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**Differential expression of progesterone receptor, FOXA1, GATA3 and p53
between pre- and postmenopausal women with estrogen receptor-positive
breast cancer**

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Abstract

Estrogen receptor (ER) is essential for estrogen-dependent growth, and its level of expression is considered a crucial determinant of response to endocrine therapy and prognosis in ER-positive breast cancer. On the other hand, the clinical role of progesterone receptor (PgR) in ER-positive breast cancer remains controversial, although testing of PgR by immunohistochemistry (IHC) has become routine. Recent studies indicated that plasma estradiol levels were related to expression levels of estrogen-responsive genes in ER-positive breast cancer tissues in both pre- and postmenopausal women. In this study, we analyzed expression levels of estrogen-responsive genes (PgR and TFF1), a progesterone-responsive gene (RANKL), ER-related genes (FOXA1 and GATA3), HER2, Ki67 and p53 in ER-positive, HER2-negative breast cancer tissues by IHC. Correlations between expression levels of these molecular markers and clinicopathological factors, including prognosis, were compared between pre- and postmenopausal women. Serum levels of estrone, estradiol, progesterone and testosterone were also measured. Expression levels of PgR, TFF1, RANKL and GATA3 were significantly higher in premenopausal women than in postmenopausal women. Serum estradiol levels were positively correlated with Ki67 labeling index (LI) in premenopausal women, but not in postmenopausal women. High expression of FOXA1 and GATA3 was significantly associated with improved disease-free survival in premenopausal women, but not in postmenopausal women, whereas high expression of PgR and low expression of p53 was significantly correlated with improved disease-free survival in postmenopausal women, but not in premenopausal women. Moreover, the best cutoff points of

Ki67 LI for disease-free survival were 30% for premenopausal women and 14% for postmenopausal women. Expression levels of ER, TFF1 and RANKL were not associated with disease-free survival in either pre- or postmenopausal women. Our results suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

Keywords

breast cancer, progesterone receptor, FOXA1, GATA3, Ki67, p53

Abbreviations

ER, estrogen receptor; PgR, progesterone receptor; TFF1, trefoil factor 1; RANKL, receptor activator of NF-kappa B ligand; FOXA1, forkhead box A1; GATA3, GATA binding protein 3; HER2, human epidermal growth factor receptor type 2; Ki67 LI, Ki67 labeling index; IHC, immunohistochemistry; BMI, body mass index

Introduction

Estrogen receptor (ER) is essential for estrogen-dependent growth, and its level of expression is considered a crucial determinant of response to endocrine therapy and prognosis in ER-positive breast cancer [1-4]. On the other hand, the clinical role of progesterone receptor (PgR) in ER-positive breast cancer remains controversial, although testing of PgR by immunohistochemistry (IHC) has become routine [5].

It was reported that quantitative analysis of ER and PgR expression provided highly significant information on risk of early relapse in postmenopausal patients in the Tamoxifen and Exemestane Adjuvant Multinational trial [4]. It has been demonstrated that ER-positive, PgR-negative tumors are a distinct subset of breast cancers characterized by aggressive behavior and tamoxifen resistance [6]. Canello and colleagues analyzed 4,837 women with breast cancer defined as luminal B (ER-positive and/or PgR-positive, HER2-positive and/or Ki67 $\geq$ 14% by IHC classification [7]) and found that PgR loss identified luminal B breast cancer subgroups at higher risk of relapse and death regardless of HER2 status [8]. It was recently reported that semiquantitative IHC expression of PgR added prognostic value in luminal A breast cancer [9]. In contrast, the EBCTCG study showed the benefit of adjuvant tamoxifen in reducing risk of recurrence regardless of PgR status in ER-positive breast cancer [10].

We previously reported that expression levels of PgR in pretreatment biopsies were not predictive of the response to neoadjuvant exemestane therapy, and that expression levels of PgR were decreased in posttreatment tumors compared to the levels in pretreatment specimens

regardless of the treatment response [11]. PgR is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes, such as *PGR* and trefoil factor 1 (*TFF1*)/*pS2*, in ER-positive breast cancer in both pre- and postmenopausal women [12, 13]. We previously showed that the incidence of ER-positive, PgR-negative breast cancer in women aged 50 years or younger and in those older than 50 years were 6% and 15%, respectively, whereas for ER-positive, PgR-positive tumors, incidences were 81% and 64%, respectively [14]. Moreover, most tumors had high PgR expression in women aged 50 or younger, while the distribution of PgR expression levels was evenly spread in tumors in women over 50 years of age. It is suggested that reduced circulating estrogens after menopause could be the cause of the increase of ER-positive/PgR-negative or ER-positive/low-PgR tumors in postmenopausal women [15]. On the other hand, PgR does not fully reflect estrogen dependence: many PgR-negative tumors respond to endocrine therapy, such as tamoxifen or aromatase inhibitors [11, 16-18].

We hypothesized that the clinical role of expression of estrogen-responsive genes may differ between pre- and postmenopausal women with ER-positive breast cancer. The present study was undertaken to assess expression of estrogen-responsive genes (PgR and TFF1), a progesterone-responsive gene (RANKL), ER-related genes (FOXA1 and GATA3), HER2, Ki67 and p53 in ER-positive, HER2-negative breast cancer samples by IHC. Correlations between expression levels of these molecular markers and clinicopathological factors, including prognosis, were compared between pre- and postmenopausal women.

Materials and methods

Patients and breast cancer tissues

A total of 289 women treated for Stage I to III breast cancer between 2004 and 2010 at Hokkaido University Hospital were recruited to this study (Table 1). The study protocol was approved by the institutional review board and conformed to the guidelines of the 1996 Declaration of Helsinki. Written informed consent for the use of surgically resected tumor tissues was provided by all patients prior to treatments. The samples were chosen from a continuous series of ER-positive, HER2-negative breast cancer. All patients had undergone mastectomy or lumpectomy. Patients treated with neoadjuvant chemotherapy were excluded. Tumor samples were obtained during surgery. Patients received adequate endocrine or chemotherapy as adjuvant therapy, and patients who had positive results of axillary lymph node dissection received adjuvant chemotherapy, as well as adjuvant endocrine therapy. The median follow-up period was 66.2 months (range, 3 to 114 months).

Immunohistochemical (IHC) analysis

One 4- μ m section of each submitted paraffin block was stained first with hematoxylin-eosin to verify that an adequate number of carcinoma cells were present and that the fixation quality was adequate for IHC analysis. Serial sections (4 μ m) were then prepared from selected blocks and float-mounted on adhesive-coated glass slides for IHC. Details of antibodies and evaluation methods are described in Table 2, and representative images of staining for TFF1, RANKL,

FOXA1 and GATA3 are shown in Figure 1. p53 was considered positive if there was $\geq 10\%$ positive nuclear staining regardless of the intensity [19-21]. The Ki67 labeling index (LI) was assessed as the percentage of tumor cells showing definite nuclear staining among >1000 invasive tumor cells using NanoZoomer 2.0-HT (Hamamatsu photonics, Hamamatsu, Japan) for slide scanning and Tissue Studio (Definiens, Munich, Germany) for automated scoring [22]. HER2-positive tumors were excluded from this study.

Measurements of serum samples

Blood samples were taken at the time of surgery and centrifuged at 1300g for 10 minutes, then the separated sera were stored in aliquots at $-20\text{ }^{\circ}\text{C}$. Serum samples were obtained from 19 premenopausal patients and 49 postmenopausal patients. The concentration of serum estrone (E1) was measured by radioimmunoassay (dextran coated charcoal method) using anti-estrone-antiserum (ASKA Pharma Medical Co., Kawasaki, Japan) according to the manufacturer's instructions. The concentration of estradiol (E2) was measured using a commercially available direct radioimmunoassay; RIACoAT ESTRADIOL US (CIS bio international, Gif-sur-Yvette, France) for estradiol [14]. Serum progesterone and testosterone levels were measured by electrochemiluminescence immunoassay using Ecrusis progesterone II and Ecrusis Testosterone, respectively (Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Mann-Whitney U tests were used to compare expression of biological markers and serum hormone levels between pre- and postmenopausal women. Spearman's rank correlation test was used to study relationships between expression levels of biological markers and clinicopathological factors. Spearman's correlation coefficient $> +0.40$ or < -0.40 and $P < 0.05$ in Spearman's rank correlation test were considered significant. Estimation of disease-free survival was performed using the Kaplan-Meier method, and differences between survival curves were assessed with the log-rank test. Cox's proportional hazards model was used for univariate and multivariate analyses of prognostic values.

Results

Comparison of expression levels of biological markers between pre- and postmenopausal women

We first examined expression levels of biological factors, including ER, PgR, TFF1, RANKL, FOXA1, GATA3, HER2, Ki67 and p53 in pre- and postmenopausal breast cancer tissues (Table 3). Expression levels of PgR, TFF1, RANKL and GATA3 were significantly higher in premenopausal women than in postmenopausal women ($P < 0.0001$, $P = 0.0002$, $P = 0.0005$ and $P < 0.0001$, respectively). In contrast, expression levels of ER, FOXA1, HER2, Ki67 and p53 did not differ between pre- and postmenopausal women.

Expression levels of ER, PgR and Ki67 were then analyzed in detail in pre- and postmenopausal women. Most tumors in both pre- and postmenopausal women showed high ER expression (90% or more positive cells) (Fig. 2a, b). In contrast, in premenopausal women, most tumors had high PgR expression, with 90% or more positive cells (Fig. 2a), whereas in postmenopausal women 30% of the tumors were PgR-negative and the distribution of frequencies of PgR positive cells was evenly spread (Fig. 2b). On the other hand, the distribution of frequencies of Ki67 LI was similar between pre- and postmenopausal women (Fig. 2c, d).

Comparison of serum hormone levels between pre- and postmenopausal women

Serum levels of E1, E2, progesterone and testosterone were measured in 19 premenopausal and 49 postmenopausal women (Table 4). E1, E2 and progesterone levels were significantly higher in

premenopausal women than in postmenopausal women ($P = 0.001$, $P = 0.002$ and $P = 0.039$, respectively). Serum testosterone levels did not differ between pre- and postmenopausal women.

Correlations between expression levels of biological markers in pre- and postmenopausal women

Links between expression levels of biological markers were analyzed using Spearman's rank correlation test (Table 5). In premenopausal women, expression levels of ER were positively correlated with those of PgR, FOXA1 and GATA3, although those were not statistically significant (Spearman's correlation coefficient; +0.364 ($P = 0.0004$), +0.255 ($P = 0.019$) and +0.313 ($P = 0.0035$), respectively). On the other hand, expression levels of ER were positively correlated with those of PgR, FOXA1, GATA3 and HER2 in postmenopausal women (Spearman's correlation coefficient; +0.272 ($P = 0.0001$), +0.338 ($P < 0.0001$), +0.305 ($P < 0.0001$) and +0.233 ($P = 0.0009$), respectively). PgR expression was positively correlated with FOXA1 and GATA3 expression in pre-menopausal women (Spearman's correlation coefficient; +0.262 ($P = 0.016$) and +0.224 ($P = 0.039$), respectively), whereas PgR expression was not correlated with expression of other biological markers except ER in postmenopausal women. TFF1 expression was positively correlated with RANKL expression in premenopausal women (Spearman's correlation coefficient; +0.347 ($P = 0.0011$)), whereas TFF1 expression was positively correlated with FOXA1 and Ki67 expression in postmenopausal women (Spearman's correlation coefficient; +0.260 ($P = 0.0002$) and +0.215 ($P = 0.003$), respectively). Expression

levels of FOXA1 and GATA3 were strongly and positively correlated in both pre- and postmenopausal women (Spearman's correlation coefficient; +0.436 ($P < 0.0001$) and +0.479 ($P < 0.0001$), respectively). FOXA1 expression was positively correlated with RANKL expression in postmenopausal women (Spearman's correlation coefficient; +0.253 ($P = 0.0003$)), but not in premenopausal women. Expression levels of Ki67 and p53 were positively correlated in both pre- and postmenopausal women (Spearman's correlation coefficient; +0.322 ($P = 0.0030$) and +0.390 ($P < 0.0001$), respectively).

Correlations between expression levels of biological markers and clinicopathological factors in pre- and postmenopausal women

We next analyzed the correlation between expression levels of biological markers and clinicopathological factors, such as age, body mass index (BMI), tumor size, nodal status and tumor grade in pre- and postmenopausal women (Table 6). RANKL expression was negatively associated with tumor size in both pre- and postmenopausal women (Spearman's correlation coefficient; -0.362 ($P = 0.0007$) and -0.207 ($P = 0.0035$), respectively). GATA3 expression was negatively correlated with tumor size and lymph node status in premenopausal women (Spearman's correlation coefficient; -0.239 ($P = 0.028$) and -0.304 ($P = 0.0050$), respectively), but not in postmenopausal women. Ki67 LI was negatively correlated with age (Spearman's correlation coefficient; -0.326 ($P = 0.0027$)) and positively associated with tumor grade (Spearman's correlation coefficient; +0.356 ($P = 0.010$)) in premenopausal women. On the other

hand, Ki67 LI was positively correlated with tumor size and grade in postmenopausal women (Spearman's correlation coefficient; +0.295 ($P < 0.0001$) and +0.262 ($P = 0.0003$), respectively). p53 expression was negatively correlated with age (Spearman's correlation coefficient; -0.230 ($P = 0.032$)) in premenopausal women, whereas p53 expression was positively correlated with tumor grade in postmenopausal women (Spearman's correlation coefficient; +0.270 ($P = 0.0001$)). Expression of ER, PgR, TFF1, FOXA1 and HER2 was not associated with clinicopathological factors in either pre- or postmenopausal women.

Correlations between serum hormone levels and biological and clinicopathological factors

Serum hormone levels, such as E1, E2, progesterone and testosterone, were compared with expression levels of biological and clinicopathological factors in pre- and postmenopausal women. Serum E2 was positively correlated with tumor size and Ki67 LI (Spearman's correlation coefficient; +0.429 and +0.444, respectively) and negatively correlated with age (Spearman's correlation coefficient; -0.515) in premenopausal women. Serum testosterone was negatively correlated with age (Spearman's correlation coefficient; -0.555) in premenopausal women. Serum E2 was negatively correlated with age (Spearman's correlation coefficient; -0.314) in postmenopausal women. Other serum hormone levels analyzed in this study were not correlated with biological or clinicopathological factors in either pre- or postmenopausal women.

Biological factors related to survival differ between pre- and postmenopausal women

To identify a clinically meaningful cutoff point for each biological factor, various levels of expression were tested using Kaplan-Meier analysis and verified by the log-rank test for disease-free survival. When the cutoff points for determining the division between negative, low and high expression of PgR were set as 1% and 50% in postmenopausal women, Kaplan-Meier analysis showed that high PgR expression was significantly correlated with increased disease-free survival compared with no PgR expression ($P = 0.031$; Fig. 3b). The survival curve of low PgR expression (1%-50%) was almost identical to that of high PgR expression (>50%). In contrast, PgR expression was not correlated with disease-free survival in premenopausal women (Fig. 3a). When the cutoff points for determining the division between low and high expression were set at 70% for FOXA1, 80% for GATA3 and 30% for Ki67 in premenopausal women, high FOXA1 (Fig. 3c), high GATA3 (Fig. 3e) and low Ki67 (Fig. 3i) expression was strongly associated with increased disease-free survival ($P < 0.0001$, $P = 0.0059$ and $P = 0.0004$, respectively). On the other hand, in postmenopausal women, when the cutoff point for determining the division between low and high Ki67 LI was set at 14%, low Ki67 LI was strongly associated with increased disease-free survival ($P = 0.0005$; Fig. 3h). In contrast, expression of FOXA1 (Fig. 3d) and GATA3 (Fig. 3f) was not correlated with disease-free survival in postmenopausal women. When the cutoff point for determining the division between low and high expression of p53 was set at 10%, low p53 expression was strongly associated with increased disease-free survival in postmenopausal women ($P < 0.0001$; Fig. 3l). Surprisingly, p53 expression levels were less than 10% in all the premenopausal women who relapsed (Fig.

3k).

We then analyzed whether expression levels of biological markers were associated with altered prognosis of premenopausal patients with ER-positive, HER2-negative breast cancer (Table 7). Univariate analysis demonstrated significant association between improved disease-free survival and expression of high FOXA1 ($P = 0.0006$), high GATA3 ($P = 0.029$) and low (<30%) Ki67 ($P = 0.0045$), as well as small tumor size ($P = 0.0003$) and lymph node status ($P < 0.0001$). In multivariate analysis, a significant association was observed between disease-free survival and tumor size ($P = 0.044$) and Ki67 expression (cut off, 30%) ($P = 0.029$). On the other hand, in postmenopausal women, univariate analysis showed a significant association between improved disease-free survival and expression of low (<14%) Ki67 ($P = 0.0014$) and low p53 ($P = 0.0001$), as well as lymph node status ($P = 0.047$) and tumor grade ($P = 0.0085$). In multivariate analysis, a significant correlation was observed between disease-free survival and p53 expression ($P = 0.0061$). Expression levels of ER, TFF1 and RANKL were not associated with disease-free survival in either pre- or postmenopausal women.

Discussion

In this study, we analyzed expression of ER, PgR, TFF1, RANKL, FOXA1, GATA3, HER2, Ki67 and p53 in ER-positive, HER2-negative breast cancer in pre- and postmenopausal women. Among our patients, expression levels of PgR, TFF1, RANKL and GATA3 were significantly higher in premenopausal women than in postmenopausal women. Expression of high FOXA1 and high GATA3 was significantly associated with improved disease-free survival in premenopausal women, but not in postmenopausal women, whereas expression of high PgR and low p53 was significantly correlated with improved disease-free survival in postmenopausal women, but not in premenopausal women. Moreover, the best cutoff points of Ki67 LI for disease-free survival were 30% for premenopausal women and 14% for postmenopausal women.

Most studies analyzing predictive or prognostic factors in ER-positive breast cancer have been performed in postmenopausal women, mainly using patients and samples in adjuvant aromatase inhibitor trials [3, 4, 23]. In premenopausal women, however, even the clinical role of PgR has been little analyzed so far. We previously analyzed genetic and environmental predictors, endogenous hormones and growth factors, and risk of ER-positive breast cancer, and demonstrated that risk factors differed between women of different menopausal status [24]. It has been reported that plasma estradiol levels are significantly associated with estrogen-responsive gene expression of ER-positive breast cancers in both pre- and postmenopausal women [12, 13]. Because plasma E1, E2 and progesterone levels are much higher in premenopausal women compared with those in postmenopausal women as shown in this study, risk factors and

biological characteristics of ER-positive breast cancer might differ according to menopausal status. We have shown that expression levels of PgR and TFF1 in breast cancer tissues were significantly higher in premenopausal women than in postmenopausal women. TFF1, originally known as pS2, was the gene which correlated most significantly with plasma estradiol concentration in postmenopausal ER-positive breast cancer tissues [13]. It was also reported that expression levels of TFF1, similar to PgR, in ER-positive breast tumors differed across the menstrual cycle in premenopausal women, although expression of proliferation genes, such as Ki67, did not differ during the cycle [12].

RANKL, involved in the control of bone remodeling [25], is one of the progesterone-responsive genes [26]. Previous studies identified RANKL as a pivotal paracrine mediator of progesterone function in mouse mammary gland development and mammary carcinogenesis [27]. It was recently reported that RANKL expression responded to progesterone and was required for progesterone-induced breast tissue proliferation [28]. In this study we found that expression levels of RANKL in breast cancer tissues were significantly higher in premenopausal women than in postmenopausal women, as serum progesterone levels were higher in premenopausal women compared with postmenopausal women. However, our results also indicate that RANKL expression is inversely correlated with tumor size in both pre- and postmenopausal women, suggesting that the progesterone-RANKL pathway may not be involved in estrogen-dependent growth in ER-positive breast cancer.

FOXA1, a forkhead family transcription factor, is reportedly expressed predominantly

in luminal A breast cancer with favorable prognosis [29-32]. We recently demonstrated that FOXA1 expression is much higher in ER-high, Ki67-low tumors than in ER-low, Ki67-high tumors [33]. GATA3 is also a transcription factor which is critical for normal mammary development [34]. Recent whole genome analysis identified a GATA3 mutation which exists in 14% of luminal A breast cancer [35]. It was reported that ER α , FOXA1 and GATA3 form a functional enhanceosome to drive the transcription of ER α -regulated genes in breast cancer cells [36, 37]. High FOXA1 and GATA3 expression might therefore affect endocrine responsiveness and prognosis in ER-positive breast cancer [38]. However, menopausal status has not been considered in previous studies on FOXA1 and GATA3 in ER-positive breast cancer. We demonstrate in this study that expression of GATA3 is significantly higher in premenopausal women than in postmenopausal women. Moreover, expression of high FOXA1 and high GATA3 is significantly associated with improved disease-free survival in premenopausal women, but not in postmenopausal women, although FOXA1 and GATA3 expression is strongly correlated in both pre- and postmenopausal women. The mechanism of estrogen-dependent growth and the clinical role of FOXA1 and GATA3 in ER-positive breast cancer might differ with differing menopausal status.

We also identified that the best cutoff points of the Ki67 LI for disease-free survival were 30% for premenopausal women and 14% for postmenopausal women. Moreover, serum estradiol levels were positively associated with Ki67 LI in premenopausal women, but not in postmenopausal women. Furthermore, our results indicate that p53 accumulation is significantly

correlated with decreased disease-free survival in postmenopausal women, but not in premenopausal women. Surprisingly, p53 expression levels were less than 10% in all premenopausal women who relapsed. Because the clinical significance of Ki67 LI and p53 alteration in premenopausal ER-positive breast cancer alone has not been reported, further studies will be necessary to confirm this point.

Finally, our data demonstrate that expression levels of ER correlate positively with HER2 expression score evaluated by IHC in postmenopausal women, but not in premenopausal women. Moreover, HER2 expression is not correlated with expression of other biological factors analyzed in this study in either pre- or postmenopausal women. It was recently reported that ER and HER2 expression, of both mRNA and protein, is positively correlated in HER2-negative tumors, validated by a TransATAC study for postmenopausal women [39].

In conclusion, the present study indicates that the clinical role of expression of estrogen-responsive genes (PgR and TFF1), a progesterone-responsive gene (RANKL), ER-related genes (FOXA1 and GATA3), HER2, Ki67 and p53 in ER-positive, HER2-negative breast cancer differs between pre- and postmenopausal women. Our results suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

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Figure legends

Fig. 1 Immunohistochemical examination of TFF1 (**a**), RANKL (**b**), FOXA1 (**c**) and GATA3 (**d**) in invasive breast carcinoma. Representative results of positive staining are shown. Cytoplasmic staining of TFF1 (**a**) and RANKL (**b**) and nuclear staining of FOXA1 (**c**) and GATA3 (**d**) are seen in breast cancer cells.

Fig. 2 Expression levels of estrogen receptor (ER) and progesterone receptor (PgR) assessed by percentage of positive cells in ER-positive, HER2-negative breast cancer in premenopausal (**a**) and postmenopausal (**b**) women. Ki67 labeling index (LI) in ER-positive, HER2-negative breast cancer in premenopausal (**c**) and postmenopausal (**d**) women.

Fig. 3 Disease-free survival according to expression of PgR (**a, b**), FOXA1 (**c, d**), GATA3 (**e, f**), Ki67 (**g, h** cut off, 14%; **i, j** cut off, 30%) and p53 (**k, l**) in ER-positive, HER2-negative breast cancer in premenopausal (**a, c, e, g, i, k**) and postmenopausal (**b, d, f, h, j, l**) women.

Table 1: Clinicopathological characteristics of patients and tumors

		Premenopausal	Postmenopausal
No. of patients		89	200
age (years)	mean $\pm$ SD (range)	44.7 $\pm$ 5.1 (27-56)	63.4 $\pm$ 8.5 (42-92)
Body mass index	mean $\pm$ SD (range)	22.1 $\pm$ 3.8 (15.2-34.9)	24.4 $\pm$ 4.3 (13.6-40.9)
Tumor size (cm)	≤ 2.0	72 (81%)	142 (71%)
	2.1 – 5.0	15 (17%)	50 (25%)
	> 5.0	3 (3%)	8 (4%)
No. of positive lymph nodes	0	63 (71%)	159 (80%)
	1-3	21 (24%)	31 (16%)
	≥ 4	5 (6%)	10 (5%)
Tumor grade	1	32 (36%)	50 (25%)
	2	49 (55%)	134 (67%)
	3	8 (9%)	16 (8%)
Histology	Invasive ductal carcinoma	73 (82%)	177 (87%)
	Others	16 (18%)	23 (13%)
Surgery type	Mastectomy	35 (39%)	102 (51%)
	Lumpectomy	54 (61%)	98 (49%)
Postoperative adjuvant therapy			
None		8 (9%)	9 (4%)
Any endocrine therapy		81 (91%)	191 (96%)
	Tamoxifen	30	14
	Tamoxifen + LHRH agonist	39	0
	LHRH agonist	6	0
	Aromatase inhibitors	6	187
Combined endocrine therapy and chemotherapy		23 (26%)	33 (17%)
Follow-up (months)	mean $\pm$ SD (range)	63 $\pm$ 29 (7-112)	68 $\pm$ 26 (3-114)

LHRH agonist, luteinizing hormone-releasing hormone agonist for a minimum of 2 years

Table 2: List of antibodies used for immunohistochemical analysis

	Antibody	Species (dilution)	2 nd antibody	Evaluation
ER	Ventana Medical Systems ^a , SP1	rabbit monoclonal (prediluted)	Ventana iVIEW DAB Detection Kit	percentage of cells showing positive nuclear staining
PgR	Ventana Medical Systems, 1E2	rabbit monoclonal (prediluted)	Ventana iVIEW DAB Detection Kit	percentage of cells showing positive nuclear staining
TFF1	Abcam ^b , SPM313	mouse monoclonal (prediluted)	Ventana iVIEW DAB Detection Kit	average cytoplasmic staining intensity of positive cells (0, 1, 2, 3)
RANKL	Santa Cruz Biotechnology ^c , FL-317	rabbit polyclonal (1:100)	DakoEnVison FLEX system	average cytoplasmic staining intensity of positive cells (0, 1, 2, 3)
FOXA1	Santa Cruz Biotechnology ^c , Q-6	mouse monoclonal (1:400)	Dako EnVison FLEX system	percentage of cells showing positive nuclear staining
GATA3	Biocare Medical ^d , L50-823	mouse monoclonal (1:400)	Dako EnVison FLEX system	percentage of cells showing positive nuclear staining
HER2	Ventana Medical Systems, 4B5	rabbit monoclonal (prediluted)	Ventana iVIEW DAB Detection Kit	0, 1+, 2+, 3+
Ki67	DAKO ^e , MIB-1	mouse monoclonal (1:200)	Dako EnVison FLEX system	labeling index
p53	DAKO, DO-7	mouse monoclonal (1:200)	Dako EnVison FLEX system	percentage of cells showing positive nuclear staining

^a Ventana Medical Systems, Tucson, USA^b Abcam, Cambridge, UK^c Santa Cruz Biotechnology, Dallas, USA^d Biocare Medical, Concord, USA^e DAKO, Glostrup, Denmark

Table 3. Comparison of expression levels of biological markers between pre- and postmenopausal women

	Premenopausal (mean $\pm$ SD)	Postmenopausal (mean $\pm$ SD)	<i>P</i>
ER	79.8 $\pm$ 23.0%	83.0 $\pm$ 20.9%	0.17
PgR	64.2 $\pm$ 34.8%	31.1 $\pm$ 32.5%	<0.0001*
TFF1	1.97 $\pm$ 0.72	1.63 $\pm$ 0.70	0.0002*
RANKL	0.85 $\pm$ 0.83	0.48 $\pm$ 0.68	0.0005*
FOXA1	91.8 $\pm$ 16.5%	88.5 $\pm$ 22.5%	0.17
GATA3	81.2 $\pm$ 26.5%	63.8 $\pm$ 37.0%	<0.0001*
HER2	0.78 $\pm$ 0.75	0.63 $\pm$ 0.75	0.11
Ki67	13.9 $\pm$ 12.6%	12.2 $\pm$ 9.6%	0.27
p53	6.84 $\pm$ 15.7%	5.23 $\pm$ 15.6%	0.43

**P* < 0.05 is considered significant.

Table 4. Comparison of serum hormone levels between pre- and postmenopausal women

	Premenopausal (mean $\pm$ SD)	Postmenopausal (mean $\pm$ SD)	<i>P</i>
Estrone (E1) (pg/ml)	197.3 $\pm$ 115.8	103.7 $\pm$ 33.0	0.001*
Estradiol (E2) (pg/ml)	158.5 $\pm$ 207.3	3.67 $\pm$ 2.29	0.002*
Progesterone (ng/ml)	2.86 $\pm$ 6.19	0.203 $\pm$ 0.121	0.039*
Testosterone (ng/ml)	0.273 $\pm$ 0.131	0.245 $\pm$ 0.135	0.227

* $P < 0.05$ is considered significant.

Table 5. Correlations between expression levels of biological markers in pre- and postmenopausal women

Premenopausal women								
	ER	PgR	TFF1	RANKL	FOXA1	GATA3	HER2	Ki67
PgR	+0.364 ^a 0.0004* ^b							
TFF1	+0.018 0.87	-0.016 0.88						
RANKL	+0.103 0.35	+0.009 0.93	+0.347 0.0011*					
FOXA1	+0.255 0.019*	+0.262 0.016*	+0.067 0.54	-0.006 0.96				
GATA3	+0.313 0.0035*	+0.224 0.039*	-0.051 0.64	-0.022 0.84	+0.436* <0.0001*			
HER2	+0.094 0.39	-0.064 0.56	-0.175 0.11	-0.077 0.49	+0.190 0.085	+0.114 0.30		
Ki67	-0.004 0.968	+0.023 0.84	+0.097 0.38	-0.101 0.36	-0.020 0.86	-0.129 0.25	+0.100 0.37	
p53	-0.098 0.37	-0.080 0.46	+0.065 0.55	-0.042 0.70	-0.014 0.90	-0.094 0.39	-0.001 0.99	+0.322 0.0030*
Postmenopausal women								
	ER	PgR	TFF1	RANKL	FOXA1	GATA3	HER2	Ki67
PgR	+0.272 ^a 0.0001* ^b							
TFF1	+0.0003 0.99	-0.037 0.60						
RANKL	+0.102 0.16	-0.019 0.79	+0.061 0.40					
FOXA1	+0.338 <0.0001*	+0.118 0.098	+0.260 0.0002*	+0.253 0.0003*				
GATA3	+0.305 <0.0001*	+0.093 0.196	+0.132 0.065	+0.125 0.080	+0.479* <0.0001*			
HER2	+0.233 0.0009*	+0.034 0.635	-0.087 0.22	-0.0068 0.92	+0.137 0.055	+0.138 0.053		
Ki67	+0.073 0.315	-0.014 0.853	+0.215 0.003*	+0.095 0.20	+0.170 0.019	+0.128 0.079	+0.136 0.062	
p53	-0.037 0.61	-0.093 0.19	+0.107 0.13	+0.022 0.76	+0.002 0.98	-0.096 0.18	+0.147 0.038	+0.390 <0.0001*

^a Spearman's correlation coefficient^b *P*, Spearman's rank correlation test*Spearman's correlation coefficient > +0.40 or < -0.40 and *P* < 0.05 is considered significant.

Table 6. Correlations between expression levels of biological markers and clinicopathological factors in pre- and postmenopausal women

Premenopausal women					
	age	BMI	Tumor size	Lymph node status	Tumor grade
ER	-0.030 ^a	-0.073	-0.069	-0.173	-0.160
	0.78 ^b	0.50	0.52	0.11	0.13
PgR	-0.090	-0.165	-0.087	-0.151	-0.079
	0.40	0.12	0.42	0.16	0.46
TFF1	+0.166	+0.154	-0.146	-0.085	+0.138
	0.13	0.15	0.18	0.44	0.20
RANKL	+0.121	+0.020	-0.362	-0.208	+0.011
	0.27	0.86	0.0007*	0.058	0.92
FOXA1	+0.048	-0.190	-0.047	-0.074	-0.191
	0.66	0.082	0.67	0.50	0.08
GATA3	-0.013	-0.091	-0.239	-0.304	-0.210
	0.91	0.41	0.028*	0.0050*	0.054
HER2	+0.012	-0.135	+0.013	-0.082	+0.211
	0.91	0.21	0.90	0.46	0.050
Ki67	-0.326	-0.204	+0.216	+0.144	+0.356
	0.0027*	0.065	0.050	0.20	0.0010*
p53	-0.230	-0.174	+0.059	-0.059	+0.121
	0.032*	0.11	0.58	0.59	0.26
Postmenopausal women					
	age	BMI	Tumor size	Lymph node status	Tumor grade
ER	+0.127 ^a	-0.006	-0.064	-0.085	-0.134
	0.073 ^b	0.94	0.37	0.24	0.059
PgR	+0.092	+0.160	-0.008	-0.127	-0.029
	0.19	0.024	0.91	0.077	0.69
TFF1	+0.088	-0.007	+0.076	-0.031	-0.035
	0.22	0.92	0.28	0.67	0.63
RANKL	-0.086	+0.021	-0.207	-0.031	-0.199
	0.23	0.77	0.0035*	0.67	0.0051
FOXA1	+0.071	-0.049	-0.055	-0.028	-0.113
	0.32	0.49	0.44	0.70	0.11
GATA3	+0.079	-0.113	-0.112	-0.084	-0.188
	0.27	0.11	0.12	0.25	0.0083
HER2	-0.023	+0.001	-0.108	+0.087	-0.041
	0.75	0.99	0.13	0.23	0.57
Ki67	+0.084	+0.045	+0.295	+0.127	+0.262
	0.25	0.54	<0.0001*	0.084	0.0003*
p53	-0.020	+0.030	+0.024	-0.020	+0.270
	0.78	0.68	0.74	0.79	0.0001*

^a Spearman's correlation coefficient

^b *P*, Spearman's rank correlation test

*Spearman's correlation coefficient > +0.40 or < -0.40 and *P* < 0.05 is considered significant.

Table 7. Univariate and multivariate analysis of factors predicting disease-free survival in pre- and postmenopausal women

Premenopausal women						
Factor	Univariate			Multivariate		
	RR ^a	95% CI ^b	<i>P</i>	RR ^a	95% CI ^b	<i>P</i>
BMI	0.83	0.63 to 1.10				
Tumor size	1.48	1.20 to 1.84	0.0003*	1.64	1.01 to 2.67	0.044*
Lymph node status	1.19	1.10 to 1.30	<0.0001*	1.13	0.98 to 1.32	0.10
Tumor grade	1.97	0.56 to 6.92	0.29			
ER	1.00	0.96 to 1.03	0.95			
PgR	0.99	0.97 to 1.01	0.25			
TFF1	0.70	0.13 to 3.83	0.68			
RANKL	0.53	0.06 to 4.60	0.57			
FOXA1 (≤70%, >70%)	0.06	0.01 to 0.30	0.0006*	0.73	0.03 to 17.40	0.84
GATA3 (≤80%, >80%)	0.09	0.01 to 0.78	0.029*	0.075	0.0015 to 3.66	0.19
HER2	0.86	0.30 to 2.50	0.79			
Ki67 (<30%, ≥30%)	10.30	2.06 to 51.46	0.0045*	15.27	1.32 to 177.05	0.029*
p53 (<10%, ≥10%)	-	-	-			
Postmenopausal women						
Factor	Univariate			Multivariate		
	RR ^a	95% CI ^b	<i>P</i>	RR ^a	95% CI ^b	<i>P</i>
BMI	0.96	0.86 to 1.07	0.48			
Tumor size	1.25	1.00 to 1.56	0.051			
Lymph node status	1.19	1.00 to 1.41	0.047*	1.00	0.84 to 1.19	0.98
Tumor grade	2.98	1.32 to 6.74	0.0085*	2.17	0.85 to 5.56	0.11
ER	1.01	0.98 to 1.03	0.58			
PgR	0.99	0.97 to 1.00	0.10			
TFF1	2.29	0.88 to 5.95	0.09			
RANKL	0.65	0.09 to 4.83	0.67			
FOXA1 (≤70%, >70%)	1.39	0.32 to 5.98	0.66			
GATA3 (≤80%, >80%)	0.83	0.34 to 2.05	0.69			
HER2	0.87	0.48 to 1.59	0.66			
Ki67 (<15%, ≥15%)	4.46	1.78 to 11.18	0.0014*	2.22	0.79 to 6.28	0.13
p53 (<10%, ≥10%)	6.01	2.49 to 14.51	0.0001*	4.03	1.49 to 10.92	0.0061*

^a relative risk

^b confidence interval

**P* < 0.05 is considered significant.

50 μm

50 μ m

Figure 2

Figure 2

Figure 3

Figure 3

Figure 3

Figure 3

Figure 3

Figure 3