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Evaluation of oestrogen receptor β wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers

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Abstract

We addressed the clinicopathological significance of the oestrogen receptor (ER) β protein, including an ERβ variant, ERβcx, in normal human breast and breast cancer. The reverse transcriptase-polymerase chain reaction (RT-PCR) showed that wild-type ERβ (*ERβw*) mRNA expression was higher in normal than cancer tissues, and that *ERβcx* mRNA was higher in cancer than in normal tissues. Immunohistochemistry of 22 normal breast tissues and 57 breast cancers was performed with three different ERβ antibodies and one ERβcx antibody. All normal breast samples showed staining with the three ERβ antibodies, suggesting that ERβw might have a physiological role in oestrogen signalling in the normal breast. In breast cancer, expression of the ERβw protein correlated well with the expression of the ERα and progesterone receptor (PgR), as well as histological grade (HG), and tended to indicate a better prognosis than when ERβw was absent. Thirty-one (54%) breast cancer samples contained ERβcx, whereas the corresponding tissue for normal breast samples stained positive in only two (9%).

Keywords: Breast cancer; Oestrogen receptor β; Oestrogen receptor βcx; Immunohistochemistry; Prognosis

1. Introduction

The second oestrogen receptor (ER) subtype, ERβ, recently identified [1], has been found to be expressed in various tissues of the rat [1], mouse [2] and human [3]. A human *ERβ* cDNA encoding 530 amino acids has also been cloned and reported previously [4]. ERβ shows high homology with the DNA binding domain (DBD) (96%) and ligand binding domain (LBD) (53%) of ERα [4], and specifically binds oestrogen with high affinity [5]. Many groups have assessed the expression of ERβ in human breast cancer using the reverse transcriptase-polymerase chain reaction (RT-PCR) [6–9]. However, there are only a few reports of ERβ protein expression in breast tumours [10,11,12]. We have previously studied wild-type ERβ (ERβw) protein expression using immunohistochemistry (IHC) and reported that patients with ERβ-positive tumour have a better prognosis than patients with ERβ-negative tumour, and suggested that ERβw protein expression in breast cancers might possibly be a good prognostic marker [13]. Different ERβ variants with exon deletion [7,14], insertion [14–16], or C-terminal splicing [5,17] have been described more recently. Some splice variants, *ERβcx* and *ERβ5* mRNAs, are increasing during tumour growth and progression and these variant mRNAs are more highly expressed than *ERβw* mRNA [18]. However, expression of ERβ variant proteins and its functional significance in human breast cancer has yet to be assessed.

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We therefore investigated *ERβw*, *ERβcx*, and *ERβ5* mRNA expression using RT-PCR, and protein expression using IHC with three different ERβ antibodies and one ERβcx antibody in 22 normal breast tissues and 57 breast cancers, and examined relationships with clinicopathological factors.

2. Patients and methods

2.1. Patients and tissues

The subjects were 57 primary breast cancer patients who underwent surgery at the Department of Surgery II of Nagoya City University Medical School (Nagoya, Japan) between April 1992 and July 1997. Clinical information is listed in Table 1. Twenty-two normal breast specimens located more than 3 cm from the edge of the tumour, were obtained at mastectomy for primary breast cancer, and confirmed to be normal histopathologically. Patients were followed postoperatively every 3 months with clinical and radiological examinations. Stage I patients without nodal metastasis did not receive any adjuvant therapy. Stage II and stage III patients mainly received adjuvant chemoendocrine therapy using cyclophosphamide, methotrexate, 5-fluorouracil and tamoxifen for 2 years. ERα and progesterone receptor (PgR) status was determined by enzyme immunoassay (EIA) (ERα-EIA and PgR-EIA kits, Dynabott, Tokyo, Japan). A positive ERα status was defined as more than 13 fmol/mg protein, and that of PgR as more than 10 fmol/mg protein. Histological grade (HG) was assessed according to the modified Bloom and Richardson method described by Elston and Ellis in Ref. [19].

2.2. RNA extraction and reverse transcriptase-polymerase chain reaction conditions

Total RNA was extracted from approximately 500 mg of frozen tissue using TRIZOL reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcriptase reactions were performed as previously described in Ref. [20]. The triple primer-PCR method for detection of 3′-terminal splicing variants was performed according to Leygue and colleagues [18] with a slight modification. Briefly, primers located at the 3′-terminus consisted of ERU (5′-CGA TGC TTT GGT TTG GGT GAT-3′; sense, in exon 7, 1400-1420), ERL1 (5′-GCC CTC TTT GCT TTT ACT GT-3′; antisense, in exon 8, 1667-1649), and ERL2 (5′-CTT TAG GCC ACC GAG TTG ATT-3′; antisense, consensus sequence in *ERβcx* and *ERβ5*) primers, for wild-type and variant *ERβ* mRNA independent amplification, respectively. PCR conditions were as follows: 1 μl of reverse transcriptase reaction mixture was amplified in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 7.5 pmol of each respective primer, and 0.2 unit of Taq DNA polymerase (Takara, Ohtsu, Japan). Each reaction consisted of 30 cycles (30 s at 94 °C 30 s at 60 °C and 30 s at 72 °C). PCR products were separated on 6% polyacrylamide gels containing 7 M urea, and visualised by ethidium bromide staining under ultraviolet (UV) illumination. Reverse transcriptase efficiency was

Table 1
Clinicopathological characteristics of primary breast cancers

Characteristics	No. of cases (%)	Characteristics	No. of cases (%)
Total	57	Histology	
ER		Papillotubular	23 (40)
+	39 (68)	Scirrhous	22 (39)
−	18 (32)	Solid-tubular	5 (9)
PgR		Invasive lobular	4 (7)
+	38 (67)	Medullary	1 (2)
−	19 (33)	Mucinous	1 (2)
Age		DCIS	1 (2)
Mean±S.D.	60.9±8.8 (years)		
Range	33–79 (years)	Histological grade	
⩾50	32 (56)	1	13 (23)
<50	25 (44)	2	30 (53)
Tumour size		3	7 (12)
⩽2 cm	12 (21)	Unknown	7 (12)
2 cm< to ⩽5 cm	40 (70)		
>5 cm	5 (9)		
Lymph node metastasis			
−	35 (61)		
+	21 (37)		
Unknown	1 (2)		

ER, oestrogen receptor; PgR, progesterone receptor; S.D., standard deviation; DCIS, ductal carcinoma *in situ*.

controlled by assessment of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene amplification in parallel, as previously described in Ref. [20]. PCR products were subcloned into the TA cloning vector, pGEM-T-Easy (Promega, Madison, WI, USA), and we confirmed the sequence using a Takara Taq Cycle Sequencing Kit (Takara) for fluorescent sequencing with a Shimadzu DSQ-1000L DNA sequencer (Shimadzu, Kyoto, Japan). Each isolated double-stranded DNA was sequenced in both the forward and reverse directions. The amplified band intensities were measured with the PC software NIH image. Validation of triple primer-PCR was performed according to the method of Leygue and colleagues [18].

The Mann–Whitney U-test was used for the statistical analysis of correlations between ERβ and clinicopathological factors. Differences were considered significant when a P value of <0.05 was obtained.

2.3. Immunohistochemical staining

We used three different ERβ antibodies and one ERβcx specific antibody. The anti-ERβ rabbit polyclonal antibody (βN) (Upstate Biotechnology, Lake Placid, NY, USA) reacts with the N-terminal region (YAEPQKSPWCEARSLEHT, amino acids 46–63). The anti-ERβ chicken IgY polyclonal antibody (βT) recognises the whole ERβ protein, including an 18 amino acid insertion variant [21]. The anti-ERβ rabbit polyclonal antibody (βC) reacts with the C-terminus of ERβ (CSPAEDSKSKEGSQNPQSQ, amino acids 512–530) [5]. The anti-ERβcx rabbit polyclonal antibody (βCX) reacts with a part of the ERβcx-specific sequence at the C-terminus of ERβcx (MKMETLLPEATMEQ), and does not react with ERβw [5]. The procedure for immunohistochemical staining using βC was as previously described in Ref. [13]. For βN, βT and βCX, representative blocks of paraffin-embedded tissues were cut at 4-μm thickness and the sections were autoclaved for 15 min at 120 °C, and blocked for endogenous peroxidase activity with hydrogen peroxidase. After prevention of non-specific reactions with Block-ace solution (Dai-nippon Pharmaceutical, Osaka, Japan), sections were incubated with 1:50 dilution of βN, 1:400 of βT, or 1:50 of βCX overnight at 4 °C. For βN staining, Envision solution (Dako, Kyoto, Japan) was applied for 45 min at room temperature. For βT staining, peroxidase-conjugated anti-chicken IgY antibody (Cosmobio, Tokyo, Japan) was then applied to the sections for 30 min at room temperature before Envision solution was applied for 45min at room temperature. For βCX staining, the CSA system (Dako) was used according to the manufacturer's instructions. Peroxidase activity was visualised with 3,3′-diaminobenzidine and 0.03 mol/l hydrogen peroxide for 5 min. The sections were lightly counterstained with haematoxylin for microscopy. As a negative control, duplicate sections were immunostained without exposure to primary antibodies.

2.4. Immunohistochemical assessment

IHC samples were evaluated blindly (and independently) by two of our investigators. For normal mammary epithelium, if brown nuclei were observed in more than one-quarter of the epithelial cells, the epithelium was considered positive. For cancers, if cells with brown nuclei occupied more than one-quarter of a tumour, they were considered positive.

Fisher's exact probability or Chi-square tests were used for the statistical analysis of correlations between ERβ IHC and any clinicopathological status. Disease-free survival (DFS) curves were obtained by the Kaplan–Meier method and verified by the logrank (Mantel–Cox) and Breslow–Gehan–Wilcoxon tests. Differences were considered significant with a P value of less than 5%.

3. Results

3.1. The relative expression of wild-type ERβ, ERβcx and ERβ5 mRNAs in normal breast and breast cancer tissues

To evaluate the proportion of *ERβw*, *ERβcx* and *ERβ5* expression at the mRNA level, we performed a triplet RT-PCR using Leygue's method [18] to detect each form separately (Fig. 1). Expression ratios of *ERβw*, *ERβcx* and *ERβ5* were 17% (16.9±3.7%, mean±standard error of the mean (S.E.M.)), 41% (41.5±3.5%), and 42% (41.8±3.8%) in normal breast tissues, and 7% (6.9±0.8%), 56% (55.6±3.2%) and 36% (36.1±3.0%) in breast cancers, respectively. The two splice variants, *ERβcx* and ERβ5, were thus more highly expressed than *ERβw*. *ERβcx* expression was significantly higher in the cancers than in normal breast tissue ($P=0.026$, Mann–Whitney U-test). However, *ERβw* expression was higher in the normal tissues than cancers ($P=0.004$, Mann–Whitney U-test). These data suggest that the expression of *ERβw* mRNA is low and that of ERβ variant mRNA is high in breast tissue. Furthermore, expression of ERβ variants, especially *ERβcx*, increased during growth and progression of the breast cancer.

3.2. ERβ protein expression in normal breast and breast cancer tissues assessed using three different ERβ- and one ERβcx-specific antibodies

We performed IHC to investigate the expression of various ERβ proteins using three different ERβ- and one ERβcx-specific antibodies. The antibodies βN and βT are

expected to detect all types of ERβ, while βC is expected to detect mainly ERβw and possibly insertion or in-frame deletion variants. βCX detects only ERβcx protein.

Normal mammary duct or lobular epithelium in all 22 cases demonstrated specific nuclear staining with all three ERβ antibodies (Fig. 2a), whereas only 2 cases showed staining with βCX (Fig. 2b).

Out of 57 breast cancers, 22 (39%), 28 (49%) and 15 (26%) stained positive with βN, βT and βC, respectively (Fig. 2c–e). Staining with βN and βT correlated significantly ($P=0.001$), whereas no correlation between βC staining and other stainings was seen. With βCX, 31 (54%) of the breast cancer samples were positive, whereas 26 (46%) were negative (Fig. 2f). βCX staining did not correlate with any other ERβ staining.

3.3. Association of ERβ expression and clinicopathological factors

Results regarding the significance of ERβ expression with reference to clinicopathological parameters are summarised in Table 2. βC staining was well correlated with ERα ($P=0.022$) and PgR ($P=0.001$) status, as well as with low HG ($P=0.029$), and βN-positive cases correlated with low HG ($P=0.003$). βT staining did not exhibit correlation with any clinicopathological factor.

The patients with βC staining tumours tended to have a better prognosis than those with βC-negative tumours (Logrank (Mantel–Cox) test, $P=0.084$; Breslow–Gehan–Wilcoxon test, $P=0.059$) (Fig. 3), whereas βN or βT positivity in the tumours was not associated with any prognosis.

4. Discussion

Evaluation of ERα expression is one of the most important indicators for endocrine dependence and prognosis of breast cancer. However, only approximately 60% of ERα-positive tumours respond to endocrine therapies, whereas less than 10% of ERα-negative cases also respond [22]. Although combination of ERα with PgR offers some improvement in the prediction of endocrine response, it still does not serve as an absolute predictor for responsiveness to endocrine therapy. Other possible additional predictive factors, pS2 [23], *bcl2* [24], EGFR [25,26], and *c-erbB-2* [27,28] have been reported. However, at present, these factors do not appear to be more advantageous to use than ER and PgR. It is necessary to obtain additional novel predictors for responsiveness of breast cancer to endocrine therapy. When the novel subtype of ER, ERβ, was discovered, we hoped that this new receptor would be helpful in this regard. However, the significance of ERβ expression in breast cancer is still somewhat unclear. Some authors have reported that *ERβ* mRNA expression is increased in ERα-negative cases [7,8]. Others have found upregulated *ERβ* mRNA expression in tamoxifen-resistant tumours, correlating with a poor prognosis [9]. More recently, *ERβw* and its variants have been assessed separately. Leygue and colleagues [18] showed that *ERβw* mRNA expression in breast cancer was inversely correlated with the tumour grade, and two ERβ variants, *ERβcx* and *ERβ5* were upregulated during tumour growth and progression. Iwao and colleagues [29] reported that *ERβw* was downregulated during carcinogenesis, using real-time PCR. In this study, we used Leygue's experimental protocol and confirmed much lower *ERβw* mRNA expression in breast cancer than that of *ERβcx* or *ERβ5* mRNA, with higher expression of these variants in cancer than in normal breast tissue. These data indicate that it is important to assess *ERβw* and *ERβ* variants separately in breast cancer tissue.

Generally, protein expression is not in direct proportion to its mRNA expression, perhaps due to varying

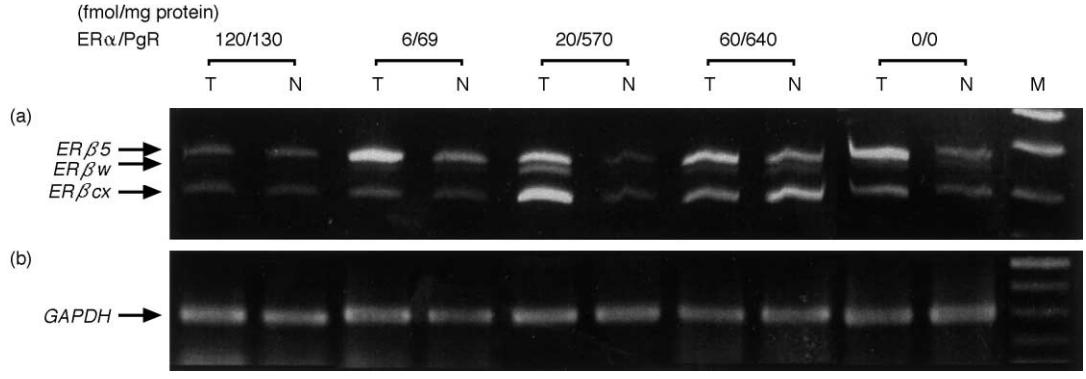

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *ERβw*, *ERβcx* and *ERβ5* expression within matched normal (N) and tumour (T) compartments of human breast tissues. (a) Triplet PCR analysis of *ERβw*, *ERβcx* and *ERβ5*. PCR products were separated on 6% polyacrylamide gels containing 7 M urea. (b) For each sample, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was amplified in parallel, and separated on an agarose gel. M, molecular weight marker (100 bp ladder; New England Biolabs, Beverly, MA, USA). ERα, oestrogen receptor α; PgR, progesterone receptor.

translation efficiency or the speed of degradation of the mRNA and its protein in cancer. It is therefore necessary to assess the expression of the ERβ protein, including that of the ERβ variants, and to compare the expression levels. However, so far there have only been a few studies of ERβ protein expression in breast tissues [10–12]. Jarvinen and colleagues [10], on the basis of IHC findings, suggested that ERβ was associated with ERα, PgR, axillary node status, low grade and premenopausal status. We previously examined the expression of ERβw protein by IHC, and found that patients with ERβw-positive tumours have a better prognosis than those with negative tumours [13]. Our data was consistent with Jarvinen's report, although these authors did not assess actual prognostic outcome. Fuqua and colleagues focused on both ERβw and ERβ variant proteins using western blotting analysis, and found that all five breast cancer samples and one cell line with a positive ERα status expressed ERβw, while two cell lines with a negative ERα status expressed a C-terminal splicing variant of ERβ [12]. We previously reported that the ERβcx protein, one of the carboxy-terminal splicing variants, inhibited the transcriptional activity of ERα rather than of ERβw, without binding to oestrogen *in vitro* [5]. If ERβcx protein was expressed to the extent indicated by its mRNA levels, it might therefore have an important role in breast cancer by modulating ERα. We thus used multiple antibodies to

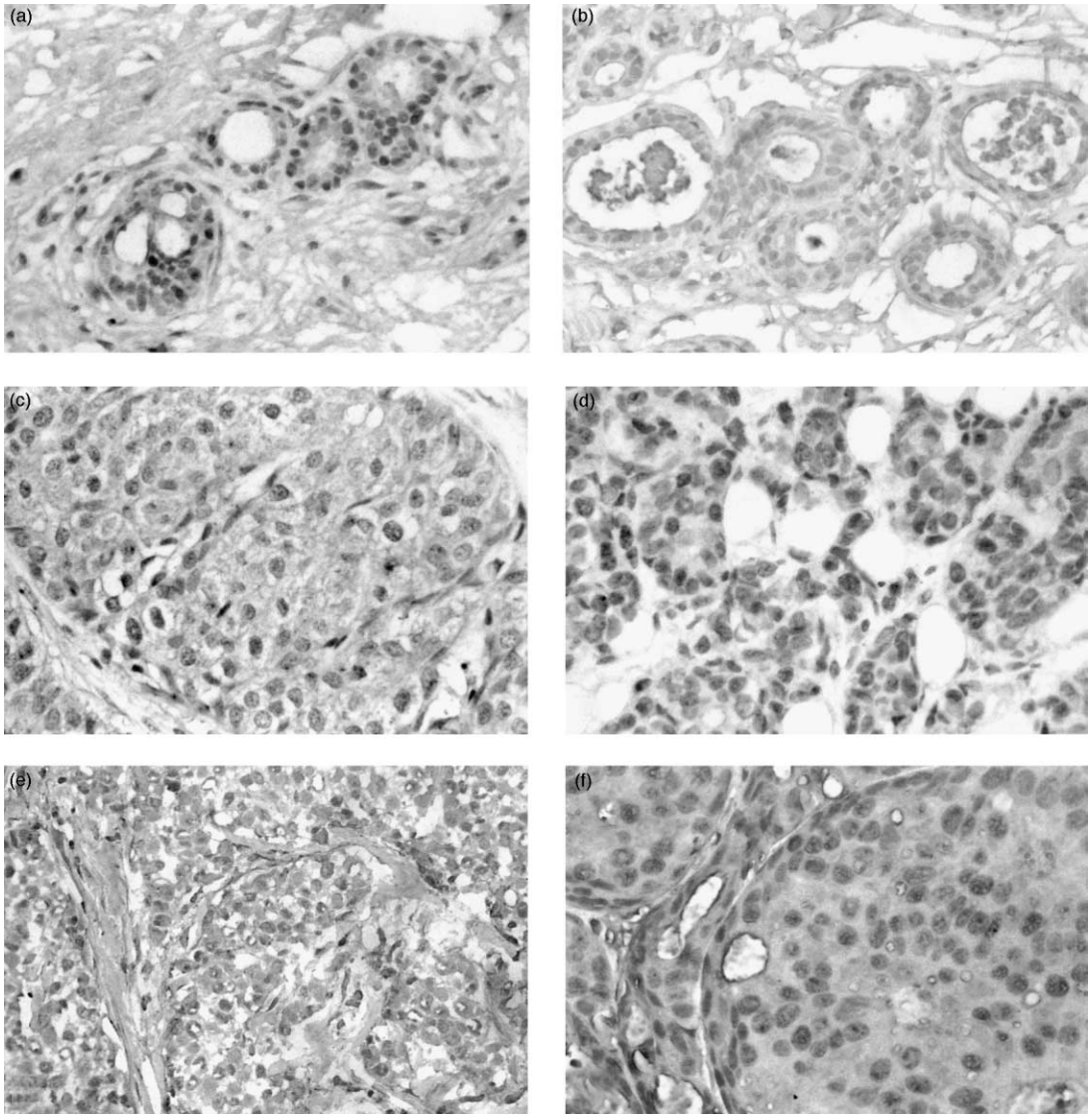

Fig. 2. Immunohistochemical staining of human breast normal and cancer tissue samples using three different ERβ and one ERβcx antibodies. Blocks of paraffin-embedded tissues were cut at 4-μm thickness and βN, βT and βCX were applied, and blocks of frozen tumour tissues were cut at 6-μm thickness for employment with βC. Magnification, ×400. (a) Normal mammary epithelium with βT. Nuclei of epithelial cells are stained brown with βT, considered as positive. (b) Normal mammary epithelium with βCX. Nuclei of epithelial cells show negative staining. (c) Breast cancer staining with βN. Nuclei of cancer cells are positive. (d) Breast cancer staining with βT. Positive staining. (e) Breast cancer staining with βC. Positive staining. (f) Breast cancer staining with βCX. Positive staining.

Table 2
Statistical analysis of ERβ staining and clinicopathological factors

Antibody	βN			βT			βC		
	+	–		+	–		+	–	
	22	35	*P* value	28	29	*P* value	15	42	*P* value
ER									
+	18	21	0.143	20	19	0.777	14	25	**0.022**
–	4	14		8	10		1	17	
PgR									
+	16	22	0.567	19	19	0.999	15	23	**0.001**
–	6	13		9	10		0	19	
Age									
⩾50	15	17	0.178	18	14	0.289	8	24	0.999
<50	7	18		10	15		7	18	
t									
⩽2 cm	5	7	0.117	9	3	0.091	4	8	0.801
<2 cm to ⩽5 cm	13	27		16	24		10	30	
>5 cm	4	1		3	2		1	4	
ly ($n=56$)									
–	13	22	0.780	19	16	0.582	12	23	0.128
+	9	12		9	12		3	18	
HG ($n=50$)									
1	10	3	**0.003**	8	5	0.362	7	6	**0.029**
2	8	22		14	16		5	25	
3	1	6		2	5		1	6	

ER, oestrogen receptor; PgR, progesterone receptor; t, tumour size; HG, histological grade; ly, lymph node metastasis. βN and βT react with all types of ERβ, and βC with ERβw. Significant *P* value in bold

assess the expression of various ERβ proteins including its variants. To our knowledge, this is the first report to address ERβcx protein expression in human breast cancers using IHC.

Among the three ERβ antibodies we used, βN and βT are expected to react with all forms of ERβ, whereas βC mainly recognises ERβw protein and some in-frame insertion or deletion variants. On the other hand, βCX is specific for ERβcx. Normal breast tissues stained positive for ERβ in all 22 cases with all three ERβ antibodies. Accordingly, ERβw might be important in oestrogen signalling in normal breast tissue. In breast cancers, positive staining with βN and βT showed good correlation, whereas staining with βC did not correlate

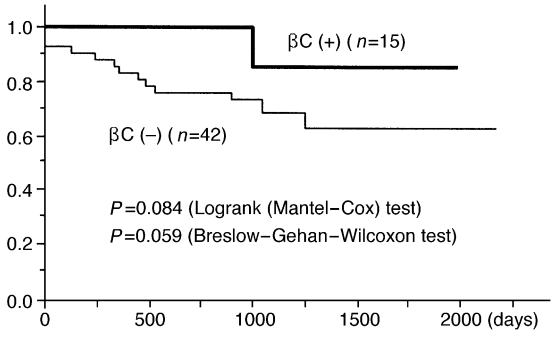

Fig. 3. Statistical analysis of disease-free survival with reference to ERβ staining with βC by the Kaplan–Meier Method. βC is expected to detect mainly ERβw. The patients with ERβ-stained tumours demonstrated a better prognosis than their negative counterparts (Logrank (Mantel–Cox) test, $P=0.084$; Breslow–Gehan–Wilcoxon test, $P=0.059$).

with the other ERβ stainings. This data indicated that these stainings were appropriate for the separate detection of the different ERβ proteins and suggested the existence of ERβ variant proteins in breast cancer. Furthermore, βC-positive cases showed a good correlation with the expression of ERα and PgR, or HG, and also tended to be associated with a better prognosis. This data indicated that ERβw protein was expressed in normal breast and in ERα- and PgR-positive or low HG cases of breast cancer. Although ERβw-positive cases tend to show better disease-free survival compared with ERβw-negative cases, we need more studies on a larger number of patients to confirm the use of ERβw as a good prognostic factor. 31 (54%) out of 57 breast cancer cases showed positive βCX staining, whereas only 2 (9%) out of 22 cases showed staining in normal breast tissue. This data indicated that ERβcx is more expressed in cancer than in normal tissue, although ERβcx did not show any relationship with clinicopathological factors (data not shown).

Separate detection of the ERβ variants might lead to a deeper understanding of ERβ function in breast cancer, but this is a difficult task since these variants are distinguished only by small differences in their amino acid sequences. Obviously, more studies are needed to investigate the expression of ERβ variant proteins in breast cancer.

In conclusion, we demonstrate ERβw protein expression in normal breast tissue suggesting that ERβw may have a physiological role in oestrogen signalling in the

normal breast. ERβcx is more expressed in cancer than in normal tissues. Expression of ERβw was higher in ERα- and PgR-positive or low HG breast cancer, suggesting that ERβw tends to be expressed in cases with a better prognosis.

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