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Distinct mRNA, protein expression patterns and distribution of oestrogen receptors α and β in human primary breast cancer: Correlation with proliferation marker Ki-67 and clinicopathological factors

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Abstract

To elucidate the molecular profile of oestrogen receptors α and β (ERα, ERβ) we studied ERα and ERβ expression at the mRNA and protein levels using real-time polymerase chain reaction (RT-PCR), Western blot analysis and immunohistochemical (IHC) methods in 41 primary breast cancers and surrounding tissues. ERα mRNA and ERβ mRNA were detected in all of the breast cancer and normal matched tissues analysed. ERα mRNA levels showed greater diversity than ERβ mRNA levels and the range of amount of ERβ transcripts was far smaller than that of ERα. At the protein level, the percentage of ERα- or ERβ-positive cases changed. Seventy percent of the tumours studied produced full-length 65 kDa ERα protein in Western blot analysis and 67% of assessed cases were positive in IHC. Full-length 57 kDa ERβ protein was detected by Western blotting in 97% of analysed breast cancers, while 67% were ERβ-positive using IHC. ERα was localised in the nucleus, while cytoplasmic and perinuclear localisation of ERβ was observed in normal as well as in breast cancer cells. The amount of ERα (but not ERβ) increased with age. The expression of ERα correlated positively with progesterone receptor and negatively with proliferation marker Ki-67. These results confirm the previous observations that the lack of ERα protein expression is not due to lack of ERα gene expression or methylation of ERα promoter, but due to post-transcriptional or post-translational mechanisms. Our investigation also suggests that ERα is more dysregulated in breast cancer, and thereby ERβ is more tightly regulated in the tumour.

Keywords: Oestrogen receptor α; Oestrogen receptor β; Ki-67; Breast cancer

1. Introduction

Oestrogens play a key role in the genesis and progression of breast cancer, activating two oestrogen receptors alpha and beta (ERα and ERβ). Immunohistochemical (IHC) studies showed that in normal mammary glands ERβ was a predominant receptor while ERα was expressed only in 5–15% of normal epithelial cells [1]. In the early stage of breast cancer the overexpression of ERα was observed in 70% of breast tumours, and it is thought that this upregulation is an initial significant event in breast cancer biology. The molecular mechanism leading to overexpression of ERα is still not clear. de Graffenried *et al.* [2,3] proposed a model that is

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responsible for high expression levels of the ERα gene in breast cancer cells. This model is based on the regulation of transcription through *cis*-acting elements and *trans*-acting factors, such as USF-1 transcriptional factor, SP-1 transcriptional factor and ERα, which together are responsible for transactivating the minimal promoter and the enhanced ERα transcription. There are some studies that have demonstrated that ERα expression is regulated at the mRNA level, partly by changes in mRNA stability, as well as at the post-translational level. Recent studies have shown constitutive expression of ERα mRNA in all breast tumours, even those that are ERα-negative in IHC study [4]. This suggests that epigenetic events, such as methylation of CpG islands in the ER promoter, are not the major regulatory mechanism responsible for ERα gene inactivation.

There are a few studies addressing a possible role for ERβ in human breast cancer. ERβ was expressed at much lower rates than ERα [5]. The expression of ERα protein in breast cancer predicts a favourable disease outcome and selects patients for anti-oestrogen treatment. Approximately one-third of primary breast cancers do not express ERα and are classified as ER-negative. Lack of ERα expression often reveals more aggressive phenotypes [6]. Whether ERβ plays an important role in human breast cancer development or response to endocrine therapy remains an open question. Although in some studies it has been shown that ERβ is significantly associated with poor prognostic features including lymph node-positive tumours and resistance to tamoxifen therapy [7], other investigations have suggested no correlation between ERβ expression and tumour size, grade and node status [8,9].

The clinical importance expression of ERs in breast cancer prompted us to characterise the pattern of both ERα and ERβ in primary breast cancer tissues and matched tissues at mRNA level using real-time polymerase chain reaction (RT-PCR) and protein levels by Western blotting and IHC techniques. The expression of ERs was correlated with expression of progesterone receptor (PGR) and Ki-67, and with known clinicopathological features of malignant potential.

2. Materials and methods

2.1. Patients and samples

The study subjects comprised 41 women treated surgically for primary breast cancer. A total of 41 tumour samples and 20 matched (adjacent) normal mammary gland tissues were frozen in liquid nitrogen and kept at −80 °C until use. Tumour samples, adjacent normal tissue and lymph nodes were also fixed in 10% (v/v) buffered formaldehyde solution for 48 h and then embedded in paraffin blocks at 56 °C according to standard procedures.

Tumour samples were cut into 5-μm thick sections and stained with haematoxylin-eosin. Histopathological examination was based on the World Health Organization (WHO) and pTN classification of breast tumours [10]. The study comprised only invasive ductal carcinomas, representing G2 (19 patients, cases) and G3 (22 patients) grade. Histopathological grading (G) was performed according to the Bloom and Richardson system [11]. There were 53.7% (22/41) tumours in pT1 stage and 46.3% (19/41) in pT2 stage. 48.8% (20/41) of women had involved lymph nodes at the time of diagnosis. Patients had not received any preoperative chemotherapy or hormonotherapy. The age of patients ranged from 33 to 83 years, with a mean of 51.9 years. The local ethical committee approved the protocol of this study.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen breast and matched tissues according to the Chomczynski and Sacchi method [12]. RNA integrity was verified by electrophoresis in 1.5% (w/v) agarose gel and staining with ethidium bromide, and by amplification of the housekeeping gene, GAPDH. RNA was quantified spectrophotometrically at 260 nm. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP mix (Promega), 2.5 μM oligo dT15, 20 U RNasin Ribonulease Inhibitor (Promega), 100 U Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega) in a final volume of 40 μl using MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA). For reverse transcription, the mixtures were incubated at 42 °C for 60 min and then heated at 95 °C for 5 min and finally cooled rapidly at 4 °C.

2.3. RT-PCR

To determine the amounts of ERα and ERβ, two standard curves were constructed with serially diluted PCR products. PCR products were obtained by amplification cDNA from normal mammary gland using specific primers as follows: sense: 5′TGC TTC AGG CTA CCA TTA TGG AGT CTG3′; antisense: 5′GTC AGG GAC AAG GCC AGG CTG3′ for ERα; sense: 5′TTT AAA GAA GCA TTC AAG GAC ATA ATG3′, antisense: 5′GAA GTG TGG CTC CCG GAG AGA GAG A3′ for ERβ. PCR was carried out in a final volume of 50 μl using 25 pmol of each of the primers, 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer and 5 μl cDNA. PCR was carried out under the following conditions: 5 min at 95 °C, 1 min denaturation at 95 °C, 1 min

annealing at 60 °C, 1 min extension at 72 °C for 40 cycles, with an additional 10 min extension for the last cycle. Amplified products were separated on a 2% (w/v) agarose gel, extracted and purified from agarose slices using DNA Gel Extraction Kit (Millipore, USA), quantified by the use of One Dscan/Zero Dscan software (Scanalytics Inc., USA) and then serially diluted in sterile water.

All RT-PCR reactions were performed using ABI Prism 7000 Sequence Detection System (Perkin–Elmer Applied Biosystems, USA). For each PCR run, a master mix was prepared with 10 ml 2× Taq Man Universal PCR Master Mix (Applied Biosystems), 1 μl 20× Assays-on Demand Gene Expression Assay Mix (Applied Biosystems), 5 μl cDNA or diluted standard and sterile water to final volume of 20 μl.

2.4. Quantitative competitive RT-PCR

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard was prepared as described previously by Bouraima *et al.* [13] using pBR322 vector as a template. The specific primers (sense Bene 1P1B: 5′TCA TCC ATG ACA ACT TTG GTA TCG TGC GGC ATC AGA GCA GAT TGT ACT GAG 3′; antisense Bene 2M1B: 5′GTG CTC AGT GTA GCC CAG GAT GCG GGG AAA CGC CTG GTA TCT TTA TAG TCC3′) were designed so that the 5′ ends of sense and antisense primers were complementary to the GAPDH mRNA sequence and the 3′ ends of these primers were complementary to the pBR322 sequence. At the 5′ and 3′ ends the PCR product contained complementary sequences to specific GAPDH primers used in quantitative competitive PCR. PCR was carried out in a final volume of 50 μl, using 25 pmol of each of the primers, 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer and 1 ng pBR322 vector. PCR was carried out under the following conditions: 5 min at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 60 °C, 1 min extension at 72 °C for 30 cycles, with an additional 10 min extension for the last cycle. Amplified products were subjected to electrophoresis on a 2% (w/v) agarose gel, extracted and purified from agarose slices using DNA Gel Extraction Kit, quantified by the use of One Dscan/Zero Dscan software and then diluted in sterile water.

To determine amounts of GAPDH mRNA, quantitative competitive PCR was performed in a final volume of 50 μl using 25 pmol of each of the primers (sense Bene1: 5′TCA TCC ATG ACA ACT TTG GTA TCG TG3′, antisense Bene2: 5′GTG CTC AGT GTA GCC CGG ATG C3′), 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer, 5 μl cDNA from studied tissues and 5 μl diluted standard in the same tube. For each analysed tissue two dilutions of GAPDH standard were used (0.5 and 50 pg). All the results obtained (in pg) were converted into pmol of GAPDH per μg total RNA. PCR was carried out under the same conditions as described for GAPDH standard building. Amplified products were separated on a 4% (w/v) agarose Metafor gel and quantified by the use of One Dscan/Zero Dscan software.

2.5. Gel electrophoresis and Western blot analysis

Western blot analysis was performed on 41 breast cancer samples and 20 matched tissues. Patient tissues were pulverised in a mortar and homogenised further on ice in lysis solution consisting of 50 mM Tris–HCl, pH 7.4; 1% (v/v) Nonidet P-40; 0.25% (w/v) deoxycholic acid (DOC); 150 mM NaCl; 1 mM Na_3VO_4, 1 mM EDTA, 1 mM NaF and protease inhibitors (10 μg/ml leupeptin, 2 μg/ml aprotinin and 200 mM phenylmethylsulphonyl fluoride (PMSF)). After the lysis samples were boiled for 5 min in 62.5 mM Tris–HCl, pH 6.8 containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001 % (w/v) bromophenol blue. The samples were electrophoresed on 0.1% (v/v) SDS–polyacrylamide gel (37.5/1 acrylamide/bisacrylamide, composed of 4% (v/v) stacking gel and a 10% (v/v) separating gel) at 50 V/gel for 1.5 h at room temperature in a pH 8.3 running buffer containing 25 mM Tris, 192 mM glycine and 0.1% (w/v) sodium dodecyl sulphate (SDS).

After SDS–PAGE, the protein was transferred to 0.2 μm pore sized nitrocellulose at 100 mA for 1 h by the use of an LKB 2117 Multiphor II electrophoresis unit according to the method described in the manual accompanying the unit. Nitrocellulose was blocked with 5% (w/v) dried milk in TBS-T buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.005% (v/v) Tween 20) for 1 h at room temperature, shaking slowly. Then the nitrocellulose was incubated with the rabbit polyclonal antibodies against ERα (HC-20, Santa Cruz Biotechnology, SCBt, USA) and against ERβ (H-150, SCBt, USA) diluted 1:200 in blocking buffer for 1 h. After the incubation, nitrocellulose was washed three times for 10 min in blocking buffer and then incubated with secondary antibody conjugated with alkaline phosphatase at concentration 1:1000 in TBS-T and incubated for 1 h, shaking slowly. Nitrocellulose was washed with TBS-T (three times for 10 min) and submitted to Sigma-Fast BCIP/NBT (Sigma, Germany) reagent.

2.6. Immunohistochemistry

For IHC studies we selected two representative sections from each case of breast cancer and matched normal mammary gland tissue. Four markers were investigated: ERα, ERβ, PGR and Ki-67. ERα was detected with a mouse monoclonal antibody (F-10,

SCBt, USA) at dilution 1:200. ERβ was assessed with the rabbit polyclonal antibody (H-150, SCBt, USA) at dilution 1:200. PGR was investigated using monoclonal mouse PGR antibody (clone 636, Dako, Denmark) at 1:100 dilution. Ki-67, a proliferation marker, was assessed using the monoclonal mouse antibody (MIB-1, Dako, Denmark) at 1:100 dilution.

The sections were deparaffinised in xylenes and hydrated through graded alcohols. Antigen unmasking was performed using heat treatment in a microwave oven at 750 W for 7 min in a container with 10 mM sodium citrate buffer, pH 6.0. Sections were allowed to cool in the buffer at room temperature for 30 min and were rinsed in deionised water three times for 2 min each. The endogenous peroxidase activity was blocked with 1% (v/v) hydrogen peroxide for 20 min. Then the sections were incubated with ERα or ERβ antibody at 4 °C overnight or with PGR or MIB-1 antibody for 1 h at room temperature using staining chamber (The Binding Site, UK). Primary antibodies were diluted in phosphate-buffered saline (PBS). After rinsing in three changes of PBS, an avidin–biotin–peroxidase complex (SCBt, USA) for ERα and ERβ and a streptavidin–biotin–peroxidase complex (LSAB kit, Dako, Denmark) for PGR and Ki-67 were used to reveal antibody-antigen reactions. Staining was routinely developed using 3,3′-diaminobenzidine as a chromogen (Dako, Denmark). Sections were counterstained with haematoxylin. Two independent pathologists evaluated immunostainings by the use of light microscopy (20× and 40× objectives). The evaluation of ERα, ERβ, PGR and Ki-67 expression was analysed in 10 different tumour and normal mammary gland fields and the mean percentage of tumour as well as normal mammary gland cells with positive staining was evaluated. The sections were classified as positive when at least 10% of cells expressed the antigen studied. We performed the following IHC controls: positive controls including breast cancers previously documented as positive for ERα, ERβ, PGR and Ki-67; negative controls including omission of primary antibodies.

2.7. Statistical analysis

Mean values ± standard deviations (SD) were calculated. The results were analysed using the Mann–Whitney test and Spearman's correlation test, accepting $P < 0.05$ as significant.

3. Results

3.1. Expression of ERα and ERβ mRNA

To evaluate the levels of ERα and ERβ mRNA expression, two curves (prepared as described in Section 2) were used: for ERα 100–10–1–0.1–0.001 fg (Fig. 1(a)), and for ERβ: 1–0.1–0.01–0.001 fg (Fig. 2(a)). All the results obtained (in fg) were converted to fmol of ERs per μg of total RNA, and then corrected by the level of GAPDH mRNA (calculated as described in Section 2). RT-PCR revealed the presence of transcripts of both ERα and ERβ in all the breast cancer tissues analysed. ERα and ERβ mRNAs were also detected in all matched normal mammary gland tissues. ERα values in breast cancer tissues ranged from 1.8×10^{-5} to 220.5 fg/μg of RNA (mean 21.61 fg/μg of RNA ± SD 43.8; Figs. 1(b) and 3). The expression of ERβ showed much less range in the amount of ERβ transcripts (Figs. 2(b) and 3), ERβ values for the breast tissues samples ranged from 0.01 to 0.25 fg/μg of RNA (mean 0.088 fg/μg RNA ± SD 0.096). Analysis of the matched normal tissues has revealed that the level of ERα mRNA was much lower in comparison with breast cancer tissues (mean 11.76 fg/μg of RNA ± SD 13.9; Figs. 1(c) and 3), but expression of ERβ mRNA displayed opposite trends. There were many more transcripts of ERβ in the matched tissues than in the breast cancer tissues (mean 0.54 fg/μg of RNA ± SD 1.0; Figs. 2(c) and 3). We also observed a positive correlation between both ER's transcript levels ($P < 0.002$, $r = 0.532$).

3.2. Expression of ERα and ERβ protein by Western blot analysis

Western blot analysis was performed on the same 41 breast cancer tissues and 20 matched normal mammary gland tissues to confirm the expression of ERs at the protein level. Seventy percent of analysed breast tumours and only 5% of matched normal tissues produced full-length ERα protein corresponding to approximately 65 kDa (Fig. 4(a) and (b)). Ninety-seven percent of analysed tumours and 95% of matched normal tissues showed the presence of an immunopositive protein band of approximately 57 kDa corresponding to wild-type ERβ (Fig. 4(c) and (d)).

3.3. Expression of ERα, ERβ, PGR and Ki-67 using IHC

IHC analysis of breast cancers revealed nuclear localisation of ERα (Fig. 5(a)), PGR (Fig. 5(e)) and Ki-67 (Fig. 5(f)) while the expression of ERβ was cytoplasmic and perinuclear (Fig. 5(c)). ERα and ERβ in normal breast tissues were shown in Fig. 5(b) and (d), respectively. After omission of primary antibody in negative controls, specific staining was abolished. In the tumour sections studied, 67% of cases were positive for ERα, 67% positive for ERβ, 58% positive for PGR and 93.5% expressed Ki-67. In three cases of ERα-positive breast cancers we did not find positive PGR expression.

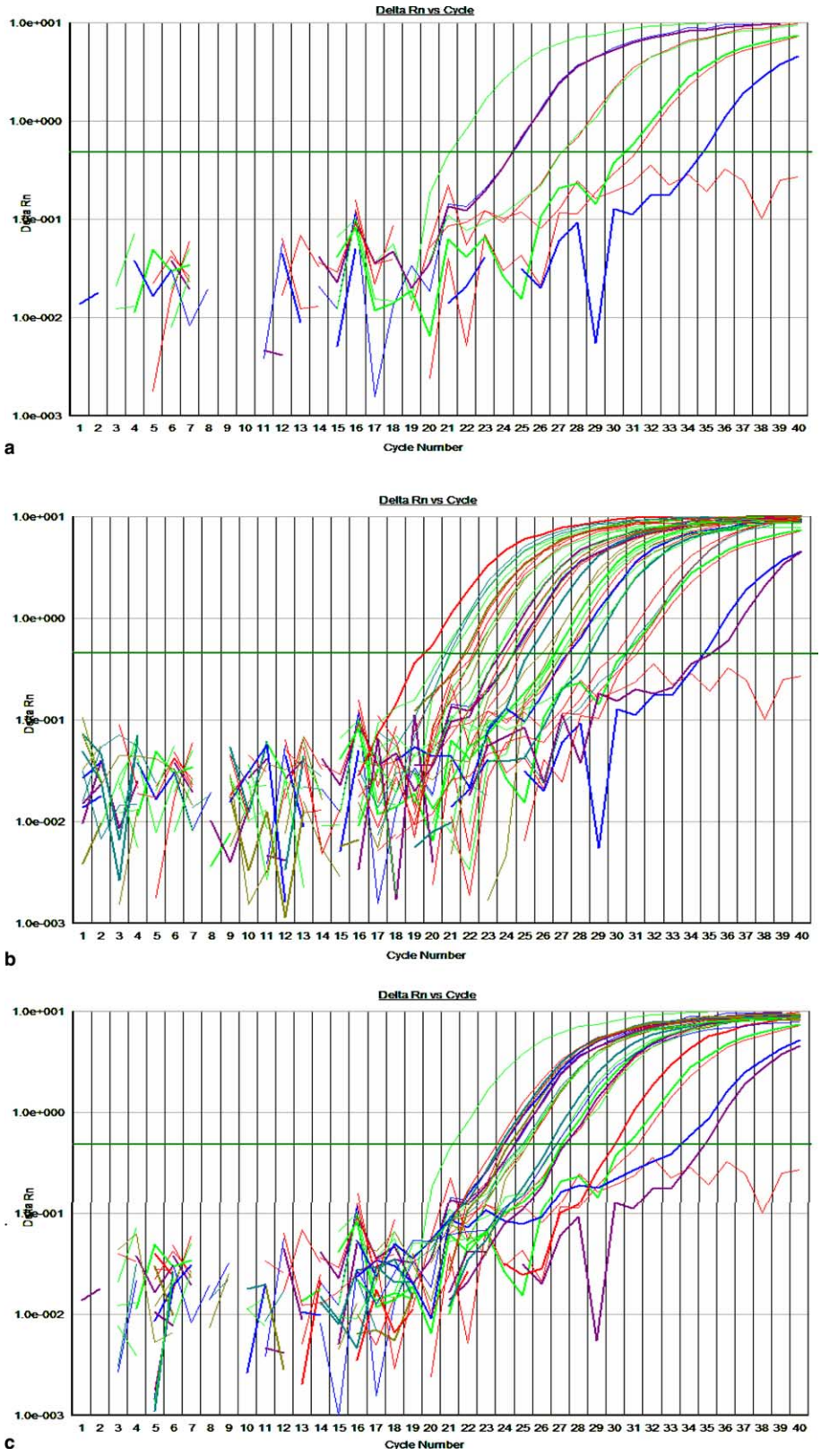

Fig. 1. Real-time amplification plot of ERα mRNA. (a) Standard curve of serial diluted polymerase chain reaction (PCR) product: 100–10–1–0.1–0.001 fg. (b) ERα transcripts were detected in all breast cancer tissues as well as in (c) matched normal tissues. ERα mRNAs levels were much lower in matched normal tissues (mean 11.76 fg/μg of RNA ± SD 13.9, $n = 20$) than in breast cancer tissues (mean 21.61 fg/μg of RNA ± SD 43.85, $n = 41$).

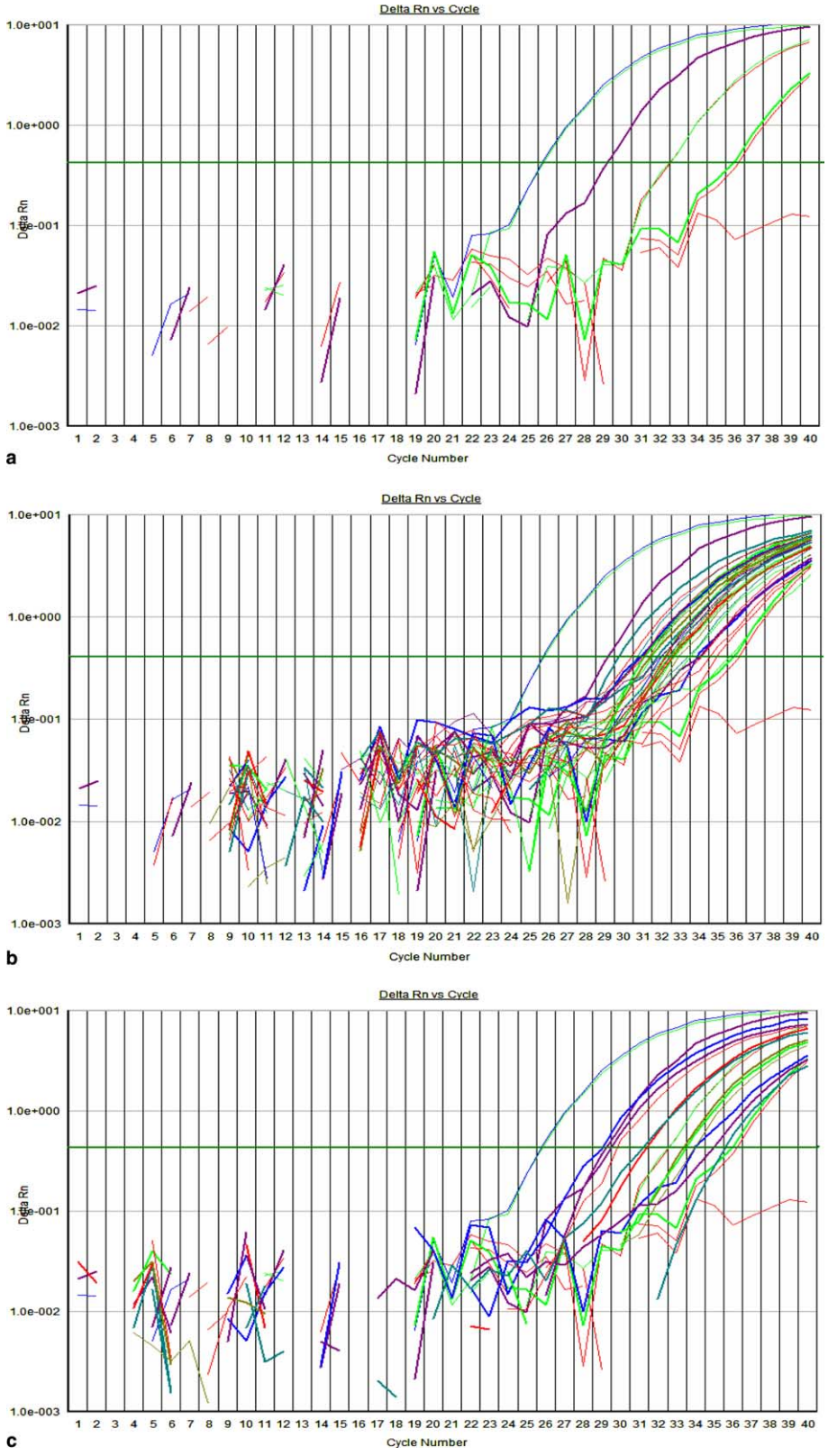

Fig. 2. Real-time amplification plot of ERβ mRNA. (a) Standard curve of serial diluted PCR product: 100–10–1–0.1–0.001 fg. (b) ERβ mRNAs were detected in all cancer tissues as well as in (c) normal matched tissues. There were more transcripts of ERβ in the matched tissues (mean 0.54 fg/μg of RNA ± SD 1.0, $n = 20$) than in the breast cancer tissues (mean 0.088 fg/μg of RNA ± SD 0.096, $n = 41$).

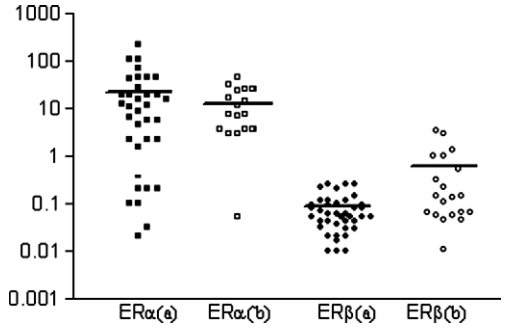

Fig. 3. Individual values on a log scale for both ERα as well as ERβ transcripts in tumours ($n = 41$) and normal matched tissues ($n = 20$) with marked mean values. Mean values for ERα mRNAs in breast tumours (ERα(a)) and in normal matched tissues (ERα(b)) were 21.61 fg/μg of RNA and 11.76 fg/μg of RNA, respectively; mean values for ERβ mRNAs in breast tumours (ERβ(a)) and in normal matched tissues (ERβ(b)) were 0.088 fg/μg of RNA and 0.54 fg/μg of RNA, respectively.

3.4. Correlation of ERα and ERβ mRNA with ERα and ERβ protein assessed by IHC

ERα expression at mRNA level correlated positively with ERα expression at protein level ($P < 0.0001$, $r = 0.848$). There was also statistically significant positive correlation, but with weak correlation coefficient between ERβ mRNA expression and ERβ protein expression ($P < 0.03$, $r = 0.390$). Thus, the tumours that failed to express the ERα as well as ERβ protein showed low levels of ERα and ERβ transcripts, respectively.

3.5. Correlation of ERα and ERβ expression with selected clinical and pathological features

Statistically significant positive correlation was found between ERα expression by IHC and the age of patients ($P < 0.006$, $r = 0.483$). We also observed a trend toward positive relationship between ERα expression assessed by RT-PCR and Western blot analysis and age ($P = 0.066$, $r = 0.334$; $P = 0.077$, $r = 0.327$, respectively). There was no association between ERα, ERβ expression and lymph node status, tumour size or tumour differentiation.

3.6. Correlation of ERα and ERβ expression with PGR status and Ki-67

ERα expression at mRNA as well as at protein level positively correlated with PGR expression ($P < 0.0009$, $r = 0.565$; $P < 0.009$, $r = 0.46$; $P < 0.0003$, $r = 0.616$ for ERα assessed by IHC, Western blot analysis and RT-PCR, respectively). There was no correlation between ERβ and PGR status. Negative correlation was found between ERα expression and Ki-67 status ($P < 0.02$, $r = -0.437$; $P < 0.0005$, $r = -0.604$; $P < 0.02$, $r = -0.451$ for ERα assessed by IHC, Western blot analysis and RT-PCR, respectively).

4. Discussion

The status of ER is routinely examined by IHC, but there are very few investigations in which ER expression has been performed at different molecular levels. By the use of RT-PCR we showed the presence of both ERα and ERβ mRNA in all analysed breast tumours and matched normal tissues. It was shown that the leader sequence 5′ of the ER transcript contains CpG islands, which could be hypermethylated in breast cancer, and therefore it might be responsible for lack of expression of the ER gene [14–16]. Our results and several recent data do not confirm this hypothesis. Alkarain *et al.* [4] examined 200 ER-negative and 50 ER-positive breast tumours assessed previously by IHC and showed that all of the 250 breast tumours analysed expressed mRNA transcripts of ERα, and the mean ERα mRNA values

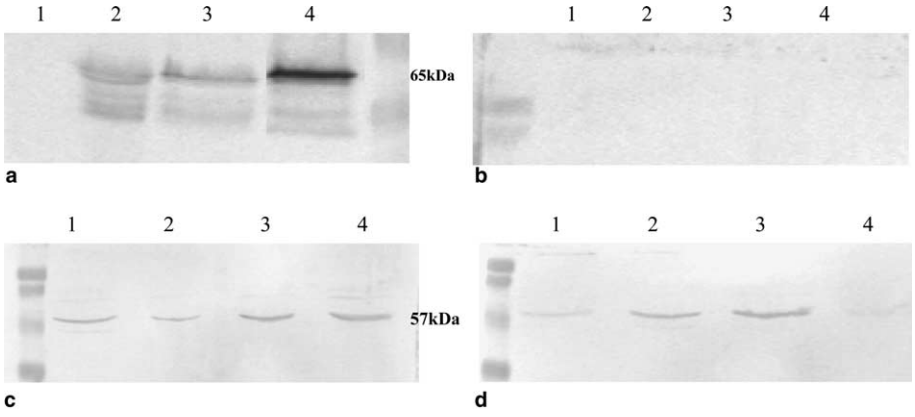

Fig. 4. Representative Western blot analysis of ERα and ERβ protein expression. (a) Full-length 65 kDa ERα protein was expressed in breast cancer tissues (lanes 2–4) and lack of expression of ERα in breast cancer tissue (lane 1). (b) Lack of expression of ERα in normal matched tissues. (c) 57 kDa ERβ protein was produced in breast cancer tissues as well as (d) in normal matched tissues.

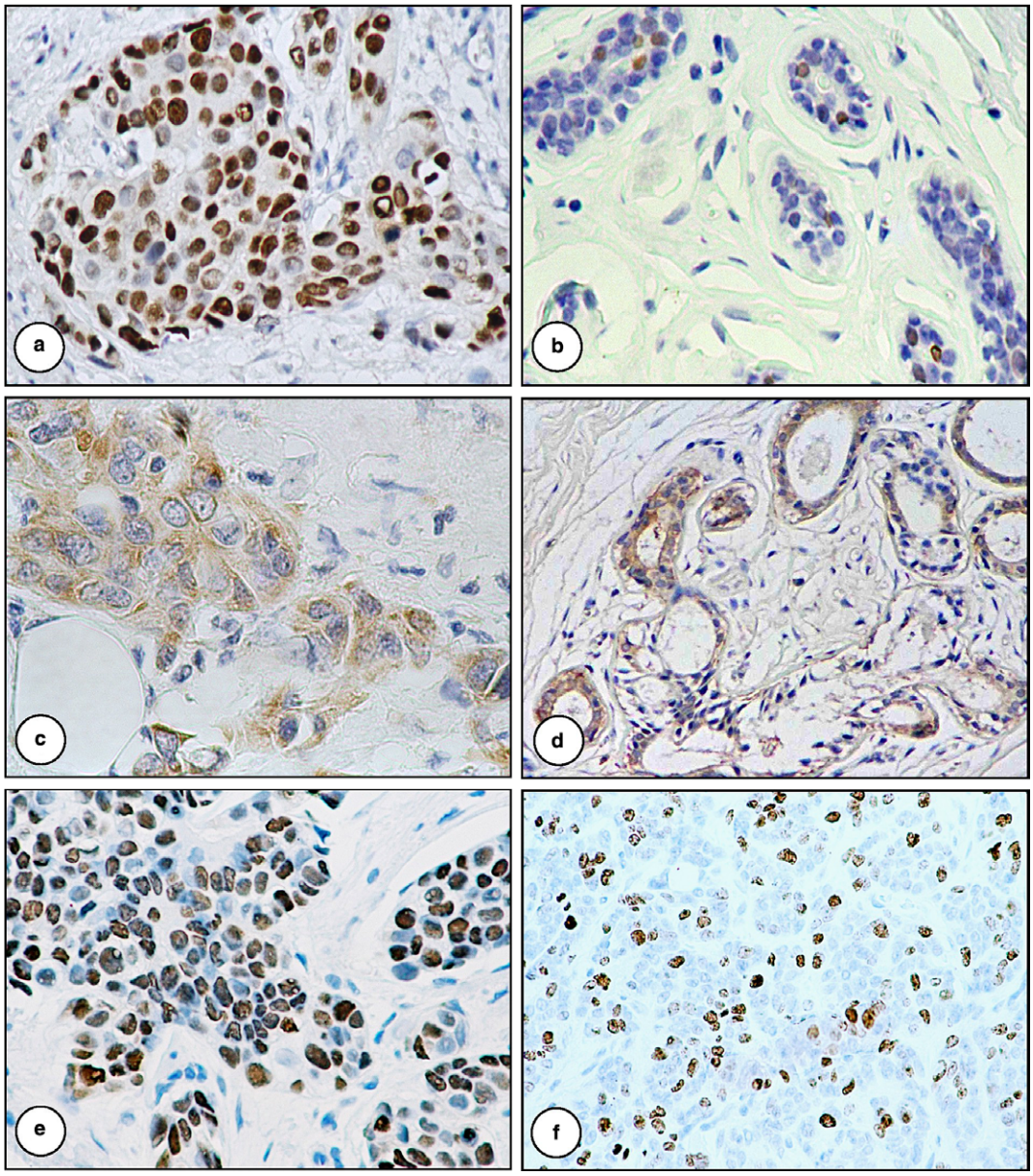

Fig. 5. (a) Strong nuclear immunostaining for ERα in majority of breast cancer cells and (b) in some cells of non-malignant tissue adjacent to tumour. (c) Positive cytoplasmic and focally perinuclear immunostaining for ERβ in breast cancer tissue and (d) in non-malignant tissue adjacent to tumour. Positive nuclear immunostainings for (e) PGR and (f) Ki-67 in breast cancer cells. Original magnifications: (a,b,d–f) 200×; (c) 400×.

did not differ significantly between two breast cancer groups. Our results seem to confirm the hypothesis that breast cancer could arise from ER-positive progenitor cells [17]. In the present study the analysed breast tumours showed a much larger amount of ERα mRNA than ERβ mRNA (means ± SD: 21.61 ± 43.85, 0.088 ± 0.096, respectively), which confirmed previous data [18,19]. Similarly to Bieche *et al.* [18], ERα transcript levels were up to 103-fold higher than ERβ. ERβ transcript levels in the breast tumours were significantly lower than in the matched normal tissues, but the amount of ERβ mRNA in the individual tumours was similar. Distribution of ERα transcripts in the tumour and matched tissues was opposite to ERβ. There were more transcripts of ERα in the tumours than in the matched tissues. ERα mRNA expression showed far larger diversity in the separate tumours. This observation might suggest that ERα is more dysregulated in breast cancer, and thereby ERβ is more tightly regulated in the tumour. Our data clearly showed, in agreement with previously results, that the majority of breast tumours overexpressed ERα mRNA [4,18,19]. The analysis of adjacent normal breast tissues also confirmed upregulation of the ERα gene in tumours, although we did not note statistical significance at ERα mRNA level between cancerous and normal tissues ($P = 0.09$). On the other

hand, ERα protein in breast cancer tissue was significantly higher compared with that in adjacent normal breast tissue, as assessed by Western blot analysis ($P < 0.005$). In most of the matched normal tissues (73%) ERα mRNA was expressed at a much lower level than in cancerous tissues, whereas ERβ mRNA showed an opposite trend. These data confirmed previous investigations [20] that ERβ mRNA was downregulated during carcinogenesis related to ERα. Although there have been some reports about expression of ERβ in breast cancer based on the analysis of mRNA using RT-PCR methods, the status of ERβ in breast tumour seems to be ambiguous. Numerous studies have reported co-expression of ERα and ERβ in breast tumour [4,7,18,21]. Dotzlaw *et al.* [22] and de Cremoux *et al.* [23] did not show any relationship between ERα mRNA and ERβ mRNA expression, whereas Knowlden *et al.* [19] and Bieche *et al.* [18] reported negative correlation between ERα and ERβ mRNA levels. However, in our study we observed a positive correlation between transcript levels of both ERs ($P < 0.002$, $r = 0.532$).

In previous reports, ERα mRNA was detected in 92% of breast cancers and ERβ mRNA in 85%, however, after protein translation the percentages of the cases of ERα(+) and ERβ(+) decreased [9]. Using Western blot analysis we showed that 70% of breast cancer expressed full-length 65 kDa ERα protein and 67% of tumours analysed were also ERα-positive in IHC assay. Only in a few adjacent tissues did we show ERα 65 kDa protein. It is accepted that 5–15% of normal epithelial cells in the mammary gland express ERα [1]. In the present study in the normal mammary gland adjacent to the breast cancer, we also observed approximately 15% of ERα-positive cells assessed by IHC (data not published).

It was shown that expression of ERβ protein decreased in neoplastic breast cells [5,24]. Our data revealed that 97% of analysed tumours produced full-length 57 kDa ERβ protein and the same percentage of 57 kDa isoform was observed in matched normal tissues. In IHC studies, 67% of assessed tumours showed cytoplasmic and perinuclear immunoreaction to this receptor. The analysis of ERβ by Western blot revealed expression of this receptor in tumours containing breast cancer cells as well as in the surrounding stromal tissue (including fibroblasts, lymphocytes and endothelial cells). In our opinion it could be one of the factors responsible for the observed differences in ERβ expression in tumours assessed by Western blot and IHC. The cytoplasmic and perinuclear localisation of ERβ may suggest that ERβ may be inactive as a classical transcriptional factor and that this receptor can probably get out rapidly from the nucleus and shuttle repeatedly between nucleus and cytoplasm [25]. In a recent study Bjornstrom and Sjoberg [26] suggested that signal transduction pathways through ERs localised in the cytoplasm could be an additional mechanism by which ERs are able to modulate the transcriptional activity of AP-1 via a non-genomic pathway. On the other hand, Chen *et al.* [27] demonstrated the presence of ERα and ERβ within the mitochondria of MCF-7 breast cancer cells and predominant receptor was ERβ. They also observed that the mitochondrial oestrogen effects were associated with increased respiratory chain activity and decreased apoptosis. It is thought that ER localised in the cytoplasm can activate E2-independent intracellular signalling cascades including MAP kinases and PI-3-K signalling pathways [28]. The evidence for presence of ERβ in most of the breast tumours analysed forced us to re-evaluate what 'oestrogen-independence' means. It seems that in breast cancer cells, E2 plays its essential role through the modification of the tumour biology and therefore the classical term of 'oestrogen-dependence' might be related to its proliferation effect. The function of ERβ independent of ERα is unknown, although it has been speculated that when both ERs are co-expressed, ERβ could negatively regulate ERα through ERα/ERβ heterodimers [29]. In the case of ERα/ERβ heterodimerisation, it has been suggested that mechanism of the oestrogen signal transduction pathway leading to proliferation of breast cancer cells can be altered.

It was established that ERα is a key transcription factor activating PGR gene containing ERE in its promoter. Since PGR is E2 regulated, it is a marker of functional ER, and lack of PGR in ERα(+) breast cancer could result in alteration of classical oestrogen-mediated pathway in such tumours [30,31]. We showed that PGR protein correlated with ERα mRNA as well as ERα protein (but not with ERβ). Using IHC we observed that 58% of breast tumours analysed co-expressed both ERα and PGR, but 3 cases of ERα(+) breast tumour did not express PGR.

It was established that proliferating cells express proliferation markers such as Ki-67 and proliferating cell nuclear antigen (PCNA) [32–34]. The relationships between ERα, ERβ and markers of proliferation presented in many studies are inconsistent. In our study we showed negative correlation between expression of ERα (at mRNA and protein levels) and Ki-67. This association between ERα and Ki-67 was stronger in a group of patients where ERα protein was detected. We did not observe any association between ERβ and Ki-67. In the recent study followed by Choi and Pinto, Ki-67 was almost exclusively expressed in ERβ positive cells but no statistical significant association was found between ERβ and S-phase [35]. These results could suggest that beside implication of ERα in cell proliferation, ERα itself might act as a proliferation inhibitory factor. It was observed that normal epithelial mammary cells that express ERα do not proliferate and do not produce proliferation markers. Cheng *et al.* [36] observed in mice that E2 does indeed initiate proliferation by interacting

with ERα or ERβ in the epithelial cells in the mammary gland. However, very shortly after the cell enters the cell cycle, ERα is down-regulated, and this is the reason why ERα is never colocalised in nuclei with proliferation markers. This blocking mechanism of proliferation is probably not so efficient in breast cancer cells, but nevertheless, the majority of neoplastic cells containing ERα do not enter into cell cycle. It is noteworthy that such tumours require E2 to their development and selective oestrogen receptor modulators (SERMs), oestrogen receptor down regulators (ERDR) or aromatase inhibitory treatment can block E2 action (inhibit development). It still remains an open question whether malignant breast cells that express ERα proliferate? Two hypotheses explaining why ERα-positive breast cancer cells proliferate have been proposed. One hypothesis holds that ERα might undergo proteolytic degradation via the ubiquitin pathway and then could not act as a cell cycle inhibitory factor. Another hypothesis is that ERα-dependent signal transduction pathway upregulates other autocrine factors in ERα-positive malignant cells that induce the proliferation of the surrounding of mammary epithelial cells. This first hypothesis seems likely in light of the evidence in our study that some of ERα-dependent breast tumours express ERα mRNA whereas ERα protein is not produced. These observations raise the possibility that the ubiquitination and proteolysis may cause ERα removal from the proliferating cells. It seems that the loss of ERα is probably due to progressive dedifferentiation process in cancer cells and then enhanced ubiquitination and proteolysis. Further development of such tumours may be E2-independent.

In the present study, we also analysed the relationships between studied markers and selected anatomoclinical features of breast cancer. We observed positive correlation between ERα expression at all the studied levels and the age of patients, and Ki-67 expression negatively correlated with age. These observations suggest that proliferation of breast cancer cells decreases with age. The expression of both ERs in the present study was not associated with lymph node status, tumour size or tumour grade. Jensen *et al*. [37] analysing 34 breast cancer samples also showed no relationship between the expression of ERα, ERβ proteins and tumour differentiation. Jarvinen *et al*. [38] studied 92 breast cancer tissues and observed the correlation between ERα expression, histological grade and tumour size and did not find any association between ERα and nodal status of the tumour. However, ERβ status was more common in axillary node-negative than in node-positive tumours, but no association was found with tumour size.

Our data suggest that ERα and ERβ are differently regulated in breast cancer and ERα seems to be much more deregulated than ERβ. The dysregulation of ERα expression at the gene and protein levels and oestrogen-dependent signalling pathways in breast cancer could be exploited to discover target drugs that would be more tailored to an individual tumour.

Conflict of interest statement

None declared.

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