Introduction

It is well established that estrogens are important for the growth and development of normal mammary gland, as well as for the initiation and progression of estrogen-dependent breast cancer. The effect of estrogens on breast tumorigenesis is believed to be mediated mainly through estrogen receptor (ER)-α. Breast cancer occurs more frequently in postmenopausal women than in younger women, and a higher proportion of these older patients have tumors that are sensitive to hormones. In postmenopausal women the concentration of estradiol in breast cancer tissue is reported to be higher than in plasma and normal breast tissue [1]. The high concentration of estradiol in breast cancer tissues of postmenopausal women may be due to in situ synthesis of estrogen by breast tissues, which is believed to be catalyzed mainly by aromatase [2]. Reports of the contribution to in situ estrogen production by stromal cells as opposed to that by breast cancer cells, assessed immunohistochemically, are controversial [3–12]. Some previous
studies showed no consistent relationship between ER-α status and tumor aromatase levels by immunohistochemistry [3,4,8,12].

The human aromatase gene, CYP19 [13], yields an mRNA that spans nine exons with the translation start site beginning at exon II [14,15]. Its transcription is regulated in a tissue-specific manner [16–19]. However, studies of associations between aromatase gene expression and clinicopathologic factors in breast cancer have been limited and the results discordant. In the present study, using quantitative real-time LightCycler RT-PCR (Roche Molecular Biochemicals, Mannheim, Germany), we correlated aromatase mRNA expression with other clinicopathologic factors in 162 cases of invasive ductal carcinoma of the breast.

Materials and methods

Patients and sample

A total of 162 primary invasive ductal breast carcinoma specimens were obtained by surgical excision at the Department of Breast and Endocrine Surgery, Nagoya City University Medical School, Nagoya, Japan between 1992 and 2000. The research protocol for the study was approved by the ethics committee of Nagoya City University Graduate School of Medicine, Nagoya, and informed consent was obtained from all patients before surgery.

Stage I patients without nodal metastasis did not receive any adjuvant therapy. Most of the stage II and III patients, who were ER-positive and/or progesterone receptor (PgR)-positive, received adjuvant endocrine therapy using tamoxifen (20 mg/day, orally) for 5 years. The median age of the patients was 53 years (range 34–88 years), and all patients were women.

Patients were followed postoperatively every 3 months by clinical and radiologic examination. The median follow-up period was 58 months (range 22–90 months). Patients were graded histopathologically according to the modified Bloom and Richardson method proposed by Elston and Ellis [20]. Samples were snap frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Total RNA isolation and reverse transcription

Total RNA from microscopically confirmed homogeneous breast cancer tissue was isolated from approximately 500 mg of frozen specimen or from one flask of the HepG2 cell line, kindly provided by Dr N Harada [21], as a positive control and to generate standard curves. mRNA was isolated using the Trizol reagent (Life Technologies Inc., Tokyo, Japan) according to the manufacturer’s instructions. RT reactions were performed as previously described [22]. Briefly, each 20 μl cDNA synthesis mixture contained 1 μg total RNA, buffer (10 mmol/l Tris-HCl [pH 9.0], 50 mmol/l KCl, 1.5 mmol/l MgCl2), 1 mmol/l each of deoxynucleotide triphosphates, 25 units of RNA-guard RNase inhibitor (Amersham Pharmacia Biotech Inc., Tokyo, Japan), 200 units of Superscript II reverse transcriptase (Life Technologies Inc.), and 100 ng pd(N)6 random hexamer (Amersham Pharmacia Biotech Inc.).

Primers and probes

We conducted Blast searches (GenBank) to confirm the specificity of the nucleotide sequences chosen for the primers and probes, and to confirm the absence of DNA polymorphism. To avoid detection of contaminating genomic DNA, the primers were located at exons 3 and 4. The specific oligonucleotide primers were synthesized according to published information on the aromatase gene [23] as follows: sense primer 5′-TCT GGA TCT CTG GAG AGG AAA-3′ (384–404); and antisense primer 5′-GCC TTT CTC ATG CAT ACC GA-3′ (517–498). The PCR product size is 140 base pairs. The donor probe 5′-CTG CCG AAT CTA GGA CGT TAA TGA TT-3′ has a fluorescein label at its 3′ end. The acceptor probe 5′-GTC TTC ATT ATG TGG AAC ATA CTG GAG C-3′ has LC Red 640 at its 5′ end.

To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) and normalized. GAPDH primers were as follows: forward primer 5′-AAA TCA AGT GGG GCG ATG ATG CTG-3′; and reverse primer 5′-GCA GAG ATG ATG ACC CCT TTG-3′. The sequences of the GAPDH probes used for real-time LightCycler PCR were 5′-AGA AGG CTG GGG CTC ATT TGC AGG G-3′ and 5′-GTC CAC TGG CGT CTT CAC CAC CAT G-3′. All primers and probes were purchased from the Japanese Gene Institute (Saitama, Japan).

Real-time reverse transcription polymerase chain reaction

PCR was performed using a LightCycler. The PCR reaction was carried out in a 20 μl final volume containing the following: H2O up to 20 μl; 2.4 μl 25 mmol/l MgCl2; 0.5 μl 20 pmol/μl sense primer and antisense primer; 0.4 μl 10 pmol/μl donor and acceptor probe; 2 μl PCR master mix; and 1.5 μl cDNA. After an initial denaturation step at 95°C for 60 s, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 0 s, hybridization at 56°C for 5 s, and elongation at 72°C for 6 s. The fluorescence signal was acquired at the end of the hybridization step. A total of 55 cycles were performed. Cycling conditions for GAPDH were as follows: initial denaturation at 95°C for 60 s, followed by 50 cycles at 95°C for 0 s, 60°C for 5 s, and 72°C for 8 s.

Standard curves and expression of results

For each PCR run, a standard curve was constructed from serial dilutions of cDNA from the HepG2 cell line. The level

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of expression of aromatase mRNA is given as relative copy numbers normalized against GAPDH mRNA and shown as mean ± standard deviation. Relative aromatase mRNA expression was calculated using the formula (A/G) × 1000, where A is the relative copy numbers of aromatase mRNA and G is the relative copy numbers of GAPDH mRNA.

A nontemplate control was included in each experiment. All of the nontemplate controls, the standard cDNA dilutions from the HepG2 cell line, and the tumor samples were assayed in duplicate. All of the patient samples with a coefficient of variation for gene mRNA copy number data greater than 10% were retested using the method of Bieche and coworkers [24].

**Immunohistochemical staining of estrogen receptor-α and progesterone receptor**

Immunostaining of ER-α and PgR was performed as previously described [25]. Briefly, the slides were incubated with anti-ER-α primary antibody (ER1D5; Dako, Kyoto, Japan) at a 1:100 dilution or anti-PgR primary antibody (PgR636; Dako), also at a 1:100 dilution, using the streptavidin–biotin system (SAB-PO kit; Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. The immunostaining of ER-α and PgR was subjectively assessed by two independent investigators (ZZ and HI), and discordant results were resolved by consultation with a third investigator (HY), as previously described [26]. Expression of ER-α and PgR was scored by assigning a proportion score and an intensity score according to Allred’s procedure [27]. In brief, the proportion of positive staining throughout the entire slide was assessed as 0 (negative), 1 (<1%), 2 (1–10%), 3 (10–33%), 4 (33–66%) and 5 (>66%), and the average staining intensity was recorded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) under light microscopy. The immunohistochemistry score for each slide (0 or 2–8) was obtained as the sum of the proportion and intensity. ER-α and PgR status by immunohistochemistry was then assessed as negative (score 0–2) or positive (score 3–8).

**Statistical analysis**

The nonparametric Mann–Whitney U-test was used for statistical analysis of associations between aromatase expression and clinicopathologic factors. Disease free survival curves were generated using the Kaplan–Meier method and verified using log-rank (Mantel–Cox) and Breslow–Gehan–Wilcoxon tests. \( P<0.05 \) was considered statistically significant.

**Results**

**Characteristics of the patients**

Clinical characteristics are summarized in Table 1. Of the 162 patients with invasive ductal carcinoma that we examined, 104 patients were older and 58 were younger than 50 years, respectively; and 59 had axillary lymph node metastases, whereas 103 had no metastases; and in 43 cases the tumor was less than 2 cm and in 119 cases it was more than 2 cm in size. By ER-α immunohistochemistry, in 108 cases the tumor was ER-α positive, in 47 cases the tumor was ER-α negative, and in seven cases the ER-α status could not be determined. In comparison, immunohistochemical analysis revealed that in 87 cases the tumor was PgR positive, in 62 cases the tumor was PgR negative, and in 13 cases the PgR status could not be determined. The tumor was classified as not being high grade in 113 patients, whereas in 49 cases histologically high-grade tumor was present. The amount of aromatase mRNA in the 162 cases ranged from 0 to 486, with a median of 24 relative copy numbers.

**Level of expression of aromatase mRNA correlates with age, axillary lymph node metastasis, and tumor size**

The level of aromatase mRNA expression in the group of patients older than 50 years of age (29 ± 60) was significantly greater than that in the patients younger than 50 years (14 ± 24, \( P=0.0042; \) Fig. 1a). The expression in patients lacking axillary lymph node metastases (30 ± 62) was significantly higher than that in the group with axillary lymph node metastases (13 ± 20, \( P=0.010; \) Fig. 1b). In addition, it was found that the level of aromatase mRNA expression in the group with tumors less than 2 cm (29 ± 54) was greater than that in the group with tumors larger than 2 cm (22 ± 50, \( P=0.042; \) Fig. 1c).

| Table 1 |
| --- |
| **Aromatase mRNA expression and clinicopathologic factors in patients with invasive ductal carcinoma** |
| Factor | \( n \) | Aromatase mRNA (mean ± standard deviation) | \( P \) (Mann–Whitney U-test) |
| --- | --- | --- | --- |
| Age (years) | | | |
| >50 | 104 | 29 ± 60 | 0.0042* |
| ≤50 | 58 | 14 ± 24 | | |
| Axillary lymph node status | | | |
| – | 103 | 30 ± 62 | 0.010* |
| + | 59 | 13 ± 20 | | |
| Tumor size | | | |
| <2 cm | 43 | 29 ± 54 | 0.042* |
| ≥2 cm | 119 | 22 ± 50 | | |
| ER-α status | | | |
| + | 108 | 28 ± 59 | 0.0021* |
| – | 47 | 13 ± 20 | | |
| PgR status | | | |
| + | 87 | 24 ± 44 | 0.63 |
| – | 62 | 25 ± 63 | | |
| Histologic grade | | | |
| 1 or 2 | 113 | 24 ± 50 | 0.11 |
| 3 | 49 | 24 ± 54 | | |

Estrogen receptor (ER-α) status and progesterone receptor (PgR) status were determined histochemically. *\( P<0.05 \).
The level of expression of aromatase mRNA correlates with immunohistochemically determined estrogen receptor-α protein expression

The level of aromatase mRNA expression was also found to be higher in the immunohistochemically determined ER-α positive group (28 ± 59) than that in the negative group (13 ± 20; \( P=0.0021 \); Fig. 1d). However, there was no difference in the level of aromatase mRNA expression between the PgR positive and negative groups by immunohistochemical PgR detection. We also analyzed aromatase mRNA expression in the subgroups of women who were premenopausal and postmenopausal, but we did not find any significant correlation (data not shown), which was probably due to the small number of individuals in the subgroups.
Established, and is mediated mainly via ER-
progression of estrogen responsive breast cancer is well
The mitogenic activity of estrogens in the promotion and
Statistical analysis of aromatase mRNA expression levels and disease
free survival, using the Kaplan–Meier method. The patients with high
aromatase mRNA expression in their tumors (46 ± 68) exhibited a trend
toward better prognosis than did those with lower expression (4 ± 3;
log-rank [Mantel–Cox] test \( P = 0.073 \); Breslow–Gehan–Wilcoxon test
\( P = 0.035 \)).

Discussion
The mitogenic activity of estrogens in the promotion and
progression of estrogen responsive breast cancer is well
established, and is mediated mainly via ER-\( \alpha \). In situ estrogen synthesis catalyzed by aromatase plays an important role in breast cancer, especially in postmenopausal patients, whose ovarian function has ceased. The final, rate limiting step in estrogen biosynthesis is controlled by the cytochrome P450-type enzyme complex aromatase. Previous studies of the immunohistochemical localization of aromatase in breast cancer have been controversial. Some studies utilizing either monoclonal [6,8] or polyclonal antibodies [5,12] localized aromatase mainly to the
In one earlier study [33] ER-\( \alpha \) protein levels in breast cancer were assessed by enzyme immunoassays, and in another study [34] ER-\( \alpha \) protein levels in breast cancer were assessed using dextran coated charcoal method until 1988 and enzyme immunoassay thereafter. In the present study, however, immunohistochemical methodology was employed. Another reason for the discrepancy between studies regarding the relationship of ER-\( \alpha \) with aromatase mRNA level may be that aromatase is distributed mainly in tumor cells or stromal cells, but this is controversial.

The higher level of aromatase mRNA expression in patients older than 50 years and with tumor size less than 2 cm, but who are ER-\( \alpha \) positive and axillary lymph node
negative (taken together with a previous study [33] that documented higher aromatase mRNA expression in the group negative for lymph node metastasis group) may imply that ER-α and aromatase may be coexpressed in endocrine responsive patients. This may further suggest that aromatase gene expression could be a potential endocrine responsiveness marker and may have prognostic significance in breast cancer.

Conclusion

Despite the limited number of patients involved in this study (only 162 cases), the significant correlation between aromatase mRNA expression and age over 50 years, negative lymph node metastasis, smaller tumor size, ER-α positive status, and better prognosis imply that ER-α and aromatase may be coexpressed in endocrine responsive patients. Our findings also indicate that aromatase gene expression is a potential marker of endocrine responsiveness and may have prognostic significance in breast cancer. Further studies, including a larger number of cases, microdissected breast tumor tissue, and longer periods of follow up, are warranted to elucidate the functional significance of aromatase in breast cancer.

Competing interests

None declared.

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