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Clinical significance of the expression of estrogen receptors α and β for endocrine therapy of breast cancer

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Abstract The assessment of the estrogen receptor (ER) α and the progesterone receptor (PgR) in breast cancer tissues is important for discriminating between hormone-dependent and hormone-independent tumors. ERβ, a more recently discovered ER, may influence estrogen action through the ERα pathway. To evaluate the clinical significance of these receptors in the response to endocrine therapy, we investigated their expression in primary breast cancer tissues. ERα and PgR were evaluated using immunohistochemistry (IHC) and enzyme immunoassay (EIA) and ERβ expression was determined using IHC and reverse transcription-polymerase chain reaction. When the cut-off level of EIA was set at 13 fmol/mg protein for ERα and that for IHC was set as an IHC score between 2 and 3, a significant correlation between ERα EIA and IHC was seen (concordance rate 88.4%). This indicates that this cut-off level of ERα IHC can be adopted to quantify breast cancer prognoses. Furthermore, the tumors with positive expression of ERα IHC or PgR IHC using this criterion were significantly related to the response to endocrine therapy. Additionally, tumors with positive expression of ERβ wild-type tended to have a better response to endocrine therapy than negative ones, and tamoxifen responders tended to exhibit a lower ratio of ERβcx (one of the ERβ variants) to ERβ wild-type than nonresponders. The results concerning ERβ are not yet fully understood; further investigations and evaluations should analyze the role of ERβ wild-type and variant type in breast cancer treatment.

Keywords Estrogen receptor · Progesterone receptor · Hormone responsiveness · Immunohistochemistry · Predictive factor

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Introduction

Human breast cancer is a typical hormone-dependent tumor, and various endocrine therapies have been employed for its treatment. To block estrogen action, selective estrogen receptor (ER) modulators, such as tamoxifen, toremifene, and raloxifene, bind to the ERα, leading to attenuation of estrogen-responsive genes [4]. Pure antiestrogens antagonistically interact with the ERα to completely suppress expression of estrogen-dependent genes [4]. To block estrogen synthesis, ovarian ablation can be achieved surgically or pharmacologically using goserelin or leuprolide in premenopausal women. Aromatase is a key enzyme of estrogen synthesis, which converts androgens to estrogen in peripheral adipose tissues. Antiaromatase agents inhibit aromatase activity, resulting in decreased estrogen production in breast tissues especially in postmenopausal women. Third-generation aromatase inhibitors now exist, such as anastrozole, letrozole, and exemestane [4].

It is important to discriminate between hormone-dependent and hormone-independent tumors to determine whether endocrine therapies should be employed [14]. ERα expression in tumor cells is one of the more valuable prognostic and predictive markers for endocrine therapy in breast cancer. The progesterone

receptor (PgR), which is produced as a result of the action of ERα, is also a good predictive biological marker [6]. Guidelines for adjuvant hormonal therapy for primary breast cancer were presented at the US National Institutes of Health Consensus Development Conference on Adjuvant Therapy for Breast Cancer in November 2000 [1] and at the Seventh International Conference on Adjuvant Therapy of Primary Breast Cancer in February 2001 [2]. Adjuvant hormonal therapy should be offered to all patients with tumors expressing ERα and/or PgR, as assessed by immunohistochemistry (IHC). The consensus statements recommended 5 years of tamoxifen as standard hormonal therapy for both pre- and postmenopausal patients with ER- and/or PgR-positive tumors. Ovarian ablation or suppression of ovarian function combined with tamoxifen is the treatment of choice for premenopausal patients with high-risk endocrine-responsive tumors. The selection of hormonal therapies and their combination with chemotherapy should be decided according to the assessment of risk of relapse, side effects, and the condition and preferences of the patient.

In 1996, a second ER—referred to as ERβ—was discovered that was highly homologous to ERα and that specifically binds estrogens with high affinity [13]. ERβ may influence estrogen action through the ERα pathway and the hormone refractoriness of breast cancer. Furthermore, ERβ has more variants than ERα [10]. We have reported that the protein expression of wild-type ERβ (ERβwt) can be used as a reliable prognostic indicator for breast cancer [16]. ERβcx, the carboxy terminal splicing variant of ERβ, has been considered a dominant repressor of ERα function, because ERβcx inhibits transcriptional activity of ERα rather than ERβwt [15]. In this paper, we investigate the relationship between response to hormonal treatment and the expression of these hormone receptors in breast cancer.

Patients and methods

Patients

We determined the expression of ERα and PgR by IHC and/or enzyme immunoassay (EIA) in 489 patients with breast cancer who underwent operations at the Department of Surgery II, Nagoya City University Medical School, Nagoya, Japan, from January 1989 to May 2000. Of these patients, 77 with recurrent or locally advanced breast cancer and with assessable lesions were treated with endocrine therapies alone: tamoxifen ($n=53$), luteinizing hormone-releasing hormone analogs ($n=15$), aromatase inhibitors ($n=8$), and progestin ($n=5$). Informed written consent was obtained from the patients before treatment. The study protocol was approved by the institutional review board of the Nagoya City University Medical School. Of the 77 tumors, 72 were invasive ductal carcinomas, three were invasive lobular carcinomas, one was medullary carcinoma, and one was mucinous carcinoma.

The response to treatment was evaluated using standard criteria. Two patients had a complete response, 18 had a partial response, 26 had stable disease, which was defined as a reduction of $<50\%$ or an increase of $<25\%$ in the size of the lesion for 24 weeks, and 31 had progressive disease.

Immunohistochemical assay for ERα, PgR, and ERβ

Representative blocks of paraffin-embedded tissues were cut at 4-μm thickness. These sections were autoclaved for 15 min at 120°C, and blocked for endogenous peroxidase activity with hydrogen peroxidase. After prevention of nonspecific reactions with Blockace solution (Dainippon Pharmaceutical, Osaka, Japan), sections were incubated with anti-ERα primary antibody (ER1D5; Dako, Kyoto, Japan) at a 1:100 dilution, anti-PgR primary antibody (PgR636; Dako) at a 1:100 dilution, or anti-ERβ rabbit polyclonal antibody that reacts with the C-terminus of ERβ (CSPAEDSKSKEGSQNPQSQ, amino acids 512–530) [9, 16]. Envision solution (Dako) was applied for detection of specific staining. As a negative control, duplicate sections were immunostained without exposure to primary antibodies.

The immunostaining was evaluated following the method of Harvey et al. [3]. In brief, the proportion of positive staining throughout the entire slide was assessed as 0 (none), 1 ($<1\%$), 2 (1–10%), 3 (11–33%), 4 (34–67%) or 5 ($>67\%$), and the average staining intensity was judged 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) under light microscopy with consistent illumination conditions. The IHC score of each slide (0 or 2–8) was obtained as the sum of the proportion and intensity. Staining status by IHC was then assessed as negative (scores 0 and 2) or positive (scores 3–8); this cut-off produced the highest concordance rate. These scores were subjectively assessed on five random visual fields for each specimen by two independent investigators (Z.Z. and H.I.), with discordant slides being resolved by consultation with a third investigator (H.Y.).

EIA for ERα and PgR

ERα and PgR values were determined by EIA (ERα EIA and PgR EIA kits, Dynabott, Tokyo, Japan). Positive ERα status was defined as >13 fmol/mg protein, and that of PgR as >10 fmol/mg protein [6].

Real-time polymerase chain reaction (PCR) on a LightCycler with hybridization probes for ERβ

Total RNA was extracted from approximately 500 mg frozen breast cancer tissue using TRIZOL reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription (RT) reactions were performed as previously described [23]. To avoid detection of contaminating genomic DNA, the primers were placed at the junction between exons 7 and 8 of the ERβ mRNA. The primers and probes can recognize the ERβwt and ERβcx separately (Sugiura et al., unpublished data). All primers and probes were purchased from the Japanese Gene Institute (Saitama, Japan). All PCR procedures were performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany).

Statistical analysis

The Kruskal-Wallis test and Chi-squared test were applied for the statistical analyses of correlations between IHC scores and EIA values, and those between ERα status and response to endocrine therapies, respectively. In the relapse-free survival analysis, the Kaplan-Meier analysis and log-rank test were also applied. The Mann-Whitney *U*-test was adopted to compare the ratio of ERβ variant type and wild type.

Results

Clinical significance of ERα expression as a prognostic factor

The correlation between ERα EIA and ERα IHC was confirmed in 489 breast cancers. When the cut-off level of EIA was set at 13 fmol/mg protein and that of IHC was set as an IHC score between 2 and 3, the concordance rate was highest at 88.4% (Fig. 1, Table 1). The statistical significance of ERα IHC ($P < 0.0001$) was higher than that of EIA ($P = 0.0009$; Fig. 2A and 2B, respectively), indicating that ERα IHC is better than ERα EIA for evaluating the prognosis of breast cancer.

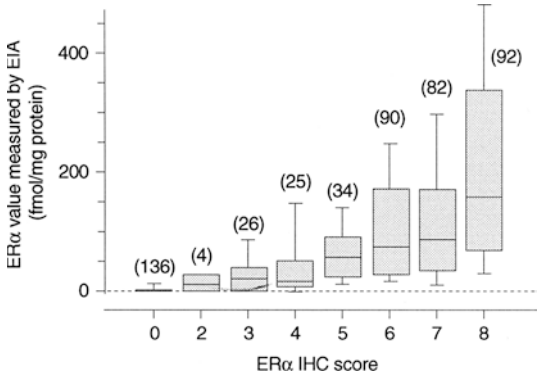

Fig. 1 The correlation between ERα EIA and ERα IHC: the ERα value increased with higher IHC score. When the cut-off level of the IHC score was set between 2 and 3, and when the cut-off of the EIA value was set at 13 fmol/mg protein, the concordance rate was highest at 88.4%. Numbers in parentheses indicate the numbers of breast cancers

Table 1 Relationship between ERα IHC and ERα EIA; concordance rate 88.4%, $P < 0.001$, Chi-squared test (*IHC* immunohistochemistry)

ERα IHC score	ERα (fmol/mg protein)	
	≥13	< 13
3–8	308	41
0, 2	12	128

Response to endocrine therapy and ERα, PgR, or ERβ expression

In the 77 recurrent or advanced breast cancers, the presence of tumors with positive expression of ERα IHC correlated significantly with the response to endocrine therapy. In the PgR IHC study, the result was similar to that for ERα IHC. The presence of tumors with positive ERβwt IHC did not correlate significantly with the response to endocrine therapy. There was a marginal significance in the correlation between ERβwt protein expression and response to endocrine therapy (Table 2).

Tamoxifen response and ratio of ERβ wt to variant type

The relationship between tamoxifen response and the mRNA levels of ERβwt and ERβcx was investigated. The efficacy of tamoxifen was evaluated in 11 patients and their mRNA was extracted. Tamoxifen responders ($n = 6$) tended to exhibit a lower ERβcx/ERβwt ratio than nonresponders ($n = 5$), but the difference was not statistically significant (Fig. 3).

Discussion

ERα and PgR are good prognostic and predictive markers for endocrine therapy for breast cancer, but there are problems with standardization of the technique and the method used to evaluate these receptors [1, 21]. We report here that ERα IHC is better than ERα EIA in the evaluation of relapse-free survival in patients with recurrent breast cancer, especially when the cut-off level was set at an Allred's IHC score between 2 and 3. Moreover, this evaluation method was also suitable for evaluating the response to endocrine therapies.

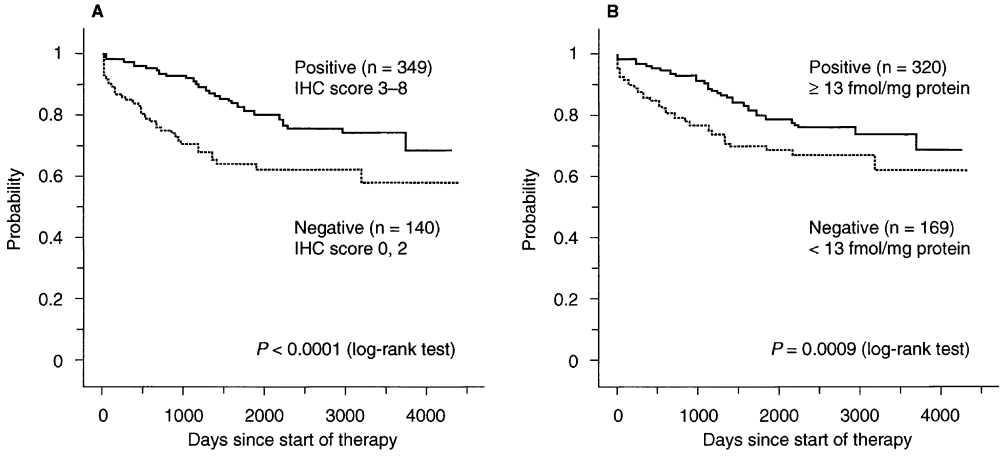

Fig. 2A, B Relapse-free survival curves estimated by (**A**) ERα IHC and (**B**) ERα EIA in 489 breast cancers: the results from ERα IHC ($P < 0.0001$) were more valuable than those from EIA ($P = 0.0009$)

Table 2 Relationship between response to endocrine therapy and ER (*IHC* immunohistochemistry, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *ER* estrogen receptor, *PgR* progesterone receptor)

	IHC score	Response to endocrine therapy			*P* value from Chi-squared test
		CR or PR	SD	PD	
ERα	0, 2	0	5	11	0.0093
	3–8	20	21	20	
PgR	0, 2	1	9	12	0.024
	3–8	19	17	19	
ERβ	0, 2	7	18	21	0.088
	3–8	13	8	10	
Total		20	26	31	

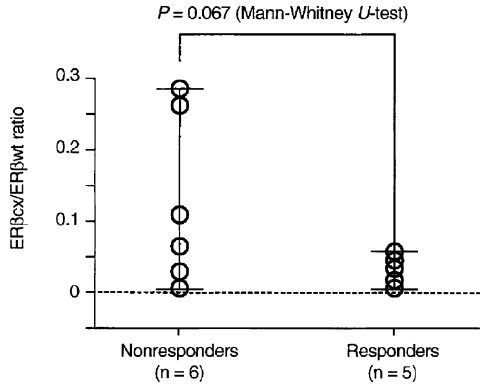

Fig. 3 Tamoxifen response and the mRNA levels of ERβ wt and ERβcx. The mRNA levels were quantitatively measured by real-time RT-PCR. Tamoxifen responders (mean ± SD, 0.023 ± 0.025) exhibited a lower ERβcx/ERβwt ratio than nonresponders (mean ± SD, 0.12 ± 0.125; $P = 0.067$, Mann-Whitney *U*-test)

Many functions have been suggested for ERβ in the breast, but its role is not yet fully understood. One group have suggested that ERβ contributes to the initiation and progression of chemical carcinogen-induced neoplastic transformation in the breast, because expression is induced in chemical carcinogen-transformed human breast epithelial cells [5]. Other groups have measured the expression of mRNAs of ERs in both normal and malignant human breast tissue by RT-PCR [22, 24]. Leygue et al. demonstrated that those tumors that co-express ERα and ERβ are node-positive and tend to be of a higher grade [10]. In another study it has been found that ERβ is often coexpressed with ERα and PgR in breast cancer and that ERβ is significantly associated with negative axillary node status and low tumor grade [7], while in a third study expression of ERβ in >10% of cancer cells was associated with better survival [11]. Thus ERβ behavior continues to have a controversial role in RNA studies.

It has been thought that these discrepancies might be attributable to the expression of ERβ variants. Table 3 summarizes studies on ERβ protein in breast cancer. In the majority of these studies, ERβ tended to be associated with low histological grade and ER expression. In one RT-PCR study, there was increased expression of ERβ mRNA in tamoxifen-resistant breast cancer patients [22], and in another study [18], there was decreased expression of ERβ protein in proliferative preinvasive breast tumors. In our experience, ERβwt is expressed in patients with good prognosis, and variant ERβ is more frequently stained in cancer tissue than in healthy tissue [17]. Saji et al., using samples from patients receiving neoadjuvant tamoxifen treatment, found that tumors positive for ERβcx (ERβ2) in IHC, especially those with poor expression of PgR, are not good candidates for tamoxifen treatment [19]. Our results showed that tamoxifen responders tended to exhibit a lower ERβcx/ERβwt ratio than nonresponders, but the difference was not statistically significant. This result may support the findings of Saji et al.

Table 3 ERβ protein studies in breast cancer (*ER* estrogen receptor, *LN* lymph node metastasis, *PgR* progesterone receptor)

Reference	Number of patients	Correlations with ERβ protein expression
7	92	LN(−), PgR, low histological grade
11	118	Response to tamoxifen
12	79	High histological grade
16	88	ERβ, low histological grade, better relapse-free survival
20	65	Low histological grade
8	29	High Ki-67
17	88	ERβwt, ERα, PgR, low histological grade

In the near future, endocrine mechanisms in the body and the molecular mechanisms of transcription by ERα/β will be more clearly elucidated, which will allow new agents and combined therapies for the endocrine treatment of breast cancer to be developed. It is currently unclear whether agonists or antagonists will be useful for ERβ as a potential novel target in the treatment of breast cancer. The potential role of ERβ splice variants—acting as natural antiestrogens and repressors of ERα function—needs further investigation and evaluation.

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