Analysis of estrogen receptor isoforms and variants in breast cancer cell lines

MAIE AL-BADER[1], CHRISTOPHER FORD[2], BUSHRA AL-AYADHY[3] and ISSAM FRANCIS[3]

Departments of [1]Physiology, [2]Surgery, and [3]Pathology, Faculty of Medicine, Kuwait University, Safat 13110, Kuwait

Received November 22, 2010; Accepted February 14, 2011

DOI: 10.3892/etm.2011.226

Abstract. In the present study, the expression of estrogen receptor (ER)α and ERβ isoforms in ER-positive (MCF7, T-47D and ZR-75-1) and ER-negative (MDA-MB-231, SK-BR-3, MDA-MB-453 and HCC1954) breast cancer cell lines was investigated. ERα mRNA was expressed in ER-positive and some ER-negative cell lines. ERα Δ3, Δ5 and Δ7 spliced variants were present in MCF7 and T-47D cells; ERα Δ5 and Δ7 spliced variants were detected in ZR-75-1 cells. MDA-MB-231 and HCC1954 cells expressed ERα Δ5 and Δ7 spliced variants. The ERβ1 variant was expressed in all of the cell lines and the ERβ2 variant in all of the ER-positive and some ER-negative cell lines (MDA-MB-231, MDA-MB-453 and SK-BR-3). MCF7, ZR-75-1, MDA-MB-453, HCC1954 and T-47D cells expressed ERβ5. All cell lines expressed an ERα 66-kDa protein band, and some expressed the truncated 42-kDa variant. ERβ1 was detected in all of the cell lines in addition to a 38-44 kDa variant. The results indicate that breast cancer cell lines widely used in research and reported as being ER-negative express ERα and/or ERβ mRNA and protein.

Introduction

Estrogen receptor (ER)α was first cloned in rats by Koike *et al* (1); almost 10 years later, a gene encoding a second type of ER, ERβ, was cloned in rats (2), humans (3) and mice (4), prompting the re-evaluation of estrogen signaling systems. ERα and ERβ are homologous, particularly in the DNA binding domain (95%) and in the C-terminal ligand binding domain (55%) (2-4). The genes for both ERα and ERβ are encoded by eight exons, located on different chromosomes, with ERα found on the long arm of chromosome 6q25.1 and ERβ on chromosome 14q22-24 (5). This confirms that each receptor is the product of independent genes. ERs have six functional domains: domain A/B, containing the N-terminal activation function-1 (AF-1); domain C, the DNA binding domain; domains D/E, bearing both the activation function-2 (AF-2) and the ligand binding domains; and finally, domain F, the C-terminal domain (6,7).

The actions of estrogens are mediated by binding to ERs (ERα and/or ERβ). These receptors, which are co-expressed in a number of tissues, form functional homodimers or heterodimers. When bound to estrogens as homodimers, the transcription of target genes is activated (8,9), while as heterodimers, ERβ exhibits an inhibitory action on ERα-mediated gene expression and, in many instances, opposes the actions of ERα (7,9). Estrogen binding to ERβ also inhibits gene transcription via AP1 sites, while binding to ERα leads to their activation (8,10,11). Thus, as several ER-negative breast cancer cell lines respond to estrogens and anti-estrogens, this suggests that these compounds may act through an alternative mechanism, not the classical ERα pathway (12), or that ER-negative cell lines are not truly ER-negative.

Much of our knowledge on breast carcinomas is based on *in vitro* studies performed with various breast cancer cell lines. These cell lines provide a source of homogenous self replicating material, free of contaminating stromal cells, that can be grown in culture in standard media. Cell lines that have retained the luminal epithelial phenotype of breast cells include MCF7, T-47D and ZR-75-1; those with a weak luminal epithelial-like phenotype include MDA-MB-453 and SK-BR-3; finally, those that do not express epitheloid markers, but exhibit a high level of vimentin (a marker found in mesenchymal cells), include MDA-MB-231 (13). Although rare, there have been reports of ER-positive cell lines converting to an ER-negative phenotype (13). However, certain breast cancer cell lines reported as being negative for ERα have since been shown to express ERβ at least at the mRNA level. In addition to the aforementioned ER isoforms, several ER variants have been identified for both receptors. A summary of the reported ERα and ERβ isoforms and their variants to date is shown in Tables I and II, respectively.

There have been some discrepancies between the results of researchers studying the mitogenic effects of estradiol and various estrogen agonists and/or antagonists using a number of breast cancer cell lines (both ER-positive and ER-negative). Although this can be attributed to many factors, in this study we aimed to determine the true ER status of breast cancer cells by studying ERβ isoform expression in breast cancer cell lines that have been reported, in the literature, to be ER-positive (MCF7, T-47D and ZR-75-1) or ER-negative (MDA-MB-231, SK-BR-3, MDA-MB-453 and HCC1954). Additionally, we

Correspondence to: Dr Maie Al-Bader, Department of Physiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait
E-mail: albader@hsc.edu.kw

Key words: breast cancer cell lines, estrogen receptor α, estrogen receptor β

Table I. Reported ERα variants in breast tissue and breast cancer cell lines.

Origin	ERα mRNA status	ERβ protein status	Refs.
T-47D$^+$ cell line	Δ2, 3 or 7		14
MCF7$^+$ cell line	Δ5		15
Breast cancer ER$^-$/PgR$^+$; ER$^+$/PgR$^-$	Δ7		16
Breast cancer ER$^-$/PgR$^+$; ER$^+$/PgR$^-$ (review)	Δ3, 5 or 7		17
BT-20 (negative cell lines)	Δ5	42 kDa	18
MCF7$^+$ cell line	Δ4 and 7		19
MCF7$^+$ cell line	Δ4 and 7		20
Breast cancer ER$^+$/PgR$^+$; ER$^+$/PgR$^-$; T-47D; ZR-75-1 (positive cell lines)	Δ5		21
Breast cancer	Δ5		22
Breast cancer	Δ7, Δ4, Δ4+7 and Δ3+4		23
Breast cancer	Δ4		24
MCF7$^+$ cell line	Duplication of exons 6 and 7	80 kDa	25
Human breast cancer	Δ2, Δ3, Δ4, Δ5 and Δ7		26
Breast cancer	Δ5	40 kDa	27
Breast cancer	ERα clone4		28
Breast cancer (relapse)	Δ5	40 kDa	29
Breast cancer	Δ4, Δ3+4, Δ5, Δ7, Δ4-7, clone 4	Δ4=54, Δ3+4=49, Δ5=40, Δ7=51, Δ4-7=39 and clone 4=24 kDa	30
Human breast epithelial cell line HMT-3522	Δ5	42 kDa	31
MCF7, T-47D and ZR-75-1 (positive cell lines)	Δ7 and 7P	Δ7=52 and 7P=60 kDa	32
MCF7$^+$ cell line	Δ1	46 kDa	33
MCF-7, T-47D, ZR-75-1, LCC1, LCC2 and LCC9 (positive cell lines)	Δ2, Δ3, Δ2+3, Δ4, Δ5, Δ6 and Δ7		34
MDA-MB-435, MDA-MB-235 and LCC6 (negative cell lines)	Δ2 and Δ4 only in MDA-MB-453		34
MCF7$^+$ cell line		130, 110, 92 and 67 kDa	35
MCF7$^+$ cell line	Δ4		36
MCF7$^+$ cell line	Δ3	61 kDa	37
Breast cancer		67+67≈134 kDa	38
MCF7$^+$ cell line		66, 46 kDa	39
Review	Δ2, Δ3, Δ4, Δ5, Δ6 and Δ7	Δ2=17, Δ3=62.3, Δ4=54.1, Δ5=41.6, Δ6=53 and Δ7=52.2 kDa	40

aimed to determine the expression of ERα and ERβ variants in these cell lines using reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting. Our results revealed that ER-positive and ER-negative cell lines used extensively in breast cancer research have variable degrees of expression of ERα and/or ERβ isoforms and variants at the mRNA and/or protein level.

Materials and methods

Materials. All media and supplements for cell culture were obtained from Invitrogen (Paisley, UK). The ERβ polyclonal antibody used corresponds to amino acids 1-150 (H-150; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Mouse monoclonal anti-ERα was raised against the steroid binding domain of ERα [amino acid residues 582-595 (referred to in this article as ERα-S)] (SRA-1010, clone C-542; Stressgen, Ann Arbor, MI, USA). For the detection of actin, a mouse monoclonal IgG1 anti-human actin antibody was used (Santa Cruz Biotechnology, Inc.). PVDF membranes were obtained from Amersham Pharmacia Biotech Ltd. (RPN303F; Buckinghamshire, UK). General laboratory chemicals were purchased from Merck (Dagenham, Essex, UK) and all fine chemicals were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). All buffers, enzymes and reagents used in the RT-PCR experiments were purchased from Invitrogen, and reagents for real-time PCR (ReT-PCR) were purchased from Applied Biosystems (Foster City, CA, USA).

Cell lines. All of the cell lines used in this study were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Seven breast cancer cell lines were used,

Table II. Reported ERβ variants in breast tissue and breast cancer cell lines.

Origin	ERβ mRNA status	ERβ protein status	Refs.
MCF7 and MDA-MB-231	ERβ and ERβΔ5 in MCF7, ERβ Δ5 in MDA-MB-231		41
Breast tissue	ERβ1, 2, 4, 5	ERβ1=54.2 kDa; ERβ2=55.5 kDa	42
Breast cancer		58-60 kDa + low mol wt (4-5 kDa); predicted 62 kDa from sequence data	43
Normal human mammary gland	ERβΔ5		44
Breast cancer		55 and 50 kDa	45
Breast – normal and cancer and cell lines	Δ2; Δ2 and Δ5-6; Δ4; Δ5; Δ5 and Δ2; Δ6; Δ6 and Δ2, Δ6 and Δ2-3; and exons Δ5-6		46
Breast cancer	ERβ1, 2, 4, 5		47
Breast cancer	ERβcx		48
Breast cancer		59, 53 and 32-45 kDa	49
Breast – normal and cancer		62, 58, 56 and 54kDa	50
Breast – normal, cancer and cell lines	ERβ1, 2, 5		51
Breast cancer	ERβcx		52
Breast cancer		59+59=118 kDa	38
Breast cancer	ERβ1, 2, 4, 5	ERβ1=54.2 kDa; ERβ2=55.5 kDa	53

Table III. Primers used for RT-PCR, expected PCR product sizes, annealing temperatures and cycle numbers.

Gene	Primers	Expected product size	Refs.	Annealing temperature (˚C)	Cycle no.
β-actin	Forward: GTCCTGTGGCATCCACGAAACT Reverse: TACTTGCGCTCAGGAGGAGCAA	201 bp	(26)	53	24
ERαΔ3	Forward: ATGGAATCTGCCAAGAAGACT Reverse: GCGCTTGTGTTTCAACATTCT	281 bp, wt; 165 bp, Δ3	(26)	45	35
ERαΔ5	Forward: CTCATGATCAAA CGCTCTAAG Reverse: ATAGATTTGAGGCACACAAAC	466 bp, wt; 328 bp, Δ5	(26)	42	32
ERαΔ6, 7, 6+7	Forward: GCTCCTAACTTGCTCTTGG Reverse: ACGGCTAGTGGGCGCATGTA	452 bp, wt; 318 bp, Δ6; 268 bp, Δ7; 134 bp, Δ6+7	(26)	53	32
ERβ-I	Forward: CGATGCTTTGGTTTGGGTGAT Reverse: GCCCTCTTTGCTTTTACTGTC	268 bp, ERβ1	(42)	51	35
ERβ-II	Forward: CGATGCTTTGGTTTGGGTGAT Reverse: CTTTAGGCCACCGAGTTGATT	214 bp, ERβ2; 295 bp ERβ5	(42)	51	35

three of which are known to be ER-positive in the literature (MCF7, T-47D and ZR-75-1) and four of which are reported to be ER-negative (MDA-MB-231, MDA-MB-453, SK-BR-3 and HCC1954). Cell lines were grown as monolayers in the following media: RPMI-1640 (T-47D, ZR-75-1 and HCC1954), Eagle's MEM (MDA-MB-231 and MCF7), McCoy's 5A (SK-BR-3) and Leibovitz's (MDA-MB-453) containing 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Other supplements were added to the medium for some of the cell lines, as per the ATCC data sheet supplied with the cell lines. When required for assays, 5 ml of a 1:10 dilution of trypsin-EDTA in phosphate buffered saline (PBS) was added to PBS-washed monolayers, followed by incubation at 37˚C for 5-10 min. Cells were centrifuged for 7 min at 130 x g, reconstituted in the medium, and counted.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from a minimum number of $5x10^6$ cells using the method of Chomczynski and Sacchi (54). Following isolation, the RNA samples were DNase-treated, then reverse transcribed using random hexamer primers (55). PCR reactions were carried out in a programmable thermal

Table IV. RT-PCR results for ERα and ERβ isoforms (and/or variants) and actin gene expression for various ER-positive and ER-negative cell lines.

Cells	β-actin	ERα Δ3 primer		ERα Δ5 primer		ERα Δ6, 7, 6+7 primer sets				ERβ1, 2, 5 primer		
		wt	Δ3	wt	Δ5	wt	Δ6	Δ7	Δ6+7	β1	β2	β5
	201 bp	281 bp	165 bp	466 bp	328 bp	452 bp	318 bp	268 bp	134 bp	268 bp	214 bp	295 bp
MCF7	++	+	+/F	+	+	+	-	+	-	+	+	++
MCF7	++	+	-	+	VF	+	-	F	-	+/F	++	-
MCF7	+	F	-	-	-	-	-	-	-	+	++	-/+?
MCF7	++	+	+/VF	++	+	+	-	+/F	-	+	+	++
MCF7	+	++	+/VF	+	F	+	-	+/F	-	+	+	+
T-47D	++	++	VF	++	F	++	-	+	-	+	+	-
T-47D	++	+++	VF	++	+	+++	-	+	-	+	+	F
T-47D	++	+	F	++	+	+++	-	+	-	+	+	F
T-47D	+	++	VF	++	+	+++	-	+	-	+	+	VF
ZR-75-1	+	+	-	-	-	-	-	-	-	-	-	-
ZR-75-1	++	+	-	++	+	++	-	+	-	-?	+	++
ZR-75-1	+	+	-	+	-	+	-	+	-	+/F	+/F	++
ZR-75-1	+	+	-	++	+/VF?	+	-	+	-	+/F	+/F	+
MDA-MB-231	+	+	-	+	-?	F	F	-	-	+	++	F
MDA-MB-231	++	+	-	+	VF	+	-	VF	-	+	++	-
MDA-MB-231	++	+	-	+	VF	+	-	VF	-	+	++	-
MDA-MB-231	++	-	-	-	-	VF	-	-	-	+	++	-
MDA-MB-453	++	-	-	VF	-	-	-	-	-	++	+/F	++
MDA-MB-453	+	-	-	-	-	-	-	-	-	-	-	+
MDA-MB-453	++	-	-	-	-	-	-	-	-	++	+	++
MDA-MB-453	++	-	-	-	-	-	-	-	-	++	+	++
SK-BR-3	+	-	-	-	-	-	-	-	-	-	-	-
SK-BR-3	+	-	-	-	-	F	-	-	-	+	+	-
SK-BR-3	++	-	-	-	-	-	-	-	-	-	-	-
SK-BR-3	++	-	-	VVVF	VVVF	-	-	-	-	+	VVF	VVF
HCC1954	++	VVF	-	-	-	-	-	-	-	-	-	+
HCC1954	++	+/F	-	+	+/F	+	-	+	-	++	-	+
HCC1954	+	+/F	-	+	+/F	+	-	+	-	++	-	+

Each experiment was repeated at least three times for each cell line, and the results are expressed as follows: +, ++ and +++, intensity of band evident; -, no band noted. F, faint; VF, very faint; VVF, very, very faint; VVVF, very, very, very faint.

cycler (Perkin Elmer, model 9700) in a reaction mixture consisting of 1X PCR buffer (20 mM Tris/50 mM KCl), 3 mM $MgCl_2$, 0.5 mM dNTPs and 0.3 μM each of forward and reverse primers (primer sets are shown in Table III), 0.5 μl template and 1.25 units recombinant *Taq* DNA polymerase in a final volume of 25 μl. The PCR reactions were then cycled as follows: 5 min at 94˚C (1 cycle); 30 sec at 94˚C (denaturation step), 30 sec (annealing step) and 1 min (extension step) at 72˚C for the required number of cycles (Table III). Tubes were then incubated for a further 7 min at 72˚C (1 cycle).

Protein analysis using Western blotting and immunodetection. Trypsinized cells (~2.65x10^6) were centrifuged at 1,000 x g at 4˚C for 10 min to remove the medium and then washed twice with PBS buffer. The pellet was resuspended in homogenization buffer (20-50 μl) and then vortexed, sonicated for 30 min at 4˚C, and frozen for 15 min. This step was repeated twice. Finally, the samples were centrifuged at 4˚C for 30 min at 20,000 x g, the supernatant was collected, and the total protein concentration was measured (20 μg of protein was loaded per lane). Proteins were separated using SDS-PAGE. A monoclonal antibody for ERα raised against the steroid binding domain (ERα-S) and a polyclonal antibody against ERβ were used. Western blot analysis and immunodetection of total ER proteins together with analysis of protein sizes were performed as previously described (55). In preliminary experiments, the primary antibody was omitted and filters were incubated with the secondary antibody only. No bands were detected with this antibody. Once the membranes were probed with the anti-ER antibodies, they were stripped and re-probed with actin, which was present equally in all the samples (data not shown). The results obtained from this experiment were compiled for each

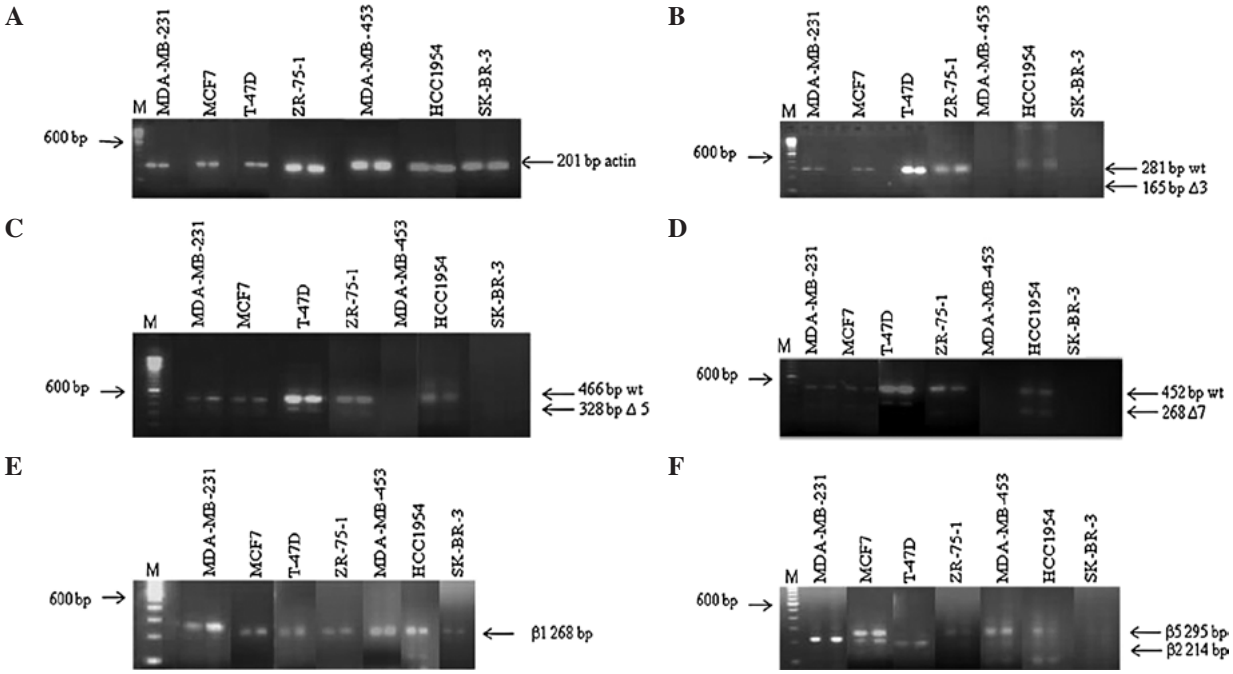

Figure 1. RT-PCR results. Representative ethidium bromide-stained gels for all positive and negative cell lines showing ERα and ERβ isoform and variant expression. The migration of the 100-bp marker (M) is shown at the left-hand side, and the expected size of the product is indicated at the right-hand side of the gel. Both RT and water samples were negative (data not shown). (A) All the cell lines expressed a 201-bp product for actin. Expression of the (B) ERα wild-type (281 bp) and Δ3 (165 bp) variant; (C) ERα wild-type (466 bp) and Δ5 (328 bp) variant; (D) ERα wild-type (452 bp) and Δ7 (268 bp) variant; (E) ERβ1 (268 bp) variant; (F) ERβ2 (214 bp) and ERβ5 (295 bp) variants. For more details, refer to Table IV.

Table V. Percentage of positive expression of ERα and ERβ isoform/variant protein determined by Western blotting.

Cell line	ERαS		ERβ	
	66[a]	40-44[a]	52-54[a]	38-44[a]
MCF7	90%	20%	30%	70%
T-47D	71%	29%	43%	43%
ZR-75-1	60%	40%	40%	40%
MDA-MB-231	50%	10%	40%	50%
MDA-MB-453	50%	25%	50%	50%
SK-BR-3	60%	60%	40%	40%
HCC1954	75%	25%	50%	25%

[a]Approximate size in kDa.

cell line and are shown as the percentage of expression for each band per group of study.

Results

ER mRNA expression using RT-PCR. Representative images for all cell lines using the various primers are shown in Fig. 1. Overall results for all cell lines studied are shown in Table IV. The housekeeping gene β-actin was used as a control and expression was verified in all the cell lines studied. Wild-type (wt) ERα was expressed in all the ER-positive cell lines (MCF7, T-47D and ZR-75-1), as well as in the ER-negative cell lines (MDA-MB-231 and HCC1954). The ERα Δ3, Δ5 and Δ7 spliced variants were present in both the MCF7 and T-47D ER-positive cell lines. Regarding the ZR-75-1 cell line, only the ERα Δ5 and Δ7 spliced variants were detected. Concerning the ER-negative cell lines, both MDA-MB-231 and HCC1954 showed mainly weak expression of the ERα Δ5 and Δ7 spliced variants. The ERα Δ6 and Δ6+7 variants were not expressed in any of the cell lines. The ERβ1 variant was expressed in the ER-positive and ER-negative cell lines; however, ZR-75-1 and SK-BR-3 cells exhibited weak expression. The ERβ2 variant was expressed in all of the ER-positive and two of the ER-negative cell lines (MDA-MB-231 and MDA-MB-453), with very weak expression noted in SK-BR-3. MCF7, ZR-75-1, MDA-MB-453 and HCC1954 clearly expressed ERβ5, with weak expression noted only in the T-47D cell line.

Western blotting and immunodetection. Representative images for ERα and ERβ protein expression are shown in Fig. 2. The percentage of positivity for ERα and ERβ in all the samples studied is shown in Table V. All cell lines (ER-positive and ER-negative) expressed a ~66 kDa protein corresponding to ERα (reported size for ERα). Smaller molecular weight bands (<66 kDa) were noted in some of the ER-positive and ER-negative cell lines. These may be spliced variants of ERα, as spliced variants have been reported for this gene (27,29,56). All of the cell lines were found to express a 52-54 kDa protein (the reported size for ERβ1). Certain cell lines also expressed a smaller molecular weight band that may be an ERβ spliced variant (46,57-59).

Discussion

It has been reported that breast cancer cell lines from different laboratories may differ in their sensitivity to estradiol (13).

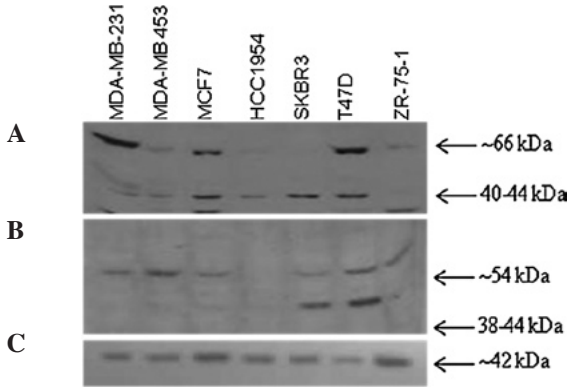

Figure 2. Results of the Western blotting. (A) Representative blot for ERα, (B) ERβ and (C) actin. The calculated size of the band is indicated at the right-hand side of the gel.

This discrepancy may be attributed to lack of proper investigation of the ER status. We demonstrated that all of the cell lines used in this study express cytoplasmic and/or membrane ER when analyzed by flow cytometry (unpublished data). In this study, we demonstrated that cell lines that have been known to be positive or negative for classical ER (ERα) show various degrees of positivity for the ERβ isoform and for the ERα and ERβ variants at both the protein and mRNA levels. This is important, as the presence of the ERβ isoforms together with the ERα isoforms in a tissue may have functional implications for binding and response to a particular ligand.

As several variants have been shown to exist for ERα (Table I) differences in estrogen responsiveness of cell lines may be due to varying ratios of wild-type to variant ER mRNA. The Δ1 variant lacking exon 1 (N terminal AF1 region) results in a 46-kDa protein that heterodimerizes with the wild-type ERα, suppressing its activity (33), and the Δ3 (60), Δ4 (61) and Δ7 variants (16) also inhibit gene transcription by interfering with the ability of the wild-type ER to initiate transcription (15,16,18). Conversely, the Δ5 variant acts in a dominant-positive manner to activate the gene transcription of an ER-regulated gene (15,16,18). Certain cell lines, misclassified as ER-negative, exhibit the Δ5 variant, which activates gene transcription in the absence of the hormone and inhibits wild-type activity by competing for steroid receptor co-activator-1e (SRC-1e) (62). Thus, the presence of this variant may explain hormone independence and tamoxifen-resistance, and may contribute to the hormone-independent proliferation of ER-negative cell lines (16).

Although the presence of variants in cell lines has been reported by several investigators, a complete analysis of variant expression has not been attempted. Two of the ER-positive cell lines, MCF7 and ZR-75-1, have been shown to exhibit the ERα wild-type Δ5 and Δ7 variants (63). However, the present results also show that in addition to these variants, MCF7 cells express Δ3, in agreement with previous reports (34,37). Strom *et al* (64) reported that the predominant ER in T-47D cell lines is ERα (9:1 with ERβ) and that estradiol stimulates growth of T-47D cells, while anti-estrogens do not induce proliferation. As we demonstrated, T-47D expresses ERα Δ5, Δ7 and Δ3 (albeit little of this variant) and ERβ (explained below), and these variants may act to inhibit or enhance wild-type ERα action. As Δ3 and Δ7 act in a dominant-negative fashion to suppress ERα wild-type activity, and Δ5 acts to enhance gene transcription, investigation of the relative expression of these variants in comparison to the wild-type gene is of critical importance. The presence of these variants may be the cause of reported discrepancies in results between different laboratories.

Reports that the MDA-MB-453 cell line is negative for ERα wild-type mRNA are in agreement with our results; however, MDA-MB-231 was found to exhibit positivity for wild-type ERα. This was confirmed by using three different sets of primers that detect the wild-type ERα and different variants. Although ERα was not expressed at the transcript level, it was detected in both cell lines at the protein level both by Western blotting, as indicated above, and by using flow cytometry (unpublished data). This may be due to a high turnover of mRNA and protein accumulation. HCC1954 was also positive for wild-type ERα and for the Δ5 and Δ7 variants. ERα Δ7 is able to form heterodimers with ERα and ERβ in a ligand-independent manner resulting in a dominant-negative effect on both ER isoforms (65,66), and the presence of ERα Δ5, which has AF-1 activity and DNA binding ability, leads to a constitutively active receptor (65). This may explain resistance to tamoxifen and hormone-independent proliferation in ER-negative cell lines (67).

Five spliced isoforms of the human ERβ, designated ERβ1-5, were cloned by Moore *et al* (10). The amino acid sequences diverge at amino acid 469 within the ligand binding domain and extend to the C-terminus (42). Longer forms of the ERβ – 485, 530 and 548 aa – have also been reported (5,10,11,68,69). In addition to the expression of wild-type ERβ of various lengths due to the use of alternative transcription start sites, a number of ERβ variants have been identified (Table II) arising from alternative splicing (10,41,42,70). As with ERα, these spliced variants, when expressed with the wild-type ERβ, alter the response of the wild-type to estradiol; thus, the relative expression levels of the wild-type vs. variant ERβ is of significance in predicting cellular responsiveness to various estrogen and anti-estrogen therapies (59,71,72).

Tumors that express ERβ2 (or ERβcx), a splice variant of ERβ that utilizes an alternative exon 8, show a poor response to tamoxifen (48,72). The ERβ2 variant does not bind ligands and heterodimerizes with ERα, having an overall dominant-negative effect on ERα reporter gene activity (10,73). In the present study, ERβ1, ERβ2 and ERβ5 mRNA expression in the cell lines was investigated; however, we did not study ERβ3 and ERβ4, as it has been indicated that they are barely detectable in breast tumor samples. However, the expression of the ERβ4 variant cannot be ruled out in breast cancer cell lines; Tong *et al* (47) used a different primer set and were able to amplify ERβ4 in MCF7, T-47D, ZR-75-1, MDA-MB-231 and SK-BR-3 cells (MDA-MB-453 and HCC1954 were not studied), although its expression was very low in comparison to the other variants, and thus it may have limited physiological significance.

The ER-positive cell lines MCF7, T-47D and ZR-75-1 were positive for ERβ1, ERβ2 and ERβ5. Other investigators have shown that MCF7 contains high levels of the ERβ2 and ERβ5 isoforms (51), and that the T-47D cell line is positive for ERβ1 and ERβ2 and negative for ERβ5 (47). Conversely, our results showed very weak ERβ5 expression in this cell line. Moreover, Tong *et al* showed that SK-BR-3 was negative

for ERβ1 and positive for ERβ2 and ERβ5, while we detected some ERβ1 and ERβ2 expression. The MDA-MB-231 cell line has been reported to express ERβ1, ERβ2 and ERβ5 (47), while our results confirm expression of ERβ1 and ERβ2 only, in agreement with other reports (42,46,51). Others have been unable to detect ERβ in SK-BR-3 (74), but in the present study SK-BR-3 cells were found to express ERβ by flow cytometry (unpublished data), as well as to express the ERβ 268 and 214-bp products at the mRNA level, and the 52-54 and 38-44 kDa products at the protein level.

Many of the ERα and ERβ variants have been shown to be translated into proteins (Tables I and II). In the present study, all of the cell lines showed wild-type ERα and ERβ1 expression, albeit to varying degrees. In addition, some cell lines clearly exhibited a 42-kDa variant that could be the translated protein product of the exon 5-deleted ERα variant. The expression of a smaller (38-44 kDa) ERβ variant by all cell lines, the significance of which is not clear at this stage, demonstrates that our level of understanding of the expression of ER variants at the functional level requires further investigation.

Acknowledgements

The authors would like to acknowledge the skillful technical assistance of Dr Beryl G. Rego and Mrs. Ani Mathew for handling the cell culture aspect of the project, and Dr Sureikah S. Mohan, Mrs. Lizamma Jacob and Ms. Jocelin Jacob for the processing of samples for protein and gene analysis. Financial support for this study was provided by the Kuwait University Grant no MY01/02. The authors would also like to acknowledge the support of the Department of Physiology, Faculty of Medicine.

References

1. Koike S, Sakai M and Muramatsu M: Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 15: 2499-2513, 1987.
2. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA: Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925-5930, 1996.
3. Mosselman S, Polman J and Dijkema R: ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392: 49-53, 1996.
4. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F and Giguere V: Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Mol Endocrinol 11: 353-365, 1997.
5. Enmark E, Pelto-Huikko M, Grandien K, *et al*: Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82: 4258-4265, 1997.
6. Montano MM, Muller V, Trobaugh A and Katzenellenbogen BS: The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. Mol Endocrinol 9: 814-825, 1995.
7. Pettersson K, Delaunay F and Gustafsson JA: Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19: 4970-4978, 2000.
8. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ and Scanlan TS: Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277: 1508-1510, 1997.
9. Cowley SM and Parker MG: A comparison of transcriptional activation by ER alpha and ER beta. J Steroid Biochem Mol Biol 69: 165-175, 1999.
10. Moore JT, McKee DD, Slentz-Kesler K, *et al*: Cloning and characterization of human estrogen receptor beta isoforms. Biochem Biophys Res Commun 247: 75-78, 1998.
11. Ogawa S, Inoue S, Watanabe T, *et al*: The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem Biophys Res Commun 243: 122-126, 1998.
12. Coradini D, Biffi A, Cappelletti V and Di Fronzo G: Activity of tamoxifen and new antiestrogens on estrogen receptor positive and negative breast cancer cells. Anticancer Res 14: 1059-1064, 1994.
13. Lacroix M and Leclercq G: Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 83: 249-289, 2004.
14. Graham ML, Krett NL, Miller LA, *et al*: T47DCO cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. Cancer Res 50: 6208-6217, 1990.
15. Fuqua SA, Fitzgerald SD, Chamness GC, *et al*: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51: 105-109, 1991.
16. Fuqua SA, Fitzgerald SD, Allred DC, *et al*: Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res 52: 483-486, 1992.
17. McGuire WL, Chamness GC and Fuqua SA: Abnormal estrogen receptor in clinical breast cancer. J Steroid Biochem Mol Biol 43: 243-247, 1992.
18. Castles CG, Fuqua SA, Klotz DM and Hill SM: Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res 53: 5934-5939, 1993.
19. Koehorst SG, Jacobs HM, Thijssen JH and Blankenstein MA: Wild type and alternatively spliced estrogen receptor messenger RNA in human meningioma tissue and MCF7 breast cancer cells. J Steroid Biochem Mol Biol 45: 227-233, 1993.
20. Pfeffer U, Fecarotta E, Castagnetta L and Vidali G: Estrogen receptor variant messenger RNA lacking exon 4 in estrogen-responsive human breast cancer cell lines. Cancer Res 53: 741-743, 1993.
21. Zhang QX, Borg A and Fuqua SA: An exon 5 deletion variant of the estrogen receptor frequently coexpressed with wild-type estrogen receptor in human breast cancer. Cancer Res 53: 5882-5884, 1993.
22. Daffada AA, Johnston SR, Smith IE, Detre S, King N and Dowsett M: Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res 55: 288-293, 1995.
23. Leygue ER, Watson PH and Murphy LC: Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst 88: 284-290, 1996.
24. Pfeffer U, Fecarotta E, Arena G, Forlani A and Vidali G: Alternative splicing of the estrogen receptor primary transcript normally occurs in estrogen receptor positive tissues and cell lines. J Steroid Biochem Mol Biol 56: 99-105, 1996.
25. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM and Jordan VC: A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res 24: 962-969, 1996.
26. Zhang QX, Hilsenbeck SG, Fuqua SA and Borg A: Multiple splicing variants of the estrogen receptor are present in individual human breast tumors. J Steroid Biochem Mol Biol 59: 251-260, 1996.
27. Desai AJ, Luqmani YA, Walters JE, *et al*: Presence of exon 5-deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. Br J Cancer 75: 1173-1184, 1997.
28. Huang A, Leygue ER, Snell L, Murphy LC and Watson PH: Expression of estrogen receptor variant messenger RNAs and determination of estrogen receptor status in human breast cancer. Am J Pathol 150: 1827-1833, 1997.
29. Gallacchi P, Schoumacher F, Eppenberger-Castori S, von Landenberg EM, Kueng W, Eppenberger U and Mueller H: Increased expression of estrogen-receptor exon-5-deletion variant in relapse tissues of human breast cancer. Int J Cancer 79: 44-48, 1998.
30. Murphy LC, Dotzlaw H, Leygue E, Coutts A and Watson P: The pathophysiological role of estrogen receptor variants in human breast cancer. J Steroid Biochem Mol Biol 65: 175-180, 1998.
31. Ohlsson H, Lykkesfeldt AE, Madsen MW and Briand P: The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. Cancer Res 58: 4264-4268, 1998.

32. Fasco MJ, Keyomarsi K, Arcaro KF and Gierthy JF: Expression of an estrogen receptor alpha variant protein in cell lines and tumors. Mol Cell Endocrinol 166: 156-169, 2000.
33. Flouriot G, Brand H, Denger S, *et al*: Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J 19: 4688-4700, 2000.
34. Poola I, Koduri S, Chatra S and Clarke R: Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. J Steroid Biochem Mol Biol 72: 249-258, 2000.
35. Powell CE, Soto AM and Sonnenschein C: Identification and characterization of membrane estrogen receptor from MCF7 estrogen-target cells. J Steroid Biochem Mol Biol 77: 97-108, 2001.
36. Ferro P, Forlani A, Muselli M and Pfeffer U: Alternative splicing of the human estrogen receptor alpha primary transcript: Mechanisms of exon skipping. Int J Mol Med 12: 355-363, 2003.
37. Han F, Miksicek R, Clarke R and Conrad SE: Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independence or tamoxifen resistance. J Mol Endocrinol 32: 935-945, 2004.
38. Razandi M, Pedram A, Merchenthaler I, Greene GL and Levin ER: Plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol 18: 2854-2865, 2004.
39. Penot G, Le PC, Merot Y, *et al*: The human estrogen receptor-alpha isoform hERalpha46 antagonizes the proliferative influence of hERalpha66 in MCF7 breast cancer cells. Endocrinology 146: 5474-5484, 2005.
40. Deroo BJ and Korach KS: Estrogen receptors and human disease. J Clin Invest 116: 561-570, 2006.
41. Vladusic EA, Hornby AE, Guerra-Vladusic FK and Lupu R: Expression of estrogen receptor beta messenger RNA variant in breast cancer. Cancer Res 58: 210-214, 1998.
42. Leygue E, Dotzlaw H, Watson PH and Murphy LC: Expression of estrogen receptor beta1, beta2, and beta5 messenger RNAs in human breast tissue. Cancer Res 59: 1175-1179, 1999.
43. Fuqua SA, Schiff R, Parra I, *et al*: Expression of wild-type estrogen receptor beta and variant isoforms in human breast cancer. Cancer Res 59: 5425-5428, 1999.
44. Speirs V, Adams IP, Walton DS and Atkin SL: Identification of wild-type and exon 5 deletion variants of estrogen receptor beta in normal human mammary gland. J Clin Endocrinol Metab 85: 1601-1605, 2000.
45. Mann S, Laucirica R, Carlson N, *et al*: Estrogen receptor beta expression in invasive breast cancer. Hum Pathol 32: 113-118, 2001.
46. Poola I, Abraham J and Liu A: Estrogen receptor beta splice variant mRNAs are differentially altered during breast carcinogenesis. J Steroid Biochem Mol Biol 82: 169-179, 2002.
47. Tong D, Schuster E, Seifert M, Czerwenka K, Leodolte S and Zeillinger R: Expression of estrogen receptor beta isoforms in human breast cancer tissues and cell lines. Breast Cancer Res Treat 71: 249-255, 2002.
48. Saji S, Omoto Y, Shimizu C, *et al*: Expression of estrogen receptor (ER) (beta)cx protein in ER(alpha)-positive breast cancer: specific correlation with progesterone receptor. Cancer Res 62: 4849-4853, 2002.
49. Saunders PT, Millar MR, Williams K, *et al*: Expression of oestrogen receptor beta (ERbeta1) protein in human breast cancer biopsies. Br J Cancer 86: 250-256, 2002.
50. Shaw JA, Udokang K, Mosquera JM, Chauhan H, Jones JL and Walker RA: Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. J Pathol 198: 450-457, 2002.
51. Girault I, Andrieu C, Tozlu S, Spyratos F, Bieche I and Lidereau R: Altered expression pattern of alternatively spliced estrogen receptor beta transcripts in breast carcinoma. Cancer Lett 215: 101-112, 2004.
52. Esslimani-Sahla M, Simony-Lafontaine J, Kramar A, *et al*: Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. Clin Cancer Res 10: 5769-5776, 2004.
53. Park BW, Kim KS, Heo MK, *et al*: The changes of estrogen receptor-beta variant expression in breast carcinogenesis: decrease of estrogen receptor-beta2 expression is the key event in breast cancer development. J Surg Oncol 93: 504-510, 2006.
54. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159, 1987.
55. Al-Bader MD: Estrogen receptors alpha and beta in rat placenta: detection by RT-PCR, real time PCR and Western blotting. Reprod Biol Endocrinol 4: 13, 2006.
56. Bukovsky A, Cekanova M, Caudle MR, Wimalasena J, Foster JS, Henley DC and Elder RF: Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. Reprod Biol Endocrinol 1: 13, 2003.
57. Choi I, Ko C, Park-Sarge OK, Nie R, Hess RA, Graves C and Katzenellenbogen BS: Human estrogen receptor beta-specific monoclonal antibodies: characterization and use in studies of estrogen receptor beta protein expression in reproductive tissues. Mol Cell Endocrinol 181: 139-150, 2001.
58. LaVoie HA, DeSimone DC, Gillio-Meina C and Hui YY: Cloning and characterization of porcine ovarian estrogen receptor beta isoforms. Biol Reprod 66: 616-623, 2002.
59. Poola I, Abraham J and Baldwin K: Identification of ten exon deleted ERbeta mRNAs in human ovary, breast, uterus and bone tissues: alternate splicing pattern of estrogen receptor beta mRNA is distinct from that of estrogen receptor alpha. FEBS Lett 516: 133-138, 2002.
60. Wang Y and Miksicek RJ: Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5: 1707-1715, 1991.
61. Park W, Choi JJ, Hwang ES and Lee JH: Identification of a variant estrogen receptor lacking exon 4 and its coexpression with wild-type estrogen receptor in ovarian carcinomas. Clin Cancer Res 2: 2029-2035, 1996.
62. Bollig A and Miksicek RJ: An estrogen receptor-alpha splicing variant mediates both positive and negative effects on gene transcription. Mol Endocrinol 14: 634-649, 2000.
63. Castles CG, Klotz DM, Fuqua SA and Hill SM: Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer 71: 974-980, 1995.
64. Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J and Gustafsson JA: Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci USA 101: 1566-1571, 2004.
65. Herynk MH and Fuqua SA: Estrogen receptor mutations in human disease. Endocr Rev 25: 869-898, 2004.
66. Garcia Pedrero JM, Zuazua P, Martinez-Campa C, Lazo PS and Ramos S: The naturally occurring variant of estrogen receptor (ER) ERDeltaE7 suppresses estrogen-dependent transcriptional activation by both wild-type ERalpha and ERbeta. Endocrinology 144: 2967-2976, 2003.
67. Chaidarun SS and Alexander JM: A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol Endocrinol 12: 1355-1366, 1998.
68. Bhat RA, Harnish DC, Stevis PE, Lyttle CR and Komm BS: A novel human estrogen receptor beta: identification and functional analysis of additional N-terminal amino acids. J Steroid Biochem Mol Biol 67: 233-240, 1998.
69. Wilkinson HA, Dahllund J, Liu H, *et al*: Identification and characterization of a functionally distinct form of human estrogen receptor beta. Endocrinology 143: 1558-1561, 2002.
70. Hanstein B, Liu H, Yancisin MC and Brown M: Functional analysis of a novel estrogen receptor-beta isoform. Mol Endocrinol 13: 129-137, 1999.
71. Iwao K, Miyoshi Y, Egawa C, Ikeda N and Noguchi S: Quantitative analysis of estrogen receptor-beta mRNA and its variants in human breast cancers. Int J Cancer 88: 733-736, 2000.
72. Saji S, Omoto Y, Shimizu C, *et al*: Clinical impact of assay of estrogen receptor beta cx in breast cancer. Breast Cancer 9: 303-307, 2002.
73. Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y and Muramatsu M: Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human. Nucleic Acids Res 26: 3505-3512, 1998.
74. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J and Lupu R: Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep 7: 157-167, 2000.