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Studies on oestrogen receptor-α and -β mRNA in breast cancer

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Abstract

The oestrogen receptor (ER) is widely used to predict response to tamoxifen in patients with breast cancer. Recently a new form of ER known as ER-β was discovered, the original ER is now designated ER-α. In this investigation, ER-α and ER-β were measured in 107 breast carcinomas and 22 fibroadenomas. Using reverse transcriptase-polymerase chain reaction (RT-PCR), ER-β mRNA, but not ER-α mRNA was expressed more frequently in fibroadenomas than carcinomas. In the carcinomas, ER-β mRNA was present in a greater proportion of samples positive for ER-α mRNA than in those lacking this form of the receptor. ER-α, but not ER-β mRNA, was significantly associated with ER protein-positivity in the cancers. ER-α mRNA was also positively related to progesterone receptors (PR), but ER-β mRNA showed an inverse relationship with PR. We conclude that the presently used enzyme-linked immunosorbent assay (ELISA) for ER appears to be mostly measuring ER-α and is unlikely to be detecting ER-β.

Keywords: Breast cancer; Tamoxifen; Oestrogen receptor-α; Oestrogen receptor-β; Predictive marker

1. Introduction

The oestrogen receptor (ER) is a nuclear transcriptional factor which modulates the expression of multiple genes. Since the 1970s, assay of this protein has been used to select endocrine-responsiveness in patients with advanced breast cancers (for review, see [1]). More recently, the ER status of primary breast cancers was shown to predict the benefit of adjuvant tamoxifen in prolonging both disease-free interval and overall survival [2]. In addition to being a predictive marker, multiple studies have shown that breast cancer patients who possess an ER-positive tumour have a better prognosis than those lacking the receptor, at least for short-term follow-up (reviewed in [3,4]).

For years, it was generally assumed that only one gene existed for the ER as is also assumed for other steroid hormone receptors. In 1996, however, a new *ER* gene was cloned from a rat prostate cDNA library and designated ER-β to distinguish it from the classical ER which was renamed ER-α [5]. The two forms of ER share approximately 95% homology in their DNA binding region and 55% homology in the ligand binding domain, but have little homology in other regions [6]. Like ER-α, ER-β binds 17β-oestradiol with high affinity and can transactivate the expression of reporter genes at oestrogen response elements by an oestrogen-dependent mechanism [6,7]. However, at the AP-1 enhancer element, ER-α and ER-β appear to signal in opposite directions [8]. For example, with ER-α, 17β-oestradiol activates transcription, whereas with ER-β, the hormone inhibits transcription [8]. In addition, at the AP-1 site, anti-oestrogens such as tamoxifen, raloxifene and ICI 164, 384 were able to block the stimulatory effect of ER-α, but acted as agonists in the presence of ER-β [8]. These findings suggests that ER-β may be involved in mediating resistance to tamoxifen in breast cancer and that measurement of ER-β in breast cancer biopsies might predict sensitivity/resistance to tamoxifen therapy.

Dotzlaw and colleagues [9] were first to show expression of ER-β in human breast cancers. In preliminary studies, these investigators reported that expression of

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mRNA for ER-β was independent of both ER protein as determined by ligand binding assay and ER-α mRNA [9,10].

The aims of this study were to compare the expression of ER-α and ER-β in primary breast carcinomas and fibroadenomas, relate the expression of ER-α to ER-β, correlate expression of ER-α and ER-β mRNA with ER protein, progesterone receptors (PR) protein and histopathological characteristics of the cancers.

2. Patients and methods

2.1. Handling of tumours

The breast cancers used were consecutive samples ($n = 107$) sent for biochemical determination of ER and PR. The histological details and steroid receptor status of the samples are summarised in Table 1. Following histopathological examination, tumours were rapidly frozen in liquid nitrogen and then transferred to a −70°C freezer. Tissue homogenisation was carried out using a Braun Micro Dismembrator (Braun, Melsungen, Germany). Part of the powder was extracted with 50 mM Tris buffer (pH 7.4) containing 1 mM monothioglycerol and assayed for ER and PR using enzyme-linked immunosorbant assay (ELISA) (Abbott Diagnostics, North Chicago, IL, USA). For ER, the cut-off point was 200 fmol/G while for PR it was 1000 fmol/G. The residual powder was extracted for total RNA using the guanidinium thiocyanate method [11]. Fibroadenomas were stored and processed in an identical manner to the carcinomas.

Table 1
Pathological features and steroid receptor status of breast cancers

Variable	Patients *n* (%)
Nodal status	
Negative	49 (46)
Positive	48 (45)
Unknown	10 (9)
Tumour size	
⩽2 cm	30 (28)
>2 cm	61 (57)
Unknown	16 (15)
Histological type	
Ductal	81 (76)
Lobular	17 (16)
Unknown	9 (8)
ER status	
Positive	72 (67)
Negative	34 (32)
Unknown	1 (1)
PR status	
Positive	38 (36)
Negative	43 (40)
Unknown	26 (24)

ER, oestrogen receptor; PR, progesterone receptor.

2.2. Reverse-transcriptase-polymerase chain reaction (RT-PCR)

In a final volume of 20 μl, 1 μg of total RNA was reverse transcribed into cDNA. The reaction mixture contained 0.4 mM of each deoxynucleotide triphosphates (dNTP), 10 μg/ml of Oligo(dT)12-18, 10 mM dithiothreitol (DTT), 50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$. The reaction mix was incubated for 5 min at 65°C to remove secondary RNA structures, centrifuged and cooled on ice. 4.6 U of human placenta ribonuclease inhibitor (Gibco BRL) and 200 U of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) were then added followed by incubation for a further 60 min at 37°C. Finally, the samples were heated for 5 min at 70°C and then stored at −20°C until required for PCR amplification.

Amplification of cDNA was carried out using primers previously described [9,10]. For ER-α the primers were as follows [9]:

sense, 5′ CAG GGG TGA AGT GGG GTC TGC TG 3′ antisense, 5′ ATG CGG AAC CGA GAT GAT GTA GC 3′

while for ER-β, the following primers were used [10]

sense, 5′ GTC CAT CGC CAG TTA TCA CAT C 3′ antisense, 5′ GCC TTA CAT CCT TCA CAC GA 3′.

PCR was performed in 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.2 mM of each dNTP, 200 ng of each primer (Genosys, Pampisford, UK), 5 μl of cDNA and 2 U of Taq DNA polymerase (Promega) in a final volume of 50 μl. For ER-α, $MgCl_2$ was included at a concentration of 5 mM, while for ER-β it was used at a concentration of 2 mM.

The amplification conditions were as follows:

ER-α: A denaturation step for 2 min at 94°C, followed by 1 min at 94°C, 0.5 min at 60°C and 1 min at 72°C for 35 cycles, followed by 5 min at 72°C.

ER-β: A denaturation step for 2 min at 94°C, followed by 1 min at 94°C, 0.5 min at 60°C and 1 min at 72°C for 38 cycles, followed by 10 min at 72°C.

With these conditions, amplification products were obtained in the exponential phase for the two sets of primers used. Following amplification, 20 μl and 25 μl of ER-α and ER-β PCR product, respectively, were run on a 2% agarose gel. The gels were stained by ethidium bromide and visualised under ultraviolet (UV) light. The intensity of the bands was determined by densitometry (EagleEye™, Stratagene UK) and expressed as arbitrary units.

As a control, PCR with primers specific for glyceraldehyde phosphate dehydrogenase (*GAPDH*) cDNA was carried out on each sample. Results for ER-α and

Table 2
ER-α and ER-β mRNA positivity in fibroadenomas and primary breast cancers

	Fibroadenomas ($n=22$)	Cancers ($n=107$)
	No. positive (%)	No. positive (%)
ER-α	12 (55)	70 (65)
ER-β	15 (68)	43 (40)

Table 3
ER-α and ER-β mRNA in ER protein-positive and ER protein-negative breast cancers

	ER protein-positive ($n=72$)	ER protein-negative ($n=34$)
	No. positive (%)	No. positive (%)
ER-α	56 (78)	14 (41)
ER-β	29 (40)	14 (41)

ER-β were expressed relative to those for *GAPDH*. RNA isolated from endometrium and ovary was used as a positive control for ER-α and ER-β, respectively. Negative controls included (a) omission of reverse transcriptase and (b) replacement of cDNA by water.

3. Results

3.1. ER-α and ER-β mRNA in fibroadenomas and primary breast cancers

Table 2 shows the proportion of fibroadenomas and primary breast cancers expressing mRNA for ER-α and ER-β. ER-β was found more frequently in the fibroadenomas than in the cancers (Chi square = 4.70, $P=0.0301$). Although the α form was present in a greater number of cancers than fibroadenomas, the difference was not statistically significant. In the cancers, ER-β was expressed more frequently in samples positive for ER-α (35/70, 50%) than in those lacking ER-α (8/37, 22%) (Chi-square = 6.97, $P=0.0083$).

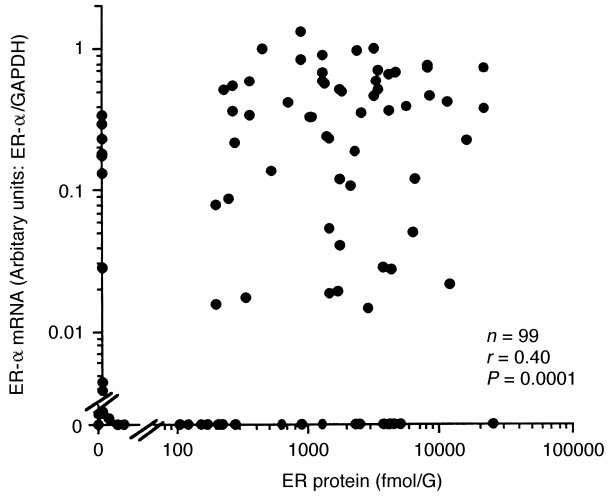

Fig. 1. Relationship between ER-α mRNA and ER protein levels. Data was analysed using the non-parametric Spearman Rank test. ER, oestrogen receptor; PR, progesterone receptor; GAPDH, glyceraldehyde phosphate dehydrogenase.

3.2. Relationship between both ER-α and ER-β mRNA and ER protein

ER-α mRNA was detected more frequently in ER protein-positive than ER protein-negative cancers (Chi square = 12.21, $P=0.0005$) (Table 3). ER-β expression, however, was not related to ER protein status. Similarly, relative levels of ER-α mRNA correlated significantly with those for ER protein (Fig. 1), whereas ER-β levels showed no such correlation (data not shown).

3.3. Relationship between both ER-α and ER-β mRNA and PR protein

As with ER protein, ER-α mRNA was present more frequently in PR protein-positive cancers than PR protein-negative cancers (Chi-square = 6.35, $P=0.0117$) (Table 4). In contrast, ER-β was expressed in a greater proportion of PR protein-negative samples than PR protein-positive samples (Chi-square = 4.45, $P=0.0350$) (Table 4). Consistent with these findings, relative levels of ER-α mRNA correlated positively with PR protein (Fig. 2a), while ER-β mRNA levels showed an inverse relationship with PR protein levels (Fig. 2b).

3.4. Relationship between both ER-α and ER-β mRNA and histopathological characteristics of the breast cancer

No significant relationship was found between either ER-α or ER-β mRNA presence and either nodal status, tumour size or histological type (Table 5). Similarly, the expression of ER-α and ER-β mRNA was not related to patient age (Table 5).

Table 4
ER-α and ER-β mRNA in PR protein-positive and PR protein-negative breast cancers

	PR protein-positive ($n=38$)	PR protein-negative ($n=38$)
	No. positive (%)	No. positive (%)
ER-α	32 (84)	24 (56)
ER-β	9 (24)	21 (49)

4. Discussion

As mentioned in the introduction, Dotzlaw and colleagues [9] were the first to detect expression of the ER-β gene in breast cancer. Using radioactive PCR, mRNA for this form of ER was found in 70% of 40 breast cancers. Using a greater number of breast cancers (i.e. 107) and staining with ethidium bromide to locate the PCR products, we found ER-β to be present in a lower proportion of cancers, i.e. 40%. However, in contrast to others [9,10] who reported no correlation between ER-α and ER-β, we found that ER-β mRNA was expressed more frequently in ER-α mRNA-containing cancers than ER-α mRNA-negative cancers.

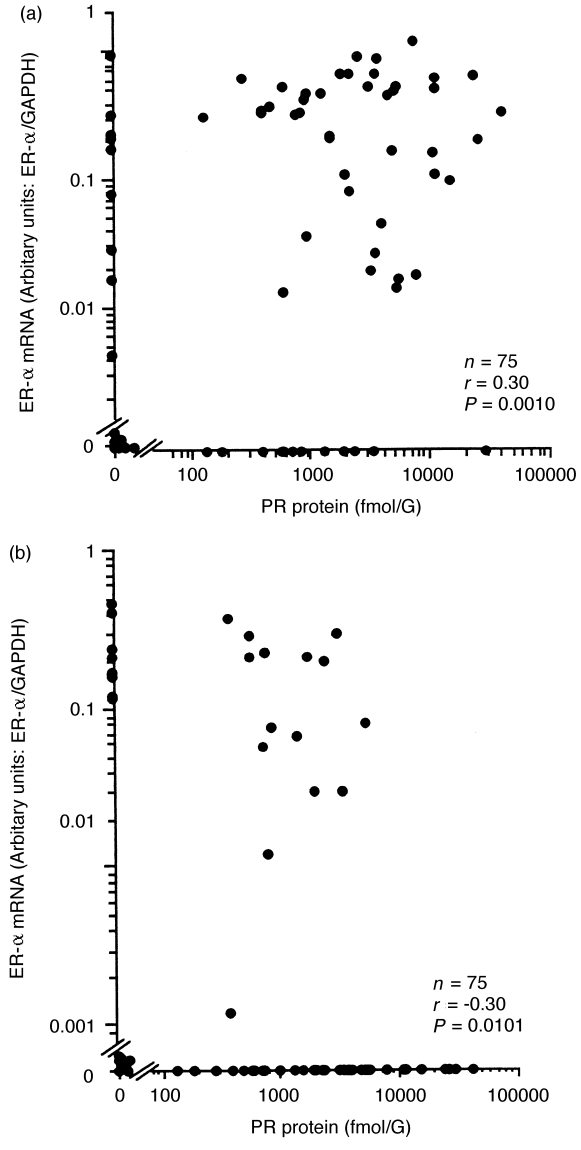

Fig. 2. (a) Relationship between ER-α mRNA and PR protein levels; (b) relationship between ER-β mRNA and PR protein levels. Data was analysed using the non-parametric Spearman Rank test. ER, oestrogen receptor; PR, progesterone receptor; GAPDH, glyceraldehyde phosphate dehydrogenase.

We also found that ER-β was expressed less frequently in cancers than fibroadenomas. Expression of ER-α, in contrast, was not significantly different in the benign and malignant groups of tumours. We are not aware of any previous publication comparing ER-α and ER-β in these two types of breast lesion. However, Speirs and colleagues [12] showed that compared with normal breast tissue, a higher proportion of cancers expressed both forms of the ER.

With the discovery of ER-β it was important to establish if the existing immunoassays for ER protein were detecting this new form of the receptor. Using a widely available ELISA to measure the ER protein, we showed that ER-α mRNA was mostly, but not exclusively, confined to ER protein-positive cancers. In contrast, ER-β mRNA showed no significant relationship with ER protein.

Consistent with these findings, relative ER-α mRNA levels correlated significantly with ER protein concentration, whereas ER-β mRNA levels showed no such relationship. Using ligand binding assays, Leygue and coworkers [10] also reported no correlation between ER-β mRNA and ER protein.

In this investigation, ER-α mRNA was also found more frequently in PR protein-positive than in PR protein-negative cancers, while the reverse was found for ER-β. The inverse relationship between ER-β mRNA and PR protein is in agreement with a previous report [13] and may be explained by the downregulation of ER-β by progesterone [13]. To our knowledge, a positive correlation between ER-α mRNA and PR protein has not previously been reported, although ER and PR proteins are well known to correlate with one another.

In contrast to studies at a mRNA level, Jarvinen and coworkers [14] using immunohistochemistry found a positive association between ER-β protein and PR levels. Furthermore, unlike our results, these authors

Table 5
Relationship between ER-α and ER-β mRNA and patient age, nodal status, tumour size and histological type

		ER-α	ER-β
Variable	*n*	No. positive (%)	No. positive (%)
Age (years)			
⩽50	44	29 (66)	18 (41)
>50	63	41 (65)	25 (40)
Nodal status			
Negative	49	33 (67)	21 (43)
Positive	48	30 (63)	15 (31)
Size			
⩽2 cm	30	18 (60)	10 (33)
>2 cm	61	42 (69)	23 (38)
Histological type			
Ductal	81	53 (65)	32 (40)
Lobular	17	11 (65)	5 (29)

reported that the ER-β protein was found more frequently in axillary node-negative than in axillary node-positive patients. The reason(s) for the different results are not clear, but it could be due to a poor correlation between ER-β mRNA and protein levels.

An important question to be addressed in the future is whether measurement of ER-β will enhance the prognostic or predictive ability of the existing ER assays. In this context, it is important to point out that Speirs and colleagues [15] recently reported that ER-β mRNA levels were significantly higher in tamoxifen-resistant than tamoxifen-sensitive breast cancers. Clearly, prospective trials are now necessary to establish if ER-β will help predict response or resistance to tamoxifen therapy in patients with breast cancer.

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