molecular significance of ER $\alpha$  expression in the efficacy of chemotherapy is not yet fully understood. To examine this issue, we first evaluated ER $\alpha$  transcriptional activity in breast cancer cells derived from pre-NAC specimens using estrogen response element–green fluorescent protein (ERE–GFP) as a reporter gene, and found that, in the cases for which ER $\alpha$  activities determined by GFP expression were not detected or low, pCR (pathological complete response) could be achieved even though ER $\alpha$  protein was expressed. Next, we examined the effects of alterations in ER $\alpha$  expression levels on sensitivity to paclitaxel, a key drug in NAC, by stable expression of ER $\alpha$  in ER-negative SKBR3 cells and by siRNA-mediated down-regulation of ER $\alpha$  in ER-positive MCF-7 cells, and showed that ER $\alpha$  expression and sensitivity to paclitaxel showed an inverse

correlation. We also established paclitaxel-resistant MCF-7 cell clones and found that they have higher estrogen-induced ER activity than parent cells. Paclitaxel is a microtubule-stabilizing agent, while HDAC6 (histone deacetylase 6), which we previously identified as an estrogen-regulated gene, enhances cell motility by destabilizing microtubules via deacetylation of  $\alpha$ -tubulin. Finally, we demonstrate herein that ER $\alpha$  knockdown in MCF-7 cells prevents deacetylation of  $\alpha$ -tubulin, thereby increasing sensitivity to paclitaxel. Taken together, these results suggest that ER $\alpha$  expression directly regulates sensitivity to paclitaxel in NAC for breast cancer via the effect on microtubule stability.

**Keywords** Breast cancer · Paclitaxel · Neoadjuvant chemotherapy · ER $\alpha$  · HDAC6

### Introduction

Several recent trials have suggested that adjuvant chemotherapy is less effective in patients with estrogen receptor (ER)-positive breast cancers [1–3]. For example, the Cancer and Leukemia Group B9344 trial evaluated the efficacy of anthracycline regimens with or without paclitaxel in adjuvant therapy and found them to be effective in 24% of ER-negative but only 11% of ER-positive patients [2]. Neoadjuvant chemotherapy (NAC) trials of anthracyclines combined with paclitaxel also showed higher pathological complete response (pCR) rates in ER-negative than in ER-positive patients [4, 5].

Recently, NAC has become the standard treatment for advanced breast cancer. NAC has numerous advantages, including down-staging of an inoperable cancer to an operable one, the availability of breast conservation for

E. Tokuda · M. Saito · F. Kasumi  
Department of Breast and Endocrine Surgery, Graduate School of Medicine, Juntendo University, Tokyo, Japan

E. Tokuda · Y. Seino · Y. Yamaguchi (✉)  
Research Institute for Clinical Oncology, Saitama Cancer Center, 818 Komuro, Ina-machi, Saitama-ken 362-0806, Japan  
e-mail: yamaguchi@cancer-c.pref.saitama.jp

Y. Seino · S. Hayashi  
Center for Regulatory Epigenome and Diseases, Department of Molecular and Functional Dynamics, Graduate School of Medicine, Tohoku University, Sendai, Japan

A. Arakawa  
Department of Human Pathology, Juntendo University Hospital, Tokyo, Japan

patients who would otherwise undergo a mastectomy, and the use of pathological response data as a surrogate marker for long-term clinical outcome [6–9]. Another advantage of NAC is that responsiveness to the particular chemotherapy regimen can be assessed, possibly allowing for individualized therapy [10].

Taxanes are potent antimicrotubule agents, which act by inducing tubulin polymerization and promoting the formation of unusually stable microtubules, thereby inhibiting the normal dynamic reorganization of the microtubular network required for mitosis and cell proliferation [11]. Paclitaxel was the first marketed taxane drug affecting the integrity of microtubules. In recent studies, paclitaxel-based combination chemotherapy has resulted in an improved pCR rate as compared with single-agent taxane treatments [12, 13]. Several prognostic markers of NAC, including ER $\alpha$ , are used to predict responses to paclitaxel. For example, patients with ER-positive breast cancers reportedly gain little benefit from the administration of paclitaxel [14, 15]. However, the molecular mechanisms underlying the role of ER $\alpha$  in the efficacy of paclitaxel are not yet fully understood.

We have studied the roles of ER $\alpha$  expression and estrogen-regulated genes as predictors of the efficacy of systemic therapy for ER-positive breast cancer. In our previous studies, we identified histone deacetylase 6 (HDAC6) as an estrogen-regulated gene using cDNA microarray analysis in the ER-positive breast cancer cell line MCF-7 [16]. In the setting of breast cancer, high HDAC6 expression was also detected [17]. HDAC6 was originally cloned as a member of the histone deacetylase family, is expressed in several tissues, and functions as a deacetylase for tubulin [18]. Furthermore, tubulin is an important component of the microtubule network that regulates cell motility, which appears to be enhanced by HDAC6. Accordingly, we hypothesized that microtubule conformation is the common target for estrogen signals and paclitaxel.

To our knowledge, this is the first study designed to clarify the significance of ER $\alpha$  in tumor sensitivity to paclitaxel, via estrogen-induced deacetylation of tubulin mediated by HDAC6.

## Materials and methods

### Cell culture and reagents

The human breast cancer cell lines MCF-7 and SKBR3 were cultured in RPMI 1640 (Sigma-Aldrich, MO) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, CA) and antibiotics. All cells were incubated at 37°C under 5% CO<sub>2</sub> in air.

MCF-7-E10 cells (E10 cells) were established from the MCF-7 by introducing a plasmid carrying the ERE (estrogen responsive element) fused with tk-GFP (green fluorescent protein) gene, as described previously [19, 20]. E2 (17 $\beta$ -estradiol) was purchased from Sigma-Aldrich. Paclitaxel was provided by Bristol-Myers Squibb (NY, USA). The paclitaxel-resistant MCF-7-E10 clones (PAC-1–PAC-3) were developed by increasing the paclitaxel dose, stepwise, from 2 to 10 nM, over 2 months.

### Assays of cell growth

The cells ( $5 \times 10^4$ /well) were seeded in 24-well plates and cultured. After 48 h, the cells were treated with paclitaxel (0–30 nmol/l) for 3 days. Cell numbers were determined using a cell counter (Coulter Counter ZBI; Beckman Coulter, CA) and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. IC50 values (drug dosages producing 50% inhibition of cell growth) were determined from growth inhibition curves.

### Knockdown of ER $\alpha$ by siRNA transfection in MCF-7 cells

Cells ( $1 \times 10^5$ /well) were cultured in RPMI medium for 24 h. In accordance with the manufacturer's instructions, 10 nmol/l small interfering RNA [ESR1-7255 (Sigma; si-1) and ESR1-7257 (Sigma; si-2) siRNAs for knockdown of ER $\alpha$ ] or scramble siRNA (SC-37007, Santa Cruz Biotechnology, CA) was mixed with siLentFect Lipid Reagent (Bio-Rad, CA) in serum-free RPMI. After 20 min, solutions were added to the cells. To investigate the effects of ER $\alpha$  knockdown, cells were harvested and ER $\alpha$  expression was determined by western blotting using anti-ER $\alpha$  antibody (sc-7207, 1/200, Santa Cruz).

### Establishment of SKBR3 cells expressing ER $\alpha$

ER-negative and HER2-positive SKBR3 cells were transfected with an expression plasmid vector for ER $\alpha$  using Trans IT-LT1 (Mirus Bio, WI) following the manufacturer's specifications, and were subjected to selection in growth medium containing geneticin (Sigma). We confirmed ER $\alpha$  expression employing real-time RT-PCR. We use the term SKBR3-cont to indicate cells transfected with control vector, while SK-ERpos cells were transfected with ER $\alpha$ .

### Western blot analysis

Cell lysates were prepared using Lysis-M Reagent (Roche Diagnostics, Germany) according to the manufacturer's

instructions. Total proteins (40 µg) were run on SDS-PAGE using 10% acrylamide gels (SuperSep<sup>TM</sup>ace; Wako, Japan) and proteins were transferred onto a PVDF membrane (Bio-Rad). After blocking the membrane with 10% dry milk for 1 h, blots were probed with an antibody to ER $\alpha$  (sc-7207),  $\alpha$ -tubulin (sc-5546, 1/200, Santa Cruz),  $\beta$ -tubulin (#2146, 1/1000, Cell Signaling Technology, MA), or acetylated  $\alpha$ -tubulin (6-11B-1, 1/2000, Sigma-Aldrich) in 0.05% Tween-20 in TBS (TTBS) at 4°C overnight. Secondary antibodies used at room temperature for 1 h were as follows: goat anti-rabbit IgG (Immun-Star Goat Anti-Rabbit-AP, 1/3000; Bio-Rad) for ER $\alpha$ ,  $\alpha$ -tubulin and  $\beta$ -tubulin, goat anti-mouse IgG (Immun-Star Goat Anti Mouse-AP, 1/3000; Bio-Rad) for anti-acetylated  $\alpha$ -tubulin. An Immun-Star<sup>TM</sup> chemiluminescent Protein Detection System (Bio-Rad) was used to detect the secondary probes. The image was captured on an LAS-4000 with accompanying Image Reader (Fujifilm, Tokyo Japan). Protein expression was analyzed using Multi Gauge v3.2 software (Fujifilm).

#### Real-time quantitative RT-PCR

RNA was extracted from whole cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNAs were synthesized from 1 µg of total RNA using a TaKaRa RNA PCR Kit Ver.3.0 (Takara Bio, Shiga, Japan). Real-time quantitative RT-PCR was performed using the LightCycler 2.0 (Roche Diagnostics) with LightCycler FastStart DNA Masterplus SYBR Green 1 kit (Roche Diagnostics). Target gene expression was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level. For PCR, the following ER $\alpha$  primer sequences were used: forward, 5'-CTCCACATC AGGCACAT-3'; reverse, 5'-CTCCAGCAGCAGGCTA TA-3'. The HDAC6 primers were as follows: forward, 5'-GTCTACTGTGGTCGTTACATC-3'; reverse, 5'-GGC CTGACAGTAACAC-3'.

#### Patients received NAC at Juntendo University Hospital

All patients who received NAC had tumors over 3 cm in diameter and/or positive axillary nodes, but no distant metastasis prior to NAC. Determinations of invasive cancer, hormone receptor status, and HER2 status were based on pre-NAC core needle biopsy results.

The patients received four cycles of fluorouracil (500 mg/m<sup>2</sup>), epirubicin (75 or 100 mg/m<sup>2</sup>), and cyclophosphamide (500 mg/m<sup>2</sup>) (FEC), followed by taxanes, namely, 12 weekly cycles of paclitaxel (80 mg/m<sup>2</sup>) or docetaxel (150 mg/m<sup>2</sup>) every 3 weeks for four cycles. Sonography plus MRI was carried out at diagnosis, then again prior to the administration of taxanes, and before

surgery. A clinical complete response (cCR) was disappearance of the tumor on these images. A clinical partial response (cPR) was more than 30% shrinkage of the tumor with NAC, and clinical progressive disease (cPD) was a more 20% increase in the tumor. Any response other than these was categorized as clinical stable disease (cSD). A pathological CR (pCR) involved no evidence of intraductal components or metastatic lesions.

We first analyzed 190 patients with advanced breast cancer who received NAC at the Breast Center of Juntendo University Hospital (Tokyo, Japan) between July 2006 and January 2008. To assess the relationships between ER activity in breast cancer cells and the clinical response to NAC, we analyzed 31 breast cancer tissues, obtained by core needle biopsy and/or surgical resection at Juntendo University Hospital from June 2009 to March 2010. All tumor specimens were obtained after informed consent from patients. The Juntendo University Hospital Ethics Committee approved this study.

#### Immunohistochemistry (IHC)

ER status was determined using IHC methods. Slides were stained and evaluated using the Ventana I-VIEW Breast Panel (Roche). Immunoreactivity greater than 10% was considered to indicate receptor-positive status. HER2 protein status, as assessed using the Herceptest (Ventana, Switzerland), was scored on a scale of 0 to 3+ according to the Dako scoring system. HER2/neu-positive status was defined as HER2 protein 3+ or 2+ and a fluorescence in situ hybridization (FISH) ratio of more than 2.2.

#### Assay of ERE activity in primary tumor cells (adenovirus ERE-GFP method)

To assess ERE activation in primary tumor cells, we used Ad-ERE-tk-GFP [20, 21]. Cancer tissue specimens were minced to ~1 mm<sup>3</sup> after rinsing with PBS and digested with collagenase solution for 20–30 min at 37°C. The cells, including tumor cells, were washed several times with PBS, and incubated in 24-well plates using 400 µl of phenol-red-free RPMI 1640 supplemented with 10% heat- and charcoal-treated fetal calf serum (DCC-FCS). The cells were then infected, either immediately or 1 day later, with  $2 \times 10^9$  PFU (plaque-forming units) (in 293A cells) of Ad-ERE-tk-GFP, and incubated for 3 days. GFP-expressing cells were counted by fluorescence microscopy after incubation. To examine the infectivity of the adenovirus in primary tumor cells, the cells were infected with  $2 \times 10^9$  PFU of Ad-CMV-DsRed, and at least 95% of cells were confirmed to be infected [21]. When more than 10% of cancer cells expressed GFP, the specimen was considered to have high ER transcriptional activity.

### 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  $\pm$  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

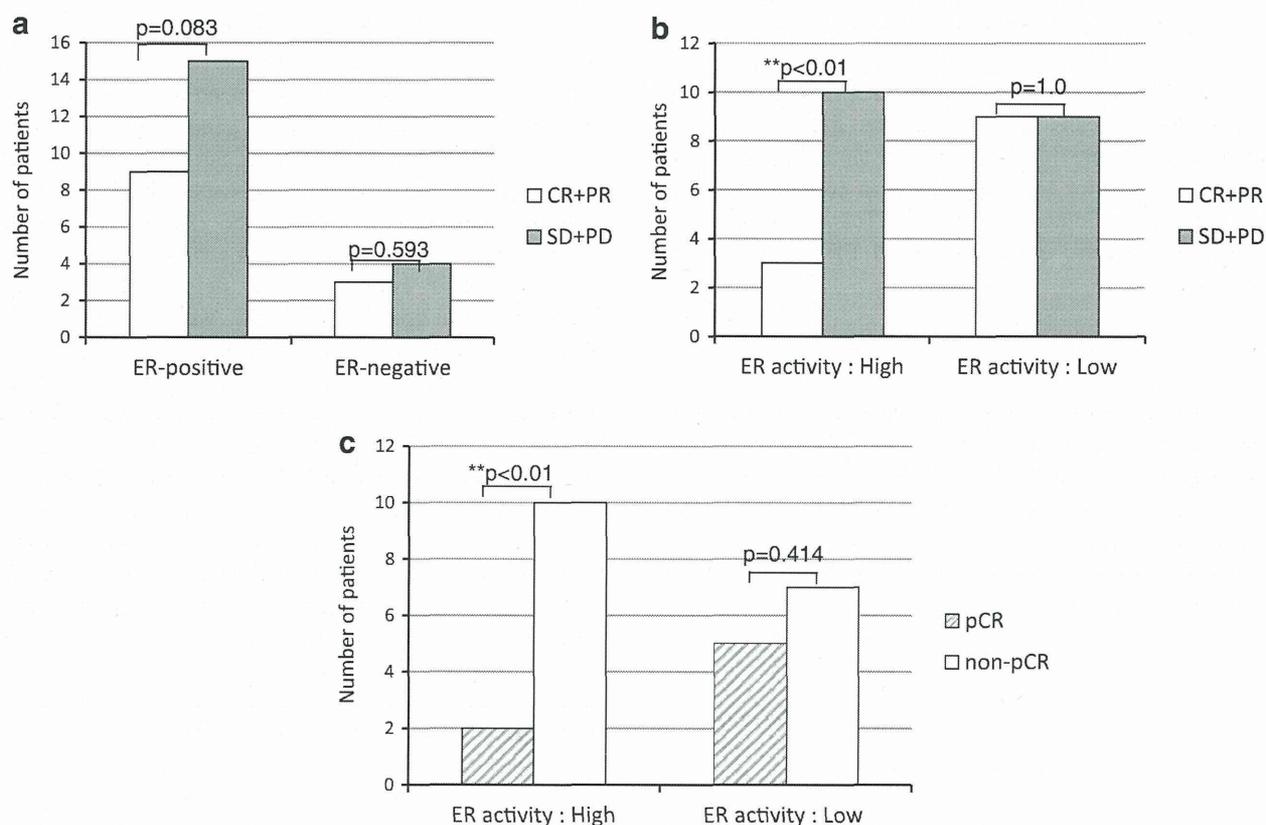
In our previous studies, we produced our own focused microarray for estrogen-regulated genes [16], and found that ER-positive breast cancers did not always show high expression of a target gene [unpublished data]. This suggests some discrepancy between the expression levels of ER protein and its function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

First, as shown in Fig. 1a and b, the relationship between response to paclitaxel and ER $\alpha$  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 ER $\alpha$  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 ER $\alpha$  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 ER $\alpha$ -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 ER $\alpha$  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 ER $\alpha$  did function as a transcription factor hardly achieve pCR. To further clarify the role of ER $\alpha$  in the sensitivity to paclitaxel, we carried out the following experiments in vitro.

### Knockdown of ER $\alpha$ of MCF-7 cells increased the sensitivity to paclitaxel

On the basis of the clinical results, we analyzed whether or not ER $\alpha$  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 ER $\alpha$  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 ER $\alpha$  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** ER $\alpha$  status were evaluated by IHC method in 31 patients. **b** ER $\alpha$  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

ER $\alpha$  expression decreased the sensitivity of ER $\alpha$ -negative SKBR3 cells to paclitaxel

Next, we examined the effect of ER $\alpha$  expression on the sensitivity of the SKBR3 cell line to paclitaxel. We generated SKBR3 cells expressing ER $\alpha$ , 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 ER $\alpha$  expression in SK-ERpos cells by real-time PCR. The IC<sub>50</sub> 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 ER $\alpha$  overexpression rendered SKBR3 cells more resistant to paclitaxel than parent SKBR3 cells.

SiRNA knockdown of ER $\alpha$  decreased deacetylation of  $\alpha$ -tubulin

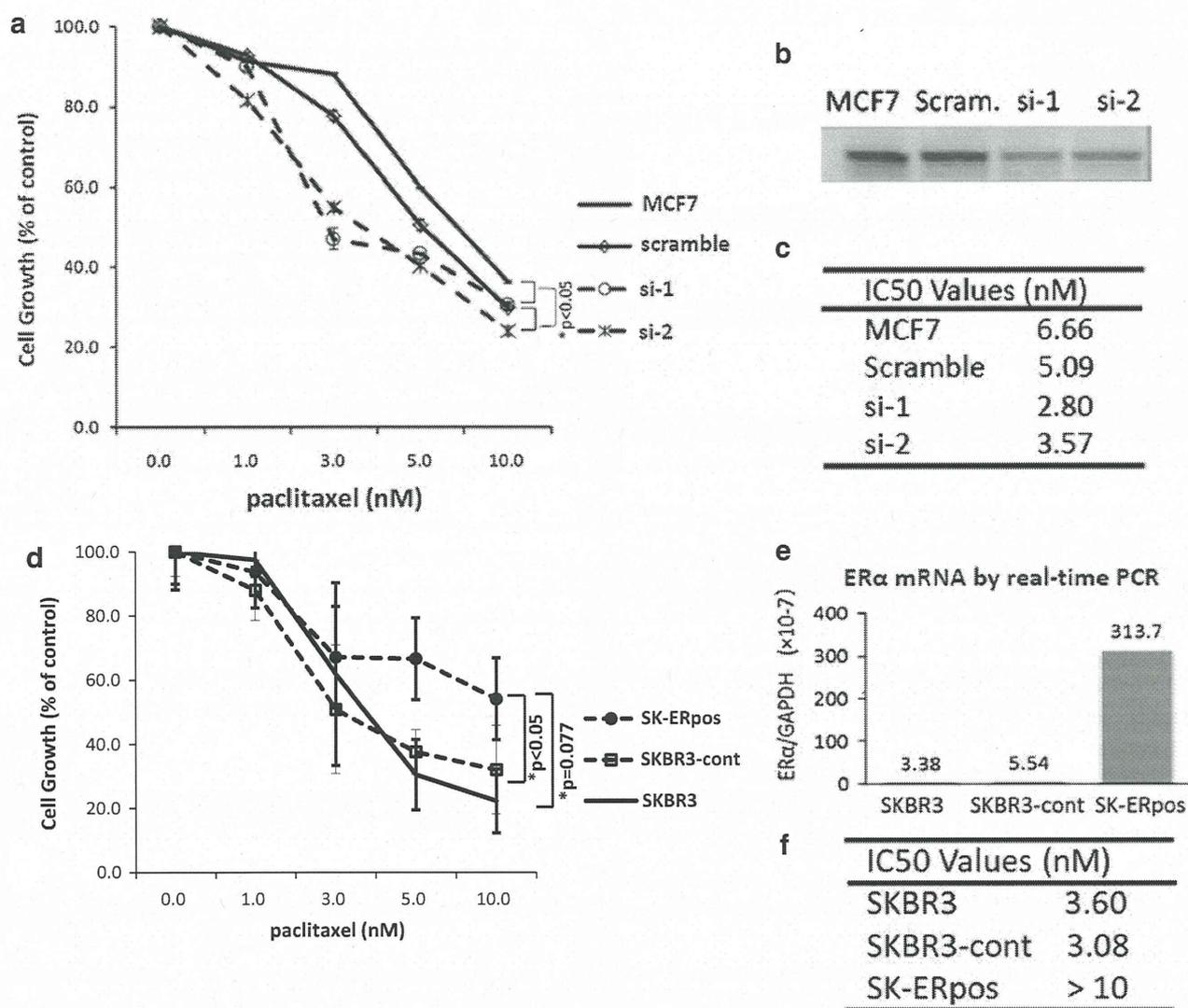
Paclitaxel promotes microtubule stabilization, which disrupts cellular processes, inhibits cell division, and ultimately induces apoptosis [23, 24]. On the other hand, estrogen promotes the deacetylation of tubulin through

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  $\alpha$ -tubulin.

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

Upregulation of ER $\alpha$  function in paclitaxel-resistant MCF-7–E10 cells

To further evaluate the function of ER $\alpha$  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 ER $\alpha$  in MCF-7 cells increased sensitivity to paclitaxel and over-expression of ER $\alpha$  decreased sensitivity to paclitaxel in SKBR3 cell lines. **a** Growth inhibition by paclitaxel in scramble and ER $\alpha$  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  $\pm$  SD of duplicate determinations of two independent experiments. Bars indicate SD. \* $P < 0.05$  for ER $\alpha$  knockdown in MCF-7 versus parent and scramble MCF-7 by ANOVA. **b** Knockdown of ER $\alpha$  in MCF-7–si-1 and MCF-7–si-2 cells. ER $\alpha$  protein

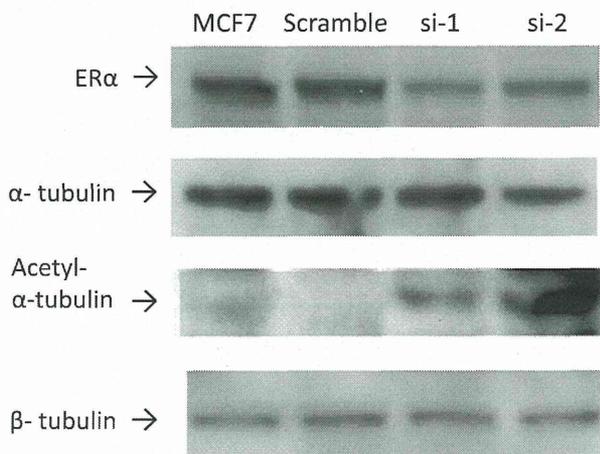
levels were assessed by western blotting. **c** IC50 values of paclitaxel in **a**. Knockdown of ER $\alpha$  rendered MCF-7 cells more sensitive to paclitaxel. **d** Growth inhibition by paclitaxel in parent, control, and ER $\alpha$  over-expressing SKBR3 cell lines. Cells were treated with paclitaxel 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  $\pm$  SD of duplicate determinations. Bars indicate SD. **e** ER $\alpha$  mRNA levels determined by real-time RT-PCR in parent, control, and ER $\alpha$  over-expressing SKBR3 cell lines. ER $\alpha$  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 ER $\alpha$  activity via GFP expression using our automated system [22, 25]. ER $\alpha$  activities in paclitaxel-resistant MCF-7–E10 cells were measured after culture with estrogen for 3 days. Estrogen-

dependent ER $\alpha$  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 ER $\alpha$  mRNA expression to be increased in paclitaxel-resistant



**Fig. 3** SiRNA knockdown of ER $\alpha$  decreased deacetylation of  $\alpha$ -tubulin. Western blotting of ER $\alpha$ ,  $\alpha$ -tubulin, and acetylated  $\alpha$ -tubulin in scramble and ER $\alpha$ -siRNA-treated MCF-7 cells and parent cells.  $\alpha$ -Tubulin was more acetylated in ER $\alpha$ -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 ER $\alpha$  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 ER $\alpha$  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 ER $\alpha$  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 ER $\alpha$ -positive and ER $\alpha$ -

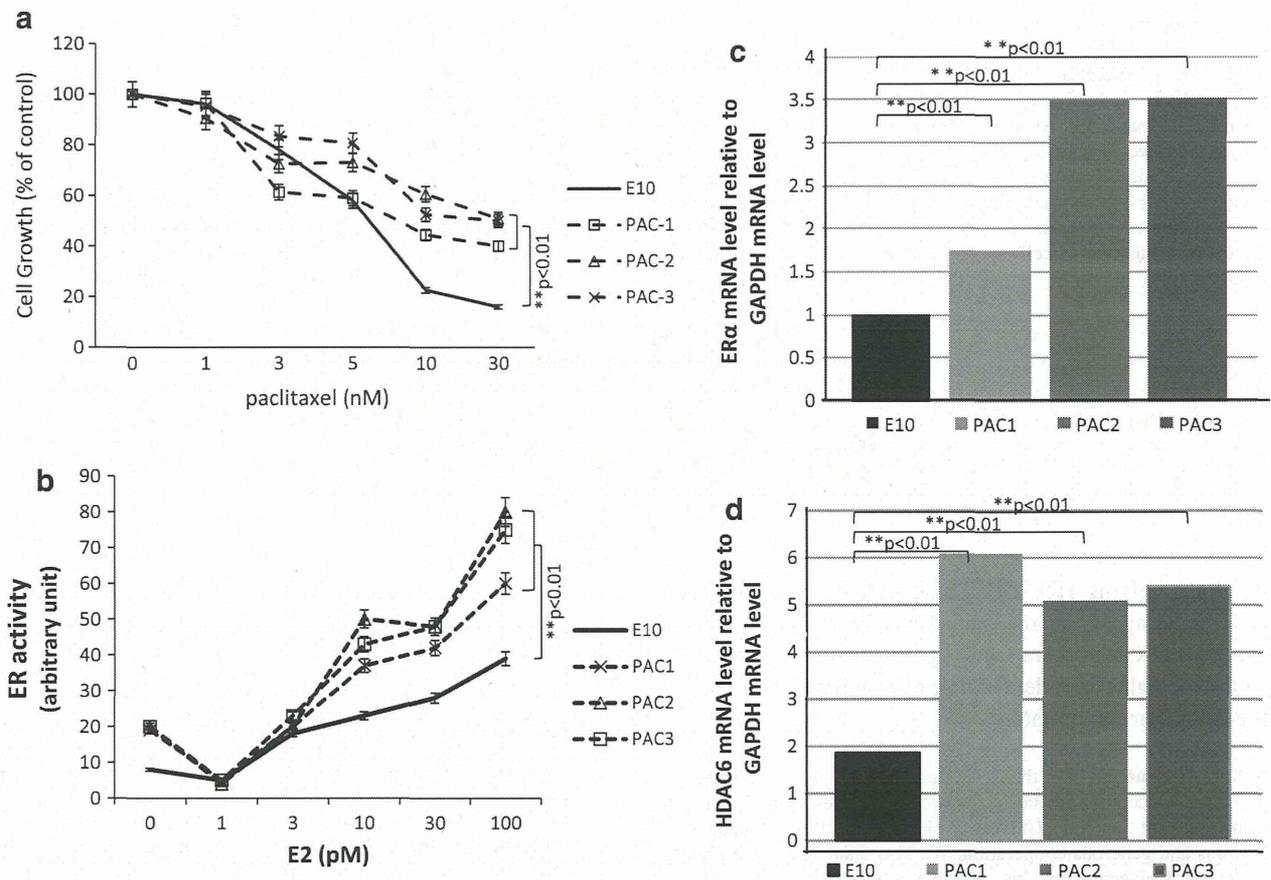
negative breast cancers, respectively, were sensitive to paclitaxel (Fig. 1a). With the ERE-GFP method, 23.0 and 50.0% of high and low ER activity breast cancers, respectively, were sensitive to paclitaxel (Fig. 1b), suggesting that ER $\alpha$ -nonfunctional breast cancers are sensitive to paclitaxel. The ERE-GFP method differs significantly from the IHC method in the selection of ER $\alpha$ -nonfunctional cases.

Among 31 cases, we next analyzed 24 ER $\alpha$  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 ER $\alpha$ -negative breast cancer, the cases for which ER $\alpha$  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 ER $\alpha$  expression and sensitivity to paclitaxel, we used two breast cancer cell lines, ER $\alpha$ -positive and HER2-negative MCF-7 cells and ER $\alpha$ -negative and HER2-positive SKBR3 cells. For each cell line, we analyzed the effects of altering in ER $\alpha$  expression levels on sensitivity to paclitaxel, and found that ER $\alpha$  expression directly affects the sensitivity to paclitaxel regardless of HER2 expression (Fig. 2a–f).

Finally, we analyzed the function of ER $\alpha$  in paclitaxel-resistant MCF-7 clones established in this study (Fig. 4a). Estrogen-dependent ER $\alpha$  activity in these PAC-resistant MCF-7 clones was higher than that in parent MCF-7 cells (Fig. 4b). Increased ER $\alpha$  expression is one mechanism whereby they showed high ER $\alpha$  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 ER $\alpha$  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  $\pm$  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 ER $\alpha$  activity in PAC-1, PAC-2, and PAC-3-MCF-7-E10 versus that in parent cells, by ANOVA. **c** ER $\alpha$  mRNA expression was increased in paclitaxel-resistant MCF-7-E10 cells (PAC-1, PAC-2, and PAC-3 cells).  $*P < 0.01$  for ER $\alpha$  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 ER $\alpha$  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  $\alpha$ -tubulin to be more acetylated in ER $\alpha$ -knockdown MCF-7 cells and that ER $\alpha$  expression induced deacetylation of  $\alpha$ -tubulin. The expression of HDAC6 was decreased in ER $\alpha$ -knockdown MCF-7 cells and increased in ER $\alpha$ -overexpressing SKBR3 cells (data not shown). HDAC6 and ER $\alpha$  mRNA expressions were also increased in paclitaxel-resistant MCF-7-E10 cells as described above. These results led us to hypothesize that ER $\alpha$  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 ER $\alpha$ , 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 ER $\alpha$  directly regulates tumor sensitivity to paclitaxel, primary via estrogen-induced deacetylation of