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ORIGINAL ARTICLE

Impact of biomarker changes during neoadjuvant chemotherapy for clinical response in patients with residual breast cancers

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Abstract
Background Residual cancer burden or Ki67 expression levels in residual tumors reportedly provided significant prognostic information for a non-pathological complete response subset after neoadjuvant chemotherapy (NAC). However, the significance of Ki67 reduction for clinical response during chemotherapy in each subtype or menopausal status is yet to be determined.
Methods A total of 183 breast cancers surgically removed after chemotherapy were recruited for this study. Expression levels of estrogen receptor (ER), progesterone receptor (PgR), and Ki67 were determined immunohistochemically for semiquantitative measurement and these biomarkers were compared in pre- and post-NAC samples from pathological non-responders ($n = 125$). Responses to chemotherapy were evaluated both clinically and pathologically.
Results Ki67 expression levels after NAC (median 5 %, range 0–70 %) were significantly reduced compared with before NAC (25, 1–80 %, $P < 0.0001$), but only in patients who attained clinical response. This significant suppression of Ki67 in clinical responders was consistently observed in breast cancers from the ER-positive subset, but not the ER-negative subset in the total test set ($n = 120$). These observations were also made in the validation set ($n = 63$). Among premenopausal, but not postmenopausal patients, a significant decrease in PgR expression levels was detected in breast cancers of patients who attained clinical response (pre-NAC 50, 0–100 %, post-NAC 5, 0–20 %; $P = 0.0003$).
Conclusion The impact of Ki67 suppression on clinical response seems to be restricted to ER-positive breast cancers. Since PgR expression levels of premenopausal ER-positive cancers were significantly reduced in clinical responders, inhibition of estrogen signaling due to chemotherapy-induced amenorrhea may be involved in this association.

Keywords Breast cancer · Neoadjuvant chemotherapy · Ki67 · Progesterone receptor

Introduction

Neoadjuvant chemotherapy (NAC) is commonly administered in daily clinical practice for locally advanced as well as operable breast cancers. The reduction of tumor volume induced by NAC facilitates the subsequent surgical procedure and has led to enhanced success rates of breast-conserving surgery [1]. In addition to this advantage for the surgical procedure, results of clinical and pathological evaluation of responses to chemotherapy yield important information for the prediction of prognosis for individual patients. It has been established that patients who attained pathological complete response (pCR) after NAC (no residual cancer in surgical specimens) showed a strong correlation with prolonged disease-free survival (DFS) and overall survival (OS) [2–6]. Although correlation between pCR and favorable prognosis has been observed irrespective

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of chemotherapy regimen [6], the prognostic significance of pCR seems to differ depending on breast cancer subtypes. In a response-guided NAC trial where, after two cycles of docetaxel, doxorubicin, and cyclophosphamide (TAC), early responders were randomly assigned to four or six additional TAC cycles, and early non-responders to four cycles of TAC or vinorelbine and capecitabine, pCR was significantly associated with DFS in hormone receptor (HR)-negative, but not in HR-positive cancers [7]. The reason why pCR does not function as a substitute for favorable prognosis for HR-positive breast cancers is currently unknown, but identification of chemotherapeutic predictive and prognostic markers for this subtype may well be warranted.

Several studies have reported that the extent of residual disease is an independent predictor of relapse-free survival and OS [8–10]. In these studies, residual cancer burden (RCB) was calculated as a continuous index by combining pathologic measurements of primary tumor (size, cellularity, and in situ disease) and nodal metastases (number and size). These RCBs were then classified into four categories, i.e., RCB 0 (complete remission), I (minimal), II (moderate), and III (extensive residual disease) (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3). In addition, Miller et al. [11] calculated tumor response ratio (TRR) as the extent of residual in-breast disease divided by size on pre-NAC imaging and concluded that TRR was a more accurate predictor of OS than pathologic stage or clinical T stage. These findings suggest that not only pCR but also residual tumor volume might be useful for predicting response and prognosis, especially for the HR-positive subset.

Bottini et al. [12] and Makris et al. [13] reported that Ki67 expression decreased after chemotherapy and that this reduction correlated significantly with clinical tumor response. Similarly, clinical responders showed a more significant reduction of Ki67 expression at day 21 than non-responders [14, 15]. Reducing Ki67 expression levels may thus be important for a better response to chemotherapy, and Ki67 after NAC is thought to provide more useful prognostic information than can be obtained pretreatment [16]. It is thus conceivable that post-NAC Ki67 expression levels could be useful for reflecting response to chemotherapy in breast cancers that have not attained pCR. However, it is not clear whether suppression of Ki67 results in clinical response during chemotherapy irrespective of breast cancer subtype. It has been well established that the expression levels of estrogen receptor (ER) and progesterone receptor (PgR) are often changed after chemotherapy [17]. Since tumor characteristics of ER-positive cancers are affected by estrogen signaling, these changes might represent a biological effect mediated through estrogen signaling induced by chemotherapies. Especially for premenopausal women, suppression of ovarian function induced by chemotherapy seems to have the effect of an endocrine therapy in addition to the direct effects of chemotherapy [18, 19].

Although a paradoxical increase of PgR expression in clinical responders has been reported, immunocytochemistry was used for samples obtained with fine-needle aspiration for a combined analysis of both pre- and postmenopausal patients [13]. Thus, it is yet to be determined whether ovarian suppression induced by chemotherapy affects reduction of Ki67 expression.

The aim of this study was therefore to assess whether reduction in Ki67 impacts clinical response irrespective of breast cancer subtype. In addition, we investigated the changes in ER and PgR expression levels between pre- and post-NAC, classified according to clinical response, in order to evaluate whether changes in these biomarkers is associated with Ki67 reduction considering menopausal status.

Patients and methods

Eligibility of patients

A total of 183 breast cancers treated with surgical procedures after various primary systemic chemotherapies at Hyogo College of Medicine ($n = 120$, between September 2005 and December 2013) or Yao Municipal Hospital ($n = 63$, between March 2010 and February 2014) were recruited for this study. The histological classification and nuclear grade were decided according to the Japanese Breast Cancer Society classification [20]. Systemic chemotherapy consisted of anthracycline-containing regimens for 8 patients, sequential administration of anthracycline and taxanes for 131 patients, and taxane-based regimens for 44 patients. Trastuzumab was used concurrently with chemotherapy for 40 patients. This study was approved by the Ethics Committee of Hyogo College of Medicine.

Evaluation of treatment efficacy

The maximum diameter of the main tumor was measured with ultrasound before and after chemotherapy. We evaluated clinical response according to the Response Evaluation Criteria in Solid Tumors criteria [21] and defined a responder as a patient attaining complete or partial response, and a non-responder as a patient showing no change or progress disease. Tumor size was also measured with magnetic resonance imaging for the majority of patients and clinical responses were the same as those evaluated with ultrasound. Pathological response was determined in accordance with the criteria of the Japanese Breast Cancer Society [20], i.e., pathological responder:

grade 3 (complete remission of invasive cancer cells) and grade 2b (minimal residual disease), and non-responder: grade 0–2a.

Immunohistochemical procedure

Expression levels of ER, PgR, human epidermal growth factor receptor 2 (HER2) and Ki67 were determined using formalin-fixed, paraffin-embedded tumor tissues obtained before chemotherapy (core needle biopsy or vacuum-assisted biopsy samples) and after chemotherapy (surgically resected samples). These tissue samples were subjected to immunohistochemical staining for quantitative determination of ER, PgR, HER2, and Ki67 expression levels in terms of the percentage of positive cancer cells except for HER2. Since post-NAC samples from pathological responders could not be immunohistochemically analyzed due to insufficient numbers of cancer cells, we examined post-NAC samples only from pathological non-responders. Primary antibodies used at the Hyogo College of Medicine for ER (1D5), PgR (PgR636) and Ki67 (MIB1) were obtained from Dako (Glostrup, Denmark). At Yao Municipal Hospital, primary antibodies used for ER, PgR and Ki67 were SP-1, 1E2 and 30-9, respectively, all of which were obtained from Ventana Medical Systems (Tucson, AZ, USA). The Hercep Test (Dako) and the PATHWAY anti-HER-2/neu (Ventana) were used, respectively, at Hyogo College of Medicine and Yao Municipal Hospital to stain membranes for immunohistochemical determination of HER2. All staining was performed by means of automated immunostainers—BOND-MAX (Leica Microsystems, Tokyo, Japan) at Hyogo College of Medicine and Bench Mark GX (Ventana) at Yao Municipal Hospital. The percentages of positive cancer cells in nuclei which stained intensely or moderately for ER, PgR and Ki67 were determined. The cut-off value for ER and PgR was set at 1 % and for HER2-negative at an immunohistochemical score of 0 or 1, and a score of 2 for FISH-negative.

Statistical analysis

The differences in clinicopathological characteristics between clinical or pathological responders and non-responders were calculated with the chi-squared or Fisher's exact test as appropriate, as well as with the Mann–Whitney test. Changes in expression levels of each biomarker before and after chemotherapy were examined with the Wilcoxon matched pairs test. Differences were considered statistically significant if $P < 0.05$. JMP Pro 10 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Relationships between clinicopathological characteristics and clinical or pathological response to chemotherapies

This study involved 104 clinical responders (86.7 %) and 16 non-responders (13.3 %) among patients treated for breast cancers at Hyogo College of Medicine. In addition, 41 patients (34.2 %) were identified as pathological responders and 79 (65.8 %) as non-responders. Similar responses were obtained for breast cancer patients treated at Yao Municipal Hospital (clinical response 87.3 %, pathological response 27.0 %). Table 1 shows the results of an analysis of the relationships between clinicopathological characteristics and responses to NAC for breast cancers treated at Hyogo College of Medicine. There was no significant difference in clinicopathological characteristics between clinical responders and non-responders. On the other hand, significant differences were observed for nuclear grade ($P = 0.01$), ER status ($P = 0.0002$), PgR status ($P = 0.0078$), HER2 status ($P = 0.0007$), Ki67 expression levels ($P = 0.043$), subtype ($P < 0.0001$) and chemotherapy regimen ($P = 0.022$).

Relationships between changes in biomarkers during neoadjuvant chemotherapy and clinical response for breast cancers of pathological non-responders

Post-NAC samples from breast cancers of pathological non-responders ($n = 132$) treated at Hyogo College of Medicine were then examined immunohistochemically. Table 2 shows a comparison of biomarker changes during NAC of clinical responders and non-responders. Although differences in changes in ER, PgR, and HER2 status between clinical responders and non-responders were observed, respectively, in 9.0, 19.2, and 7.8 % of the patients, there were no significant associations between biomarker changes and clinical response.

Changes of Ki67 expression levels after neoadjuvant chemotherapy in breast cancers of pathological non-responders

The results of a comparison of Ki67 expression levels in pre- and post-NAC samples in relation to clinical response in breast cancers treated at Hyogo College of Medicine are shown in Fig. 1. Post-NAC Ki67 expression levels were significantly reduced in breast cancers that attained clinical response [post-NAC: 5 % (median), 0–70 % (range); vs pre-NAC: 25, 1–80 %; $P < 0.0001$], but not

Table 1 Clinicopathological characteristics of breast cancers treated at Hyogo College of Medicine according to clinical and pathological response

Characteristics	cRes ($n = 104$)	cNon-Res ($n = 16$)	P value	pRes ($n = 41$)	pNon-Res ($n = 79$)	P value
Menopausal status						
Pre-	42 (80.8)[a]	10 (19.2)	0.097	16 (30.8)	36 (69.2)	0.49
Post-	62 (91.2)	6 (8.8)		25 (36.8)	43 (63.2)	
Histological type						
IDC	98 (86.7)	15 (13.3)	0.41	39 (34.5)	74 (65.5)	0.48
ILC	2 (66.7)	1 (33.3)		0 (0)	3 (100)	
Others	4 (100)	0 (0)		2 (50.0)	2 (50.0)	
Tumor size (cm)						
≤2	24 (85.7)	4 (14.3)	0.99	13 (46.4)	15 (53.6)	0.17
>2	80 (87.0)	12 (13.0)		28 (30.4)	64 (69.6)	
Clinical lymph node metastasis						
Negative	64 (85.3)	11 (14.7)	0.57	25 (33.3)	50 (66.7)	0.8
Positive	40 (88.9)	5 (11.1)		16 (35.6)	29 (64.4)	
Nuclear grade						
1 + 2	70 (86.4)	11 (13.6)	0.99	20 (24.7)	61 (75.3)	0.01
3	31 (86.1)	5 (13.9)		18 (50.0)	18 (50.0)	
Unknown	3 (100)	0 (0)		3 (100)	0 (0)	
Estrogen receptor status[b]						
Positive	58 (87.9)	8 (12.1)	0.67	13 (19.7)	53 (80.3)	0.0002
Negative	46 (85.2)	8 (14.8)		28 (51.9)	26 (48.1)	
Progesterone receptor status[b]						
Positive	40 (87.0)	6 (13.0)	0.94	9 (19.6)	37 (80.4)	0.0078
Negative	64 (53.3)	10 (8.3)		32 (43.2)	42 (56.8)	
HER2 status						
Negative	66 (82.5)	14 (17.5)	0.058	19 (23.8)	61 (76.3)	0.0007
Positive	38 (95.0)	2 (5.0)		22 (55.0)	18 (45.0)	
Ki67 expression levels (%)						
≤20	27 (96.4)	1 (3.6)	0.11	4 (14.3)	24 (85.7)	0.043
>20	65 (83.3)	13 (16.7)		27 (34.6)	51 (65.4)	
Unknown	12 (85.7)	2 (14.3)		10 (71.7)	4 (28.6)	
Subtype[c]						
Luminal	40 (87.0)	6 (13.0)	0.1	7 (15.2)	39 (84.8)	<0.0001
Luminal/HER2	18 (90.0)	2 (10.0)		6 (30.0)	14 (70.0)	
HER2	20 (100)	0 (0)		16 (80.0)	4 (20.0)	
TN	26 (76.5)	8 (23.5)		12 (35.3)	22 (64.7)	
Chemotherapy						
Anthracycline	4 (57.1)	3 (42.9)	0.086	2 (28.6)	5 (71.4)	0.022
A and T[d]	65 (87.8)	9 (12.2)		32 (43.2)	42 (56.8)	
Taxane	35 (89.7)	4 (10.3)		7 (17.9)	32 (82.1)	

cRes clinical responder (complete response and partial response), *cNon-Res* clinical non-responder (no change and progress disease), *pRes* pathological responder (Grade 2b and 3), *pNon-Res* pathological non-responder (Grade 0, 1a, 1b, 2a), *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

[a] (%)

[b] Positive ≥1 %, negative < 1 %

[c] Luminal: ER+/HER2−, luminal/HER2: ER+/HER2+, HER2: ER−/HER2+, TN: ER−/HER2−

[d] Sequential administration of anthracycline and taxane

Table 2 Biomarker changes during neoadjuvant chemotherapy on clinical response of breast cancers treated at Hyogo College of Medicine

Characteristics	cRes	cNon-Res	*P* value
Estrogen receptor status[a]			
Positive to positive	41 (87.2)	6 (12.8)	0.076
Positive to negative	4 (66.7)	2 (33.3)	
Negative to positive	0 (0)	1 (100)	
Negative to negative	18 (75.0)	6 (25.0)	
Progesterone receptor status[a]			
Positive to positive	23 (82.1)	5 (17.9)	0.91
Positive to negative	8 (88.9)	1 (11.1)	
Negative to positive	5 (83.3)	1 (16.7)	
Negative to negative	27 (77.1)	8 (22.9)	
HER2 status			
Negative to negative	46 (78.0)	13 (22.0)	0.61
Negative to positive	0 (0)	0 (0)	
Positive to negative	6 (100)	0 (0)	
Positive to positive	10 (83.3)	2 (16.7)	

cRes clinical responder (complete response and partial response), *cNon-Res* clinical non-responder (no change and progress disease)

[a] Positive ≥1 %, negative <1 %

in clinical non-responders as for all patients ($P = 0.87$) (Fig. 1a). This significant suppression of Ki67 was consistently observed in the ER-positive subset (5, 0–50 vs 20, 1–80 %; $P < 0.0001$). On the other hand, no differences were observed in Ki67 expression levels between pre- and post-NAC in the ER-negative subset even though clinical responders were included in the comparison (Fig. 1b, c).

To validate these results, we examined changes in Ki67 expression levels during NAC using tumor samples from patients treated at Yao Municipal Hospital. Similar to the findings described above, post-NAC Ki67 expression levels were significantly reduced in breast cancers that attained clinical response ($P = 0.033$), but not in clinical non-responders as for all patients (Fig. 2a). This significant suppression of Ki67 was consistently observed in the ER-positive subset ($P = 0.034$), but not in the ER-negative subset (Fig. 2b, c).

Because the number of samples from Yao Municipal Hospital was small, further analyses were performed only for breast cancers treated at Hyogo College of Medicine. Since a significant decrease in Ki67 was observed exclusively in the ER-positive subset, changes in Ki67 were further analyzed in terms of menopausal status. Significant

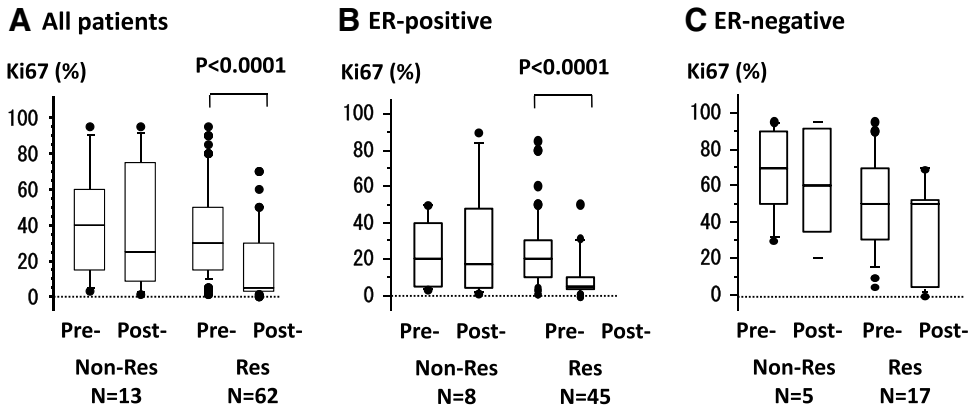

Fig. 1 Labeling indices of Ki67 for cancers treated with pre- and post-neoadjuvant chemotherapy according to clinical response for all patients (**a**), estrogen receptor (ER)-positive subset (**b**), and ER-negative subset (**c**) treated at Hyogo College of Medicine. *Res* clinical responder, *Non-Res* clinical non-responder. *Box* shows median and quartile range

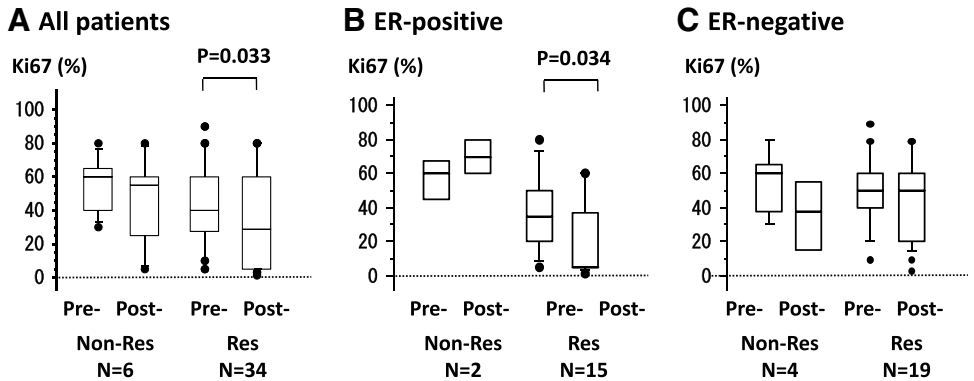

Fig. 2 Labeling indices of Ki67 for cancers treated with pre- and post-neoadjuvant chemotherapy according to clinical response for all patients (**a**), estrogen receptor (ER)-positive subset (**b**), and ER-negative subset (**c**) treated at Yao Municipal Hospital. *Res* clinical responder, *Non-Res* clinical non-responder. *Box* shows median and quartile range

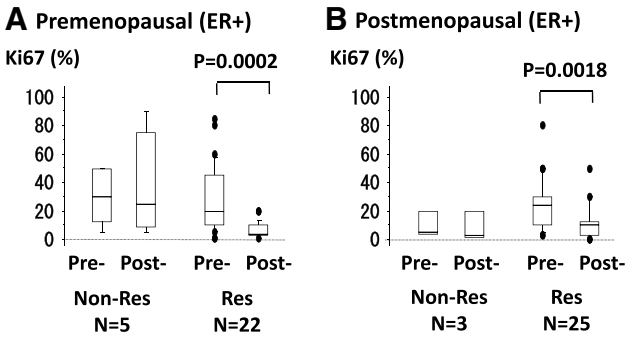

Fig. 3 Labeling indices of estrogen receptor (ER) for cancers treated with pre- and post-neoadjuvant chemotherapy for ER-positive premenopausal (**a**), and postmenopausal patients (**b**) treated at Hyogo College of Medicine. *Res* clinical responder, *Non-Res* clinical non-responder. *Box* shows median and quartile range

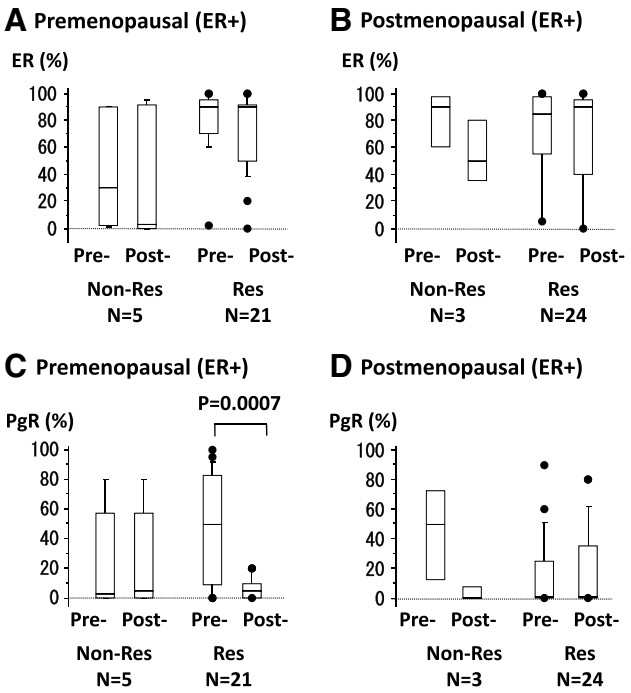

Fig. 4 Labeling indices of progesterone receptor (PgR) for cancers treated with pre- and post-neoadjuvant chemotherapy for estrogen receptor (ER)-positive premenopausal (**a**), and postmenopausal (**b**) treated at Hyogo College of Medicine. *Res* clinical responder, *Non-Res* clinical non-responder. *Box* shows median and quartile range

down-regulation of Ki67 among responders was detected in both premenopausal [post-NAC 4 (1–20) % vs. pre-NAC 17.5 (1–80) %, $P = 0.0002$; Fig. 3a] and postmenopausal [post-NAC 10 (0–50) % vs pre-NAC 23 (3–50) %, $P = 0.0018$; Fig. 3b] patients.

Changes in ER and PgR expression levels after chemotherapy for breast cancers of pathological non-responders according to menopausal status

ER expression levels after chemotherapy of the ER-positive subset of patients with residual cancers were not different from those before chemotherapy for both responders and non-responders even though menopausal status was taken into consideration (Fig. 4a, b). A significant reduction in PgR was identified in breast cancers of premenopausal patients with clinical response [post-NAC 5 (0–20) % vs pre-NAC 50 (0–100) %; $P = 0.0003$], but not in those with clinical non-response (Fig. 4c). However, there was no significant difference in PgR expression levels between pre- and post-NAC samples from postmenopausal patients even though clinical responders were included in the analysis (Fig. 4d). PgR reduction after NAC in postmenopausal clinical non-responders was remarkable (Fig. 4d), but since only 3 patients were included in this subset, no statistical significance could be established.

Discussion

The findings of the study presented here show that Ki67 expression levels in post-NAC samples were significantly reduced for clinical responders, even though the chemotherapy had not yet had a discernable pathological effect (pathological non-responders). Since a significant decrease in Ki67 after chemotherapy was restricted to clinical responders in the ER-positive but not in the ER-negative subsets, these results seem to indicate that the impact of down-regulation of Ki67 in the clinical response differs between these subsets of pathological non-responders. It has been demonstrated that post-treatment, but not pretreatment Ki67 expression levels provide additional prognostic information for patients who do not attain pCR [22, 23]. This prognostic impact of Ki67 after NAC was demonstrated elsewhere, specifically in breast cancer patients in the HR-positive subset [16].

Of the premenopausal breast cancer patients treated with adjuvant CMF (cyclophosphamide, methotrexate, and 5-fluorouracil), those with amenorrhea had significantly improved prognosis compared without amenorrhea [14]. Furthermore, in a randomized trial which compared the effect of four cycles of doxorubicin and cyclophosphamide followed by four cycles of docetaxel, four cycles of doxorubicin and docetaxel, or four cycles of doxorubicin, cyclophosphamide, and docetaxel, OS was improved for patients with amenorrhea for ≥6 months regardless of treatment [15]. These findings are a strong indication that chemotherapies function, at least in part, as endocrine therapy for premenopausal patients. In contrast to there being no associations between clinical response and difference between positive and negative PgR status (Table 2), PgR expression

levels were significantly reduced after chemotherapy in responders, but not in non-responders, while ER expression levels were similar. These observations seem to indicate that additional effects, mediated through suppression of estrogen signaling, may contribute to Ki67 reduction in clinical responders, at least among premenopausal patients. We also analyzed the relationship between pre-NAC PgR expression levels and clinical response. However, since there was no significant association between PgR expression levels at baseline and clinical response (data not shown), we believe that the reduction in PgR expression, and not the baseline expression, may contribute to clinical response. On the other hand, PgR expression levels for postmenopausal patients did not decrease even for clinical responders. This discrepancy may be explained by the low expression levels of PgR for postmenopausal patients. Additional effects linked to endocrine therapy for patients who attained Ki67 suppression may be associated with clinical response by ER-positive breast cancers of patients treated with chemotherapeutic regimens.

Although the reason is currently unknown, a decrease in Ki67 may not necessarily induce a positive response in the ER-negative subset. In this context, it has been reported that apoptotic indices increased in clinical [14] and pathological responders [15], but not in non-responders. In an in vivo study using MCF-7 cells which grew independently of estrogen, treatment with either fulvestrant or BKM120, a phosphatidylinositol 3 kinase (PI3 K) inhibitor, inhibited tumor growth, while the combination of the two drugs induced near-complete tumor regression. The combination therapy was thus significantly more effective than only one of the agents [24]. Interestingly, biological analyses disclosed that BKM120-treated tumors showed an increased expression of an apoptosis marker, cleaved caspase-3/7, but no change in Ki67-positive tumor cells. However, tumors treated with fulvestrant showed a reduction in Ki67-positive tumor cells but no change in cleaved-caspase-3/7-positive tumor cells. These results indicate that not only Ki67 reduction but also tumor cell death may be needed for a significant clinical response. One hypothesis suggests that a reduction in Ki67 can be induced by inhibition of growth factor signaling, including that by the epidermal growth factor receptor HER2, and of estrogen signaling [25–27]. Furthermore, it is well established that PI3 K/Akt and RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase pathways are involved in anti-apoptotic signaling [24]. It has also been demonstrated that, in addition to stimulation of cancer cell proliferation, estrogen signaling can inhibit apoptosis by means of estrogen-mediated mechanisms in many cell types [28, 29]. These results indicate that in addition to the effect of chemotherapy, inhibition of estrogen signaling may exert additional effects mediated through not only the inhibition of cell proliferation, but also induction of cell death for ER-positive patients.

The limitation of this study is that our analysis could not take HER2 status into consideration because the sample size was not large enough and the number of non-responders was too small. Validation of our findings is needed using larger samples as well as investigation of other aspects, including molecules associated with survival signals. Furthermore, correlations between PgR expression levels and ovarian function by means of determining serum estradiol levels after NAC as well as the prognostic significance of Ki67 reduction need to be examined.

In conclusion, we have identified a significant reduction in Ki67 expression levels after chemotherapy in breast cancer patients who demonstrated a clinical response. This reduction was observed only in the ER-positive subset. Since PgR expression levels were significantly suppressed in ER-positive breast cancers of premenopausal clinical responders, the impact of Ki67 suppression on clinical response may be associated with chemotherapeutic inhibition of estrogen signaling, at least in premenopausal patients. The suppression of estrogen signaling may have additional effects on chemotherapeutic procedures. Our findings are expected to provide useful information for considering strategies which improve the efficacy of such procedures.

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Conflict of interest The all authors declare that they have no conflict of interest.

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