Effects of serum estradiol and progesterone on estrogen-regulated gene expression in breast cancers of premenopausal patients

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Abstract

Background: Expression of estrogen receptor α in breast cancer is essential for estrogen-dependent growth and partially determines the breast cancer subtype. In premenopausal women, expression of estrogen-regulated genes in estrogen receptor-positive breast cancer tissues are reportedly influenced by the menstrual cycle.

Methods: We investigated correlations between serum estradiol (E2; tested on the day of surgery) and expression of estrogen-regulated genes and proliferation genes in strongly estrogen receptor α-positive breast cancer tissues from 91 premenopausal women by quantitative reverse transcription-polymerase chain reaction. We also investigated correlations between serum progesterone levels on the day of surgery and mRNA expression of progesterone-regulated genes and proliferation genes.

Results: The serum E2 level affected expression of estrogen-regulated genes, including progesterone receptor (P = 0.016, Rs = 0.07) but showed no correlation with expression of genes associated with proliferation. We also observed strong positive correlations between mRNA expression of ESR1 and that of estrogen-regulated genes (P < 0.0001, Rs = 0.329–0.756) and proliferation genes (P < 0.0001, Rs = 0.753–0.843). The serum progesterone level affected expression of RANKL mRNA. However, we observed no correlations between serum progesterone and expression of Wnt-4 or proliferation genes.

Conclusions: The serum E2 level on the day of surgery influences estrogen-regulated gene expression moderately in patients found to be strongly positive for estrogen receptor α by immunohistochemistry. Changes in serum E2 levels might influence the results of molecular profiling tests in premenopausal women with breast cancer.

Key words: breast cancer, estradiol, estrogen receptor α, progesterone receptor, progesterone
Introduction

Estrogen receptor (ER) α is a member of the nuclear receptor family, which regulates the transformed phenotype of more than 70% of breast cancers (1). ERα-positive tumors are recognized as genetically distinct from ERα-negative tumors (2). Estrogens are major drivers of breast cancer development and promote carcinogenesis of ERα-positive breast cancers (3,4). Treatment options for such patients include endocrine therapies that inhibit ERα signaling by antagonizing ERα-ligand binding, downregulating ERα or suppressing estrogen production (5).

Progesterone also drives breast cancer development and promotes carcinogenesis of ERα and/or progesterone receptor (PgR)-positive breast cancers (3,4). The role of PgR has not yet been elucidated in ERα-positive breast cancer. Recently, expression of both PgR protein and mRNA were reported to correlate with menstrual cycles of premenopausal patients with ERα-positive breast cancer (6). The same study also showed that mRNA expression of other estrogen-regulated genes (ERGs) differed during the menstrual cycle (6). Hosoda et al. showed that ERG protein expression was higher in premenopausal patients than in postmenopausal patients (7). These reports suggested that serum estradiol (E2) levels at surgery may affect the expression of ERGs, including PgR, in ERα-positive breast cancers of premenopausal patients.

The use of molecular profiling tests, including the 21-gene recurrence score assay OncotypeDX®, 70-gene breast cancer recurrence assay MammaPrint®, and PAM50, has recently become widespread, and they all include tests for ER, ERGs and PgR. Although PgR is considered to be an estrogen-regulated gene, its expression might change during the menstrual cycle, as described above, possibly because of changes in serum E2 and progesterone levels due to menstruation. Consequently, the results of molecular profiling tests might also change according to menstrual cycles of premenopausal women. We previously examined the characteristics of tumors in patients treated between 1982 and 2010. We found that the frequency of ER-positive breast cancer in women aged 50 years or younger was increased greatly during the studied interval (8). Because premenopausal women often desire pregnancy and childbirth after breast cancer treatment, whether chemotherapy is necessary is an important consideration for these women.

This study evaluated the effects of serum E2 and progesterone levels on ERGs and proliferation-associated genes.

Materials and methods

Patients and serum samples

We selected patients who had undergone surgery for breast cancer between January 2001 and December 2012 from the archive of the Department of Breast Surgery, Nagoya City University Hospital in Japan. Each patient had provided written informed consent before surgery. The protocol was approved by the Institutional Review Board of Nagoya City University Graduate School of Medical Sciences and executed in accordance with the Declaration of Helsinki.

The tissues were fixed in 10% buffered formalin and embedded in paraffin or snap-frozen in liquid nitrogen immediately after resection and stored at −80°C until RNA extraction. The histological grade was estimated according to the Bloom and Richardson method proposed by Elston and Ellis (9). Blood samples were centrifuged at 1300 × g for 10 min, and the separated sera were stored in aliquots at −20°C. Concentrations of serum E2 and progesterone were measured using commercially available automatic electrochemiluminescence immunoassay analyzers (Estradiol III for E2 and Progesterone II for progesterone; Roche Diagnostics GmbH, Mannheim, Germany).

RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from frozen tissue sections using an RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s procedure. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s procedure. TaqMan Gene Expression assays (Applied Biosystems) were used to measure mRNA expression of ERα (ESR1), PgR (PGR), trefoil factor 1 (TFF1), GREB1, PDZ domain-containing 1 (PDZK1), Ki67 (MKI67), cyclin D1 (CCND1), survivin, RANKL, Wnt-4 and two housekeeping genes (ACTB and GAPDH). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were amplified independently in duplicate. Results were converted into relative concentrations using an in-run standard curve. Relative concentrations were normalized to the means values of the housekeeping genes to account for variations in the amount of input cDNA.

Immunohistochemistry

One 4-μm-thick section from each paraffin-embedded specimen was first stained with hematoxylin and eosin to ascertain whether an adequate number of invasive ductal carcinoma cells were present and that the quality of fixation was adequate for immunohistochemical analysis. Serial sections (4 μm thick) were then prepared from suitable tissue blocks and float mounted on adhesive-coated glass slides for ERα, PgR and HER2 staining. Primary antibodies were mouse monoclonal antihuman antibodies against ERα (1D5; Dako, Glostrup, Denmark) at a 1:100 dilution and PgR (PgR636; Dako) at a 1:100 dilution. A rabbit antihuman c-erbB2 oncoprotein antibody (Dako) at a 1:200 dilution was used to stain HER2. The DAKO EnVision system (Dako EnVision-labeled polymer, peroxidase) was used to detect ERα, PgR and HER2. Immunostained specimens were scored after the entire section had been evaluated by light microscopy. Expression of ERα and PgR was evaluated by the percentage of cells with positive nuclear staining. A positive nuclear staining ratio of ≥1/100 was considered as positive. However, we included patients with tumors containing more than two-thirds of cells positive for ERα. HER2 immunostaining was evaluated using the same method employed by the HercepTest (Dako). Scoring of HER2 expression was based on the membrane staining pattern and scored on a scale of 0–3+. Tumors with scores of 0 or 1 were considered to be negative for HER2 overexpression, and those with a score of 3 were considered to be positive. All tumors with scores of 2 were omitted from the analysis, because they were not used for fluorescence in situ hybridization analysis.

Statistical analyses

mRNA expression and immunohistochemical analyses were performed by researchers who were blinded to the clinical data. Statistical calculations were performed using JMP 10 software (SAS Institute, Inc., Cary, NC). Relationships between serum E2 levels and clinicopathological factors were assessed by χ² and Fisher's
Results

Relationship of serum E2 with expression of ERGs

A total of 1527 surgically resected breast cancer tissue samples were selected from the archive of the Department of Breast Surgery, Nagoya City University Hospital. All samples were collected from patients treated between January 2001 and December 2012. We excluded 1030 samples because they were from postmenopausal patients. Serum samples collected on the day of each patient’s surgery and RNA samples of their breast cancer tissues were available for 183 of the 497 premenopausal patients. Tumors in 123 of the 183 premenopausal patients were positive for ERα, of which 91 had more than two-thirds ERα-positive tumor cells. We used these 91 samples for this analysis, but five samples positive for HER2 were excluded from the proliferation activity analysis.

First, we investigated the correlation between serum E2 levels on the day of surgery and mRNA and protein expression of PgR in breast cancer tissues, because PgR is a marker for estrogenicity in many studies and detection of PgR by immunohistochemistry (IHC) is a routine clinical test (10–12). Patients were classified as low E2 (serum E2 concentration: <200 pg/ml) and high E2 (≥200 pg/ml). Patient demographics indicated no major differences in the clinicopathological characteristics of low- and high-E2 groups (Table 1). High-E2 levels were positively correlated with higher levels of PGR mRNA expression in premenopausal patients with ERα-positive breast cancer, although the strength of the correlation of the two variables was very weak (Fig. 1A and B). Mean percentages of cells that showed positive nuclear staining for PgR were 58.3 and 76.7% in low- and high-E2 groups, respectively (Fig. 1C). These results indicated that high-E2 levels were positively correlated with higher PgR protein expression in these patients (P = 0.01). PGR mRNA expression was also positively correlated with PgR protein expression in this cohort (Fig. 1E). Our data demonstrated that the serum E2 level influenced PgR expression in premenopausal ERα-positive breast cancer patients. We chose 200 pg/ml as the cut-off level for the serum E2 concentration in this study, because the strongest correlation between the serum E2 level and PGR mRNA expression was observed at this concentration.

Next, we investigated the correlation between the serum E2 level and expression of other ERGs such as GREB1, TFF1 and PDZK1 (13–16). High GREB1 mRNA expression was correlated with the serum E2 level (Fig. 2A), although this relationship was marginal in Spearman correlation analysis (Fig. 2B). TFF1 mRNA expression was also associated with the serum E2 level, although the strength of the correlation of the two variables was very weak. However, PDZK1 mRNA expression was not correlated with serum E2 levels (Fig. 2C–F).

Next, we investigated whether the serum E2 level affected expression of proliferation genes including MKI67, CCND1 and survivin. We expected expression of these genes to correlate with serum E2 levels because ERα-positive breast cancer is dependent on estrogen. However, we found no correlation between them (Fig. 3) or between ESR1 expression and the serum E2 level (Fig. 4).

Table 1. Patient demographics and clinicopathological characteristics

|                | All patients n (%) | Low E2 n (%) | High E2 n (%) | P value |
|----------------|-------------------|--------------|--------------|--------|
| Number         | 91                | 75           | 16           |        |
| Age in years   | 42.9              | 43.1         | 42.4         |        |
| Tumor size     |                   |              |              |        |
| ≤2 cm          | 65 (71)           | 55 (73)      | 10 (63)      |        |
| >2 cm          | 26 (29)           | 20 (27)      | 6 (37)       | 0.384  |
| Node status    |                   |              |              |        |
| Negative       | 38 (42)           | 28 (37)      | 10 (63)      |        |
| Positive       | 53 (58)           | 47 (63)      | 6 (37)       | 0.064  |
| Tumor grade    |                   |              |              |        |
| 1              | 48 (53)           | 40 (53)      | 8 (50)       |        |
| 2              | 16 (18)           | 14 (19)      | 2 (13)       |        |
| 3              | 23 (25)           | 17 (23)      | 6 (37)       |        |
| Unknown        | 4 (4)             | 4 (5)        | 0            | 0.510  |
| Histology      |                   |              |              |        |
| IDC            | 79 (88)           | 65 (94)      | 14 (93)      |        |
| ILC            | 4 (4)             | 4 (6)        | 0            |        |
| Other          | 8 (8)             | 0            | 1 (7)        | 0.560  |
| ERα IHC status |                   |              |              |        |
| Positive       | 91 (100)          | 75 (100)     | 16 (100)     |        |
| Negative       | 0                 | 0            | 0            |        |
| PgR IHC status |                   |              |              |        |
| Positive       | 86 (95)           | 70 (93)      | 16 (100)     | 0.288  |
| Negative       | 5 (5)             | 5 (7)        | 0            |        |
| HER2 status    |                   |              |              |        |
| Positive       | 5 (5)             | 5 (7)        | 0            |        |
| Negative       | 83 (92)           | 67 (89)      | 16 (100)     |        |
| Unknown        | 3 (3)             | 3 (4)        | 0            | 0.392  |

E2, estradiol; ERα, estrogen receptor α; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; PgR, progesterone receptor.

ESR1 mRNA expression and expression of ERGs and proliferation genes

Although expression of ESR1 mRNA and ERα protein is associated, ESR1 mRNA expression also reportedly varies among tumors with high nuclear staining for ERα (17). We therefore investigated the effect of ESR1 mRNA expression on the expression of ERGs and proliferation genes in this series. We found that ESR1 mRNA expression was strongly associated with expression of ERGs including PGR, GREB1, TFF1 and PDZK1 (Fig. 5A–D). Interestingly, ESR1 expression was also associated with expression of proliferation genes such as MKI67, CCND1 and survivin (Fig. 5E–G).

PGR mRNA expression and expression of progesterone-regulated genes and proliferation genes

Tanou et al. recently reported that PgR signaling induced cell proliferation in their experimental model, and that RANKL and Wnt-4 were induced by activation of PgR signaling in human breast epithelium (18). We therefore investigated the correlation between PGR mRNA expression and expression of two progesterone-regulated genes (PRGs), RANKL and Wnt-4, in premenopausal patients with ERα-positive breast cancer. We found that PGR mRNA expression was positively correlated with both RANKL and Wnt-4 mRNA expression (Fig. 6A and B). However, the strength of the correlation of the two variables was very weak. We next examined the
relationships between PGR mRNA expression with expression of proliferation genes, including MKI67, CCND1 and survivin, and found them to be positively correlated (Fig. 6C–E).

Relationship of the serum progesterone level with RANKL mRNA expression

Progesterone has been shown to induce adult mouse mammary stem cell expansion (19, 20), and RANKL was recently reported to correlate with the serum progesterone level in human breast epithelial tissues (18). Therefore, we investigated the correlation between the serum progesterone level on the day of surgery and RANKL mRNA expression in premenopausal patients with ERα/PgR-positive breast cancers. Patients were classified into those with low progesterone (serum progesterone concentration: <1 ng/ml) or high progesterone (≥1 ng/ml). The serum progesterone level on the day of surgery was positively correlated with the RANKL mRNA expression level in this series, but the strength of the correlation of the two variables was very weak (Fig. 7). However, the serum progesterone level was not associated with expression of Wnt-4, although Wnt-4 is reportedly another progesterone target gene (21), or proliferation genes including MKI-67, CCND1 and survivin (Fig. 8). We analyzed the correlation between various progesterone concentrations and RANKL mRNA expression, and the best correlation between them was seen at a cut-off value of 1 ng/ml. Therefore, we chose 1 ng/ml as the cut-off level in this analysis.

Discussion

Our data demonstrated that the serum E2 level influenced PgR expression in premenopausal patients with strongly ERα-positive
breast cancer. Moreover, the serum E2 level influenced expression of some ERGs. These results support a report by Haynes et al., indicating that the menstrual cycle influences expression of ERGs included PgR. The present study also confirmed the dynamic relationship of serum E2 with ERG expression, which changed throughout the menstrual cycle.

Haynes and colleagues assigned patients with primary ERα-positive breast cancer into three menstrual cycle windows. They reported that the expression of ERGs and proliferation-associated genes varied across these cycle windows. However, individual differences were large in the menstrual cycle, including the cycle length. Therefore, we measured serum E2 and progesterone levels on the day of surgery and investigated correlations between them and expression of ERGs and proliferation-associated genes. Expression levels of ERGs, including PgR, were also weakly but positively correlated with serum E2 levels in our data, implying that the menstrual cycle affects estrogen-dependent tumor growth. Because PgR is one of the genes measured in OncotypeDx®, there is the possibility that the results of this test might be influenced by the menstrual cycle.

Figure 2. Correlation between the serum E2 level and ERG expression in ERα-positive breast cancer tissues from premenopausal patients. Relative mRNA expression levels of GREB1 (A, B), TFF1 (C, D) and PDZK1 (E, F) were determined by quantitative RT-PCR. A, C, E: Mann–Whitney U-test; B, D, F: Spearman correlation analysis. ERG, estrogen-regulated gene; RT-PCR, reverse transcription-polymerase chain reaction.
In this study, we examined 91 tumors that were strongly positive for ERα, i.e., more than two-thirds of their tumor cells were positive for ERα. We found a strong positive correlation between ESR1 mRNA expression and expression of ERGs and proliferation genes. However, no correlation was seen between the serum E2 level and mRNA expression of proliferation genes, and the serum E2 level and expression of ERGs were not strongly associated ($PGR$: $P = 0.016$, $Rs = 0.07$; $GREB1$: $P = 0.061$, $Rs = 0.032$; $TFF1$: $P = 0.029$, $Rs = 0.022$; $PDZK1$: $P = 0.596$, $Rs = 0.4281E-6$). Furthermore, Haynes and colleagues reported that expression levels of $ESR1$ were not influenced by serum E2 levels, which is in accordance with our results (Fig. 4). These data suggest that serum E2 levels were sufficiently high to induce ERG expression in premenopausal women and strongly suggest that $ESR1$ mRNA expression mediates expression of ERGs and proliferation genes. Badve and colleagues investigated $ESR1$ mRNA and protein expression by RT-PCR and IHC, respectively. They reported a wide variation in the expression of $ESR1$ mRNA in strongly ERα-positive tumors ($\geq 7$ Allred score) (17). Our data support their report. Effects of endocrine therapy in strongly ERα-positive tumors are known to vary. These differences in mRNA expression might be one of the causes.

Because serum progesterone expression levels also change during the menstrual cycle, we investigated its influence. PgR signaling has been shown to induce RANKL and Wnt-4 in breast epithelial cells (18). RANKL is required for mammary gland development (22), and RANKL signaling is important for mouse mammary carcinogenesis (23,24). In this study, we found that both PgR expression and serum progesterone on the day of surgery weakly correlated

![Figure 3. Correlation between the serum E2 level and mRNA expression of proliferation genes in ERα-positive breast cancer tissues from premenopausal patients. Relative mRNA expression levels of MKI67 (A), CCND1 (B) and survivin (C) were determined by quantitative RT-PCR and Spearman correlation analyses.](https://academic.oup.com/jjco/article-abstract/49/1/12/5160087)

![Figure 4. Correlation between the serum E2 level and ESR1 mRNA expression in ERα-positive breast cancer tissues from premenopausal patients. Relative ESR1 mRNA expression levels were determined by quantitative RT-PCR and Spearman correlation analyses.](https://academic.oup.com/jjco/article-abstract/49/1/12/5160087)
with RANKL mRNA expression. However, no correlation was found between the serum progesterone level and expression of proliferation genes including MKI-67, CCND1 and survivin. Serum progesterone also did not correlate with Wnt-4 expression. Therefore, the progesterone-PgR–PRGs pathway involved in the proliferation of human breast cancer requires further consideration.

Figure 5. Relationships of ESR1 mRNA expression with ERG and proliferation gene expression in ERα-positive breast cancer tissues from premenopausal patients. mRNA expression levels of PGR (A), GREB1 (B), TFF1 (C), PDZK1 (D), MKI67 (E), CCND1 (F) and surviving (G) relative to that of ESR1 were determined by quantitative RT-PCR and Spearman correlation analyses.
A previous study showed that progesterone inhibits estrogen-dependent growth of ERα-positive cell line xenografts and primary ERα-positive breast tumor explants (25). Haynes et al. also showed that expression of proliferation genes was lower in window 3 (days 17–26 of the menstrual cycle) than in window 2 (days 7–16) and window 1 (days 27–35 + 1–6). The serum progesterone level was higher in window 3 compared with the other windows (26). These data support the possibility that progesterone inhibits breast cancer growth in clinical settings. However, we observed no correlation between serum progesterone levels and expression of proliferation genes including MKI67, CCND1 and survivin.

Because the serum hormonal environment with regard to E2 and progesterone is dynamic in premenopausal women, breast cancer cells might repeatedly proliferate and pause.

ESR1 and PGR mRNA expression mediate expression of ERGs, PRGs and proliferation genes. We conclude that the serum E2 level...
Figure 7. Relationship between the serum progesterone level and RANKL mRNA expression in ERα-positive/PgR-positive breast cancer tissues from premenopausal patients. Relative RANKL mRNA expression was determined by quantitative RT-PCR (A: Mann-Whitney U-test; B: Spearman correlation analysis; low progesterone: <1 ng/ml; high progesterone: ≥1 ng/ml).

Figure 8. Correlation between the serum progesterone level and mRNA expression of Wnt-4 and proliferation genes in ERα-positive/PgR-positive breast cancer tissues from premenopausal patients. Relative mRNA expression levels of Wnt-4 (A), MKI67 (B), CCND1 (C) and survivin (D) were determined by quantitative RT-PCR and Spearman correlation analyses.
on the day of surgery influences ERG expression moderately in strongly ER-positive patients, which should be considered for premenopausal patients

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Conflict of interest statement

None declared.

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