INTRODUCTION

Endocrine therapy has been considered the most important systemic therapy for all stages of hormone-receptor-positive breast cancers for more than 100 years. A major clinical problem limiting the usefulness of this therapy is resistance [1]. In the metastatic setting especially, initially responsive tumors eventually become resistant to endocrine treatment, leading to tumor progression and death [2]. Therefore, extensive basic and clinical research is aimed at differentiating breast cancer patients into subgroups with predictable clinical outcomes. Pathological separation of breast cancers based on tumor grade and stage provides primary information on disease outcomes, but this information falls short of the accurate outcome prediction required in clinics. The status of hormone receptors including estrogen receptor (ER)-alpha and progesterone receptor (PR) are known to be good prognostic and predictive markers for endocrine therapy for breast cancer. For many years, only one gene for ER, ER-alpha, had been recognized and was known to correlate with prognosis. ER-alpha-positive tumors are frequently treated with anti-estrogen drugs such as tamoxifen and aromatase inhibitors and usually have better prognoses than do ER-alpha-negative tumors [3], but in 1996, a second ER subtype, ER-beta, was identified [4]. ER-beta showed strong homology with ER-alpha in the DNA-binding domain and the ligand-binding domain, but little elsewhere [5]. Several groups have investigated the expression of ER-beta using real-time polymerase chain reaction (RT-PCR) [6] and immunohistochemistry or Western blotting [7,8]. However, the results have been rather inconsistent. Further, predictive or prognostic significance of expression of ER-beta protein or mRNA is still controversial.

In this study, we investigated ER-beta mRNA using the branched chain QuantiGene2.0 assay, the respective protein expression (ER-beta), ER-alpha, and PR using immunohistochemistry (IHC), the status of the HER2 gene by silver in situ hybridization (SISH) in surgically treated ER-positive breast cancer following endocrine therapy. We examined their relationships with clinicopathological factors and prognosis.

METHODS

Patients

Of the patients with invasive breast carcinoma, 139 samples...
were collected from patients who underwent breast cancer surgery following treatment with endocrine therapy according to the ER-positive result and had long-term follow-up information between January 2003 and December 2005 at Seoul St. Mary’s Hospital. All cases were stage I, II, or III and diagnosed as invasive carcinoma based on the core-biopsy. Histologic types (130 invasive ductal carcinomas, not otherwise specified; 5 mucinous carcinomas; 3 lobular carcinomas; and a tubular carcinoma) were verified on paraffin-embedded slides after operation by two pathologists. All patients underwent local and systemic treatments. Local treatment included surgery and radiotherapy. Systemic treatment included chemotherapy and endocrine therapy according to routine institutional protocol, and none of the patients received neoadjuvant chemotherapy. Surgical procedures consisted of mastectomy and breast conserving surgery. We retrospectively reviewed follow-up data. The follow-up contacts were carried out at 3-month intervals over the first year, 6-month intervals during the second year, and at 12-month intervals thereafter. The medical work-up consisted of regular physical checkups. Imaging tests such as X-ray, positron emission tomography, bone scan, and/or ultrasound were used to look for recurrences, second primary breast cancers, or metastatic disease. Recurrence was defined as radiographic or pathological evidence of regional tumor recurrence or distant metastasis at any time after initial therapy. Overall survival time was defined as the interval between the date of histological confirmation of disease and death or the last observation taken. The data were censored at the last follow-up period for living patients. Disease-free survival time was calculated as the time that recurrence was first suspected. In disease-free survival analysis, the data were censored for patients without tumor recurrence. The data of ER-alpha mRNA levels and PR mRNA levels measured by a branched-chain assay were obtained from previous study [9]. Study design, data collection, and analysis followed the principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board (IRB) of the Catholic University of Korea (IRB number, KC11TIS10143).

**Tissue microarray**

To construct the tissue microarray block, a 2 mm-sized single core was taken from morphologically representative areas of formalin-fixed and paraffin-embedded (FFPE) tumor tissue and were assembled on a premade recipient block (containing 6 holes by 10 holes) using a manual tissue arrayer (Quick-Ray Manual Tissue Microarrayer; Unitma Co., Ltd., Seoul, Korea). After construction, one section was stained with hematoxylin and eosin for histology verification. Each of the recipient blocks had 2 different control cores of normal breast tissue obtained from breast reduction surgery.

**Immunohistochemistry**

For ER-beta staining, sections of the FFPE tissue arrays were deparaffinized and quenched with 3% hydrogen peroxide. Heat-induced epitope retrieval was conducted by boiling the slides in a 0.01 M citrate buffer (pH 6.0) using a microwave vacuum histoprocessor (RHS-1; Milestone, Bergamo, Italy) at a temperature of 121°C for 15 minutes. The sections of tissue array were incubated with monoclonal ER-ß antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature for 30 minutes followed by incubation with peroxidase labeled polymer conjugated to secondary antibody (EnVision™ + Kit, DAKO, Carpinteria, USA) for 30 minutes. The immunoreactions were visualized with 3-3’-diaminobenzidine (DAB) and counterstained with Mayer’s hematoxylin.

For ER and PR staining, all procedures were performed using an Ventana BenchMark XT automated slide stainer (Ventana, Tuscon, USA), with anti-ER (SP1) rabbit monoclonal antibody (Ventana) and anti-PR (clone 1E2) rabbit monoclonal antibody (Ventana).

The Allred scoring system [10] was used for ER, PR, and ER-beta staining interpretation. The proportion of positive stained cells was rated as follows: 0, no cells stained positive; 1, between 0% and 1% positive; 2, between 1% and 10% positive; 3, between 10% and 33% positive; 4, between 33% and 66% positive; and 5, between 66% and 100% positive. In addition to the proportion score, an intensity score was made on the basis of the average intensity of staining: 0, negative; 1, weak; 2, intermediate; and 3, strong. The intensity score and the proportion score were added to obtain the total score; this is referred to as the Allred score [10], and is either 0 or between 2 and 8. Scores of 0 and 2 were interpreted as negative.

**HER2 SISH**

Three-micrometer sections of the tissue arrays were stained according to the manufacturer’s protocols with the INFORM HER2 DNA probes (Ventana). The probe was labeled with di-nitrophenol (DNP) and optimally formulated for use with the ultraView SISH Detection Kit and the Ventana BenchMark XT automated slide stainer (Ventana). The black dot signals for the HER2 gene were counted in at least 20 tumor cells and classified into 3 categories according to the 2007 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [11]: The ratio of HER2/chromosome 17 was then calculated by dividing the total score for HER2 by the total score for chromosome 17. 1) Negative for HER2 gene amplification, a ratio of < 1.8; 2) positive for HER2 gene amplification, a ratio of > 2.2; 3) equivocal, a ratio between 1.8
and 2.2. For equivocal cases, signals from 20 more tumor nuclei were counted in each slide and a new ratio was calculated. This could be repeated in these border line cases until a clear decision was reached.

**The branched chain QuantiGene2.0 assay**

Tissue homogenates were prepared according to the procedure described in the QuantiGene sample-processing kit for FFPE tissues (Panomics Inc., Fremont, USA). Briefly, from a 7 μm-slide section, a total of 200 mm² of tumor area was selectively dissected and incubated for 3 hours after adding 900 μL of homogenizing solution and 9 μL of proteinase K (50 μg/μL). The tissue homogenate was separated from paraffin and debris by centrifugation and transferred to a fresh microfuge tube.

Probe design software was used to design specific oligonucleotide probe sets for target genes to be used in Quantigene2.0 reagent systems (Panomics Inc.). A probe set for a target gene consists of capture extenders, label extenders, and blocking probes, covering a continuous legion (1435-1813) of the ER-beta transcript (whole length 2169). The Quantigene2.0 assay was performed according to the recommended protocol of Quantigene2.0 reagent systems, as previously described [9,12].

**Statistical analysis**

All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, USA) for Windows. Fisher's exact probability test or the chi-square test was used to compare the mRNA and protein data for ER-beta with other clinicopathological variables. The correlation between the IHC data and RNA levels was evaluated using the Spearman correlation test. Survival curves were plotted using the Kaplan-Meier method, and the statistical significance was determined by the log-rank test. Multivariate Cox proportional hazards regression analysis was performed to obtain a model for prognostic factors using backward selection strategies. \( p \)-values of less than 0.05

![Estrogen receptor (ER)-beta, ER-alpha, and progesterone receptor (PR) immunohistochemistry (IHC) results in breast carcinomas showed examples of positive nuclear staining of (A) ER-beta (×200), (B) ER-alpha (×200), and (C) PR (×200). HER2 silver in situ hybridization (SISH) results showed (D, E) the examples of HER2 amplification in SISH (D, HER2; E, chromosome 17; HER2/chromosome 17 ratio >2.2) (×400).](http://dx.doi.org/10.4048/jbc.2012.15.1.79)
were considered significant. Using receiver operating characteristic (ROC) curve, we get the area under the ROC curve (AUC) to determine the cutoff value.

## RESULTS

The positive rates for ER-beta and PR were 81.4% and 86.4%, respectively. HER2 amplification was observed in 8.6% (12 out of 139 cases) (Figure 1). The Allred score for ER-beta IHC correlated with the Allred score for ER-alpha IHC \((p<0.001, r=0.297)\) and the Allred score for PR IHC \((p=0.022, r=0.195)\) but not with the HER2 IHC score \((p=0.242, r=1.000)\) (Figure 2).

ER-beta mRNA level by a QuantiGene2.0 assay ranges from 0 to 1,826.1 (mean, 30.49). ER-beta mRNA level was not correlated with the Allred score of ER-beta IHC \((p=0.480, r=0.060)\), ER-alpha IHC \((p=0.463, r=-0.063)\), and PR IHC \((p=0.736, r=0.029)\) nor with the HER2 IHC score \((p=0.116, r=0.134)\) (Figure 3). ER-beta mRNA level was correlated with PR mRNA level \((p<0.001, r=0.427)\) but not with ER-alpha mRNA level \((p=0.097, r=0.141)\) (Figure 4). To identify a clinically meaningful cutoff point for levels of ER-beta mRNA expression that could be used in disease prognosis analysis, various levels of ER-beta mRNA expression were tested using the Kaplan-Meier method and verified by the log-rank test. Using the ROC curve, the cutoff value of ER-beta mRNA set at 0.29 showing sensitivity and the specificity were 86.7% and 41.1%, respectively, allowing us to obtain the most significant difference between the patient groups in disease-free survival analysis. And, the AUC was 0.659 with the \(p\)-value of 0.045 (95% confidence interval, 0.525-0.793). ER-beta mRNA status was assessed as either low expression (0-2.9) or high expression (>2.9) using the cutoff level described above. High ER-beta mRNA expression was observed in 62.1% of cases (87 out of 139 cases). ER-beta IHC positivity was associated with smaller tumor size \((p=0.045)\). In contrast to ER-beta IHC, there was no significant association between ER-beta mRNA expression and clinicopathological variables (Table 1).

During the median follow-up of 48 months (range, 5-74 months), 15 patients (10.8%) experienced disease recurrence and 4 patient’s deaths (2.9%). In survival analysis, poor differ-

![Figure 2](http://ejbc.kr)

**Figure 2.** Plot error bar graph (mean, standard deviation, 95% confidence interval), correlation between immunohistochemical staining results of (A) ER-beta versus ER-alpha, (B) ER-beta versus PR, and (C) ER-beta versus HER2. ER = estrogen receptor; PR = progesterone receptor; IHC = immunohistochemistry.
entiation \( (p = 0.002) \), lymph node metastasis \( (p = 0.019) \), Stage (III) \( (p = 0.019) \), high expression of ER-beta mRNA \( (p = 0.040) \) (Figure 5), and absence of PR protein expression \( (p = 0.007) \) were associated with shorter disease-free survival in univariate analysis. Age \( (\geq 50 \text{ year}) \), poor differentiation, and lymph node metastasis tended to show worse overall survival but were not statistically significant (Table 2). Poor differentiation (hazard ratio [HR], 5.967; 95% confidence interval [CI], 1.939-18.365; \( p = 0.002 \)), lymph node metastasis (HR, 3.985; 95% CI, 1.103-14.392; \( p = 0.035 \)), high expression of ER-beta mRNA (HR, 4.640; 95% CI, 1.039-20.727; \( p = 0.044 \)), and absence of PR protein expression (HR, 5.717; 95% CI, 1.712-19.092; \( p = 0.005 \)) were also independently associated with shorter disease-free survival in multivariate analysis (Table 3).

**DISCUSSION**

Breast cancer is a typical hormone-dependant tumor. It has been shown that longer exposure to estrogen results in an increased risk of developing breast cancer, and endogenous estrogens are thought to play a major role in breast cancer carcinogenesis [13].

The accurate assessment of hormonal status is of therapeutic importance because endocrine therapy reduces risk of recurrence by more than 50% in breast cancer patients with hormone-sensitive tumors [14]. The current method used to determine breast cancer treatment is based on the evaluation of ER-alpha and PR gene status by IHC. ER-beta status could provide additional information about the therapeutic effect on or the prognostic value in breast cancer treated by various
Table 1. Distribution of ER-beta protein and mRNA status in surgically treated ER-positive breast cancer following endocrine therapy

| Characteristics          | ER-beta protein | ER-beta mRNA |
|--------------------------|-----------------|---------------|
|                          | Negative | Positive | p-value | Low  | High | p-value |
| Age (yr)                 |          |          |         |       |      |         |
| <50                      | 11       | 57       | 0.454   | 26   | 43   | 1.000   |
| ≥50                      | 15       | 56       | 0.529   | 27   | 44   |         |
| Histologic type          |          |          |         |       |      |         |
| IDC                     | 26       | 104      | 0.529   | 48   | 83   | 0.425   |
| ILC                     | 0        | 3        | 0.898   | 2    | 1    |         |
| Mucinous ca              | 0        | 5        | 0.237   | 3    | 2    |         |
| Tubular ca               | 0        | 1        | 0.465   | 0    | 1    |         |
| Differentiation          |          |          |         |       |      |         |
| Well                     | 5        | 35       | 0.045   | 17   | 23   | 0.758   |
| Moderate                 | 16       | 55       | 0.237   | 26   | 45   |         |
| Poor                     | 5        | 23       | 0.045   | 10   | 19   |         |
| Size (cm)                |          |          |         |       |      |         |
| ≤2                       | 11       | 74       | 0.045   | 30   | 56   | 0.376   |
| >2                       | 15       | 39       | 0.237   | 23   | 31   |         |
| Lymph node status        |          |          |         |       |      |         |
| Negative                 | 10       | 58       | 0.237   | 25   | 44   | 0.213   |
| N1                       | 11       | 35       | 0.045   | 20   | 26   |         |
| N2                       | 3        | 16       | 0.237   | 8    | 11   |         |
| N3                       | 2        | 4        | 0.045   | 0    | 6    |         |
| AJCC stage               |          |          |         |       |      |         |
| I                        | 6        | 45       | 0.256   | 20   | 32   | 0.794   |
| II                       | 15       | 48       | 0.256   | 25   | 38   |         |
| III                      | 5        | 20       | 0.256   | 8    | 17   |         |
| HER2 SISH                |          |          |         |       |      |         |
| Not amplified            | 25       | 103      | 0.465   | 50   | 78   | 0.535   |
| Amplified                | 1        | 11       | 0.465   | 3    | 9    |         |

ER=estrogen receptor; IDC=invasive ductal carcinoma; ILC=invasive lobular carcinoma; ca=carcinoma; AJCC=American Joint Committee on Cancer; SISH=silver in situ hybridization.
score of ER-beta was correlated with the Allred score of PR and that the mRNA level of ER-beta was correlated with the mRNA level of PR. There have been controversial results in correlation between ER-beta protein and the mRNA expression level. Jarzabek et al. [16] reported a weak but positive correlation between mRNA and protein expression of ER-beta. Most studies repeatedly found no correlation between mRNA and protein expression of ER-beta [17,18] and, in this study, there was also no correlation between ER-beta protein and the mRNA expression level.

We revealed the association of protein expression of ER-beta with smaller tumor size in breast cancer, which was indirectly supported by the theory of favorable prognostic value of protein expression of ER-beta [19-21]. The association of protein expression of ER-beta with smaller tumor size was previously documented [22]. Further, we revealed an independent association of high ER-beta-mRNA levels, quantitatively measured using the QuantiGene2.0 assay, with poor disease-free survival in ER-alpha positive and endocrine therapy treated settings. ER-beta mRNA appeared to indicate a poor response to treatment [23-25], which was supported by the correlation of ER-beta mRNA with tumor grade and the up-regulation of some subtypes of ER-beta during breast cancer tumorigenesis and tumor progression [26]; whereas positive ER-beta protein (even though we did not revealed the predictive value in this study) was thought to indicate a favorable response to anti-estrogen treatment [27,28]. Although there is no clear explanation for these differences, the more dominant regulation of ER-beta protein by a degradation process, in addition to regulation at a transcription level, was suggested [17].

ER-beta protein expression and mRNA expression, interestingly, showed close correlation with PR protein expression and quantitative PR mRNA expression level, respectively, which represents their possible association in mechanism. However, high ER-beta mRNA level had poor prognostic implication in disease-free survival in contrast to the better prognostic value of PR mRNA and protein expression.

It is becoming clear that the mechanisms of action of hormone receptor including ER, PR, and epidermal growth factor receptor are much more diverse and complicated than initially thought. Part of the pleiotropic of the ER pathway can now be explained by new discoveries regarding the intense cross-talk of ER with growth factor and other signaling pathways [29]. The expression of ER-beta mRNA level might play a more complicated and different role in disease recurrence in comparison to ER and PR.

The limits of this study are that: 1) this is a retrospective study and 2) we performed this study in TMA. However, the use of TMA has been validated in a number of tumor types by comparing the expression of specific proteins in a small number of TMA core biopsies with their expression in whole sections from the donor tissue. The good concordance reported for small sampling areas may be the result of the marker expression being abundant and/or being homogenously expressed. Indeed, a large study of breast cancer showed a high degree of concordance for hormonal receptor that a single core from representative section identifies about 95% of the information for the ER and PR. Furthermore, single core information yielded significant associations with tumor specific survival than large section analyses did [30]. So, a single core sample from a tumor was sufficient to identify associations between molecular alteration and clinical outcome, especially in the hormone receptor study of breast cancer.

As far as we know, this is the first study to assess the prognostic significance of high expression of ER-beta mRNA using QuantiGene2.0 assay in surgically treated ER-positive breast cancer following hormone therapy. The high expression of ER-beta mRNA is an independent poor predictive marker for disease-free survival.

### Table 2. Univariate analysis results of disease-free survival and overall survival in surgically treated ER-positive breast cancer following endocrine therapy

| Univariate |  \( \rho \)-value | Disease-free survival | Overall survival |
|------------|-------------------|-----------------------|-----------------|
| Age (\( \geq 50\) yr) | 0.410 | 0.057 | 0.057 |
| Tumor size | 0.579 | 0.332 | 0.332 |
| Differentiation (poor) | 0.002 | 0.073 | 0.073 |
| Histologic type (IDC) | 0.871 | 0.687 | 0.687 |
| LN metastasis (\( \geq pN2\)) | 0.019 | 0.099 | 0.099 |
| Stage (III) | 0.019 | 0.518 | 0.518 |
| Chemotherapy (+) | 0.830 | 0.952 | 0.952 |
| High ER-beta mRNA | 0.040 | 0.908 | 0.908 |
| ER-beta IHC (+) | 0.317 | 0.452 | 0.452 |
| HER2 amplification (+)* | 0.785 | 0.562 | 0.562 |
| PR IHC (-) | 0.007 | 0.492 | 0.492 |

ER=estrogen receptor; IDC= invasive ductal carcinoma; LN=lymph node; PR=progesterone receptor; IHC=immunohistochemistry. *Evaluated by silver in situ hybridization.

### Table 3. Multivariate analysis of disease-free survival of patients with breast cancer treated with endocrine therapy

| Factors |  \( \rho \)-value | HR | 95% CI |
|---------|-------------------|----|--------|
| Differentiation (poor) | 0.002 | 5.967 | 1.939-18.365 |
| LN metastasis (\( \geq pN2\)) | 0.035 | 3.985 | 1.103-14.392 |
| Stage (III) | 0.651 | 1.328 | 0.389-4.530 |
| High ER-beta mRNA | 0.044 | 4.640 | 1.039-20.727 |
| PR protein (-) | 0.005 | 5.717 | 1.712-19.092 |

HR=hazard ratio; CI=confidence interval; LN=lymph node; ER=estrogen receptor; PR=progesterone receptor.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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