Real-Time PCR: Detection of Oestrogen Receptor-Alpha and -Beta Isoforms and Variants in Breast Cancer

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Abstract. *Background: Splice variants exist for both alpha and beta oestrogen receptors (ERs). Oestrogen function results from a balance between the wild-type ERs (wt) and their variants. Patients and Methods: Forty formalin-fixed paraffin-embedded breast cancer samples were analysed by real-time PCR using ERα primer sets detecting wt and exon- deleted 3, 5, 6 and 7 variants. The ERβ primer sets detected wt ERβ1 and ERβ2 and ERβ5 variants. At the end of the PCR cycles, a dissociation curve was generated showing the peaks for each sample at specific melting temperatures (Tm); finding more than one peak indicated the presence of variants. Results: Many samples expressed both wt ER isoforms and their variants. The Tm value served as a cut-off point for determination of wt versus variant ER expression. Conclusion: This method of detection of wt and variant ER could help in patient selection for anti-oestrogen therapy and in monitoring response to therapy.*

In Kuwait, breast cancer is the commonest type of cancer among Kuwaiti women accounting for 30% of all female malignancies, and 43% of cancer-related deaths (1). Different studies have shown that the overexpression of oestrogen receptor (ER) is a common feature of breast cancer (2).

The production of two isoforms of oestrogen receptor, ERα and ERβ, in breast cells could give rise to different or opposing biological activities (3). While ERα induces cell proliferation, ERβ can inhibit ERα-stimulated transcription and cell proliferation *in vitro*, acting as a regulator of oestrogen signalling (3, 4). In addition to the ERα and ERβ isoforms, several variants exist for each isoform. Thus, oestrogen function is a consequence of the balance between wild-type ER isoforms and their functional variants (3).

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Between 30-70% of patients with ER-positive tumours that respond to endocrine therapy develop resistance during treatment despite continued expression of ER in the relapse tissue (5, 6). Such results indicate mechanisms other than loss of ER expression as being responsible for this resistance (5, 6).

Several splice variants of ERα have been identified in breast tissue (Table I). These include deletions at exons 2, 3, 4, 5, 6 and 7 (Figure 1). The ERαΔ3 variant is a receptor lacking exon 3, which encodes the second zinc finger of the DNA-binding domain preventing the protein from forming specific complexes with oestrogen response elements (EREs) (20). As a result, the protein induces dominant negative activity, suppressing oestrogen-induced transcriptional activity (20). Another ERα splice variant is an exon-5-deleted variant (ERαΔ5) giving rise to a truncated receptor lacking the hormone-binding domain (5), but still having the transcription activation function (AF-1) activity and DNA binding ability, leading to a constitutively active receptor (20). ERαΔ6, an exon 6 deleted variant, is found in breast cancer tissue and in the ER-positive cell line MCF-7 (20). A deletion in exon 6 results in loss of hormone binding and dimerization domains (20). The most observed variant in breast cancer is ERαΔ7 (20). ERαΔ7 is able to form heterodimers with ERα and ERβ in a ligand-independent manner resulting in a dominant negative effect on both ER isoforms (20, 21).

In addition to ERα variants, several variants have been reported for ERβ (Table II). ERβ variants, described as ERβ1, ERβ2, ERβ4 and ERβ5 (Figure 2) are co-expressed in human breast cancer (34, 35). ERβ2 and ERβ5 mRNAs are more highly expressed than ERβ1 mRNA in cancer tissues. ERβ2, also known as ERβcx (36) is expressed in about 54% of breast tumours (34).

Several methods have been adopted to measure the expression of ER in breast cancer tissue, the most widely used is immunohistochemistry (IHC). This method however, does not enable the detection of ER variants. The aim of this study was to determine whether real-time reverse-transcriptase-polymerase chain reaction (ReT-PCR) is a useful method for detecting ER isoforms and variants in routine formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples.

Table I. *Reported ERα variants in breast tissue.*

Origin	ERa mRNA variants	ERa protein variants	Reference
Breast cancer ER–/PgR+ ER+/PgR-	Δ3, 5 or 7		(7)
Breast cancer ER–/PgR+ ER+/PgR-	Δ3, 5 or 7		(8)
Breast cancer ER+/PgR+ ER+/PgR			
T-47D ZR-75-1 (positive cell lines)	Δ5		(9)
Breast cancer	Δ5		(10)
Breast cancer	Δ7, Δ4, Δ4+7 and Δ3+4		(11)
Breast cancer	Δ4		(12)
Breast cancer	Δ2, Δ3, Δ4, Δ5, Δ7		(13)
Breast cancer	Δ5	40 kDa	(14)
Breast cancer	ERa clone4		(15)
Breast cancer (relapse)	Δ5	40 kDa	(5)
Breast cancer	Δ4, Δ3+4, Δ5, Δ7, Δ4-7, clone 4	Δ4=54 kDa, Δ3+4=49 kDa, Δ5=40 kDa, Δ7=51 kDa, Δ4–7=39 kDa, clone 4=24 kDa	(16)
Breast cancer		67+67~134 kDa	(17)
Breast cancer	Δ4, Δ5, Δ6+7	Δ4=53 kDa, Δ5=40 kDa, Δ6+7=54 kDa	(18)

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

Origin	ERβ mRNA status	ERβ protein status	Reference
Breast tissue	ERβ1, 2, 4, 5	ERβ1~ 54.2 kDa; ERβ2 ~ 55.5 kDa	(22)
Breast cancer		58-60 kDa + low mol wt (4-5 kDa); predicted 62 kDa from sequence data	(23)
Normal breast tissue	ERβΔ5		(24)
Breast cancer		55 kDa, 50 kDa	(25)
Breast, normal, cancer and cell lines	Δ2; Δ 2 and Δ 5-6; Δ4; Δ5; Δ5 and Δ2; Δ6; Δ6 and Δ2, Δ6 and Δ2-3; Δ5-6		(26)
Breast cancer	ERβ1, 2, 4, 5		(27)
Breast cancer	ERβcx		(28)
Breast cancer		59 kDa, 53 kDa, 32-45 kDa	(29)
Breast, normal and cancer		62 kDa, 58 kDa, 56 kDa, 54 kDa	(30)
Breast, normal, cancer and cell lines	ERβ1, 2, 5		(31)
Breast cancer	ERβcx		(32)
Breast cancer		59+59 ~ 118 kDa	(17)
Breast cancer	ERβ1, 2, 4, 5	ERβ1~ 54.2 kDa; ERβ2 ~ 55.5 kDa	(33)

Materials and Methods

Materials. All buffers, enzymes and reagents used in reverse-transcription PCR experiments were purchased from Invitrogen (Carlsbad, CA, USA) and ReT-PCR reagents from Applied Biosystems Inc. (Foster City, CA, USA). All the ReT-PCR primers were purchased from SynGen Inc. (Hayward, CA, USA).

Samples. Forty archival FFPE breast cancer tissue samples were used in this study. Samples were obtained from the Department of Pathology, Faculty of Medicine, Kuwait University (Table III). An ERα-positive breast cancer cell line, MCF-7, was used as a positive control.

RNA isolation. A clean sharp microtome blade was used to cut 10-20 μm thick sections from trimmed tissue blocks. Sections were immediately placed into a sterile tube and tightly capped to minimize moisture in the sample. Two or three 10-20 μm thick sections of FFPE breast cancer samples were used for each experiment. RNA was isolated from FFPE samples using the Absolutely RNA FFPE Kit (Catalog #400809; Stratagene, CA. USA) with slight modification whereby proteinase K digestion was maintained overnight to increase the final RNA yield.

Determination of RNA concentration. Samples were diluted with diethylpyrocarbonate (DEPC)-water (1 in 500 dilution), vortexed and the absorbance was read at 260 and 280 nm using DEPC-water as blank. The spectrophotometer was zeroed at 280 nm and the blank was read at 260 nm. The ratio A_{260}/A_{280} is an indication of the purity of the preparation and ratios of ≥1.7 were used in this study.

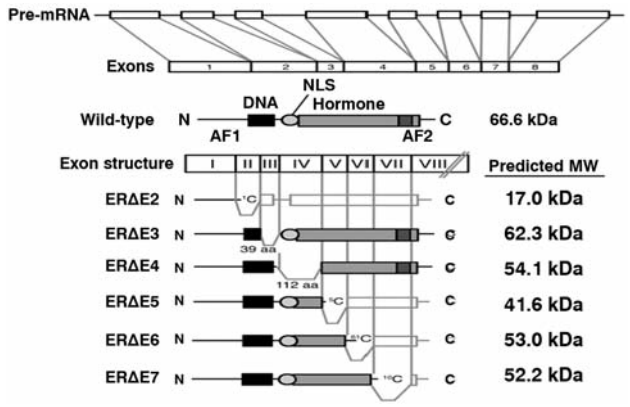

Figure 1. *ERα and its variants (reproduced with permission from (19)).*

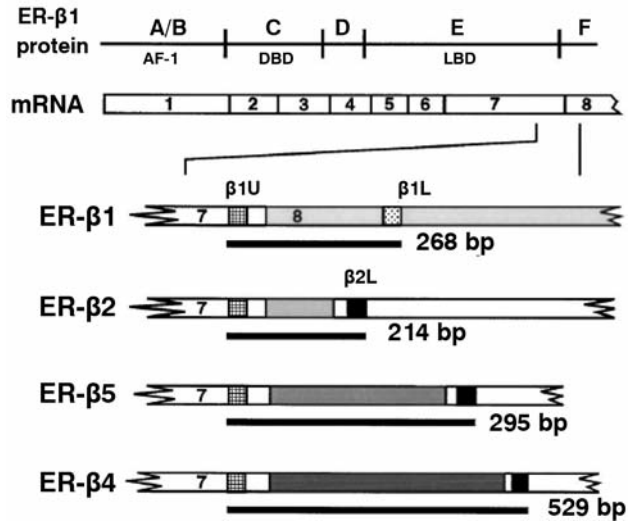

Figure 2. *ERβ and its variants (reproduced with permission from (22)).*

DNase treatment. Total RNA samples were treated with DNase enzyme to ensure the removal of genomic DNA. This was done by mixing the following on ice: the equivalent of 2 μg total RNA, 4 μl RNasin (40 U), 2 μl 10X DNase I buffer, 2 μl DNase I (1U), and DEPC-water to make up the volume to 20 μl. The mixture was incubated at room temperature for 15 min. The reaction was then terminated by adding 2 μl of 25 mM EDTA, and then heated for 10 min at 70˚C. The samples were used directly for reverse transcription.

Reverse transcription (RT). RT was carried out with the addition of 2 μl random primers (100 ng/μl) to the DNase-treated sample. The mixture was mixed, heated at 70˚C for 10 min and immediately chilled on ice for >3 min, then briefly centrifuged at 7200×*g*. The DNase-treated sample was divided into two aliquots for RT+, and for RT- control (12 μl each). On ice, the following were added: 4 μl first-strand buffer, 2 μl DTT (5 mM final concentration), 1 μl dNTP mix (500 μM final concentration), 1 μl (200 U) superscript II (RT+ reaction) or 1 μl water (RT- reaction). The sample was mixed gently using a pipette. The mixture was incubated at room temperature for 10 min, then at 42˚C for 50 min. It was then heated at 70˚C for 15 min to inactivate the enzyme. The sample was used for ReT-PCR directly.

Table III. *Samples used in the present study.*

No.	Tumour type	ER status	Tumour grade	LN metastasis
1	Invasive ductal carcinoma	Positive	I	Absent
2	Invasive ductal carcinoma	Positive	III	Present
3	Invasive ductal carcinoma	Positive	III	Present
4	Invasive ductal carcinoma	Negative	III	Present
5	Minimally invasive ductal carcinoma	Positive	II	Absent
6	Invasive ductal carcinoma	Positive	II	Absent
7	Invasive ductal carcinoma	Negative	II	Absent
8	Invasive ductal carcinoma	Positive	NA	Present
9	Minimally invasive ductal carcinoma	Positive	II	Absent
10	Invasive ductal carcinoma	Positive	II	Absent
11	Invasive ductal carcinoma	Positive	II	Absent
12	Invasive lobular carcinoma	Positive	NA	Present
13	Invasive ductal carcinoma	Negative	III	Present
14	Invasive ductal carcinoma	Positive	III	Absent
15	Invasive ductal carcinoma	Positive	I	Present
16	NA	NA	NA	NA
17	Invasive ductal carcinoma	Negative	II	Present
18	NA	Positive	NA	NA
19	Medullary carcinoma	Negative	NA	NA
20	Invasive lobular carcinoma	Positive	NA	Present
21	NA	Negative	NA	NA
22	Invasive ductal carcinoma, grade III	Negative	III	NA
23	NA	Positive	NA	NA
24	NA	Positive	NA	NA
25	Invasive ductal carcinoma	Positive	I	Absent
26	NA	Negative	NA	NA
27	Mucinous carcinoma	Positive	NA	Present
28	Invasive ductal carcinoma	Positive	III	Present
29	Invasice ductal carcinoma	Positive	III	Present
30	Medullary carcinoma	Negative	NA	Absent
31	NA	Negative	NA	NA
32	Invasive ductal carcinoma	Positive	III	Absent
33	NA	NA	NA	NA
34	Invasive ductal carcinoma	Positive	III	Absent
35	Invasive ductal carcinoma	Positive	II	Absent
36	Invasive ductal carcinoma	Positive	II	Absent
37	Invasive ductal carcinoma	Negative	III	Absent
38	Minimally invasive ductal carcinoma	Negative	II	Absent
39	Invasive ductal carcinoma	Positive	III	Absent
40	NA	NA	NA	NA

NA: Pathology report either not complete or not available, however, confirmed as breast cancer by one of the co-authors IF.

ReT-PCR. The PCR reaction was carried out in a real-time PCR system (Applied Biosystems, model 7500). The forward and reverse primer sequences for ERα and ERβ, in addition to PCR product sizes are presented in Table IV. The primers that were used allowed detection of ERα isoforms exon deleted 3 (Δ3), 5

Table IV. *Primer sets used in the present study.*

Gene	Forward primer	Reverse primer	Expected product size	Reference
β-Actin	GTCCTGTGGCATCCACGAAACT	TACTTGCGCTCAGGAGGAGCAA	201 bp	(13)
ERαΔ3	ATGGAATCTGCCAAGAAGACT	GCGCTTGTGTTTCAACATTCT	281 bp wt; 165 bp Δ3	"
ERαΔ5	CTCATGATCAAACGCTCTAAG	ATAGATTTGAGGCACACAAAC	466 bp wt; 328 bp Δ5	"
ERαΔ6, 7, 6+7	GCTCCTAACTTGCTCTTGG	ACGGCTAGTGGGCGCATGTA	452 bp wt; 318 bp Δ6; 268 bp Δ7; 134 bp Δ6+7	"
ERβ-I	CGATGCTTTGGTTTGGGTGAT	GCCCTCTTTGCTTTTACTGTC	268 bp ERβ1	(22)
ERβ-II	CGATGCTTTGGTTTGGGTGAT	CTTTAGGCCACCGAGTTGATT	214 bp ERβ2; 295 bp ERβ5	"

(Δ5), 6 (Δ6), 7 (Δ7), 6+7 (Δ6+7) variants; and ERβ1 and its variants, ERβ2 and ERβ5. The PCR reactions were prepared using SYBR GREEN master mix (#4309159, Applied Biosystems). On ice, the following were added: 12.5 μl of 2× SYBR GREEN, 1 μl of forward primer (5 μM), 1 μl of reverse primer (5 μM), 1 μl of template cDNA, 9.5 μl of sterile water. The PCR was then run as follows: 2 min at 50˚C (1 cycle); 10 min at 95˚C (1 cycle), 15 s at 95˚C and 1 min at 60˚C for 60 cycles, followed by a dissociation step for 15 s at 95˚C, 1 min at 60°C and 15 s at 95˚C for 1 cycle. In all experiments an RT-control (FFPE sample where reverse transcriptase was not added) and no template control (NTC), where water was added as sample, were included as negative controls, the former to exclude genomic DNA contamination and the latter to exclude any reagent contamination.

Results

Gene amplification curves by ReT-PCR are shown in Figure 3 A, B, E, F, I, J for the specific genes of interest. These curves show the plot of Delta Rn (ΔRn) *versus* the cycle number. ΔRn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye (ROX). Using this method it is not possible to distinguish whether the amplification plot is for the target gene or for one of its variants, thus a dissociation step (melting curve step) was added. From the dissociation curve the peak derivative for each isoform/variant relative to the melting temperature (Tm) was determined (Figure 3 C, D, G, H, K, L). If more than one peak is obtained at the higher melting temperatures then this indicates the presence of variants for the specific gene of interest (Figure 4 points a, b and c). However, one must be cautious with interpretation of results as it is important to take peak derivatives that have a melting temperature higher than that of the NTC peak derivative. For example, as point a falls at a lower temperature and the peak derivative of NTC (point d) is at the same Tm then this sample was not considered to be positive. In addition, the peak derivative provides an estimation of the extent of expression of a specific gene; the higher the peak the greater the expression, provided that the starting DNase-treated RNA is uniform for all samples and equal amounts of cDNA were used, which is the case in our study. All PCR products were initially run on ethidium bromide-stained agarose gels to verify the product sizes and compare them with the peak derivatives that were obtained (data not shown). Actin was used as an internal control; all RT samples were run for the housekeeping gene actin and showed no amplification, that is, no genomic DNA contamination was detected (data not shown).

Table V shows the different genes studied for each sample and the expression of the various ER isoforms and variants. At the bottom of the table, the percentage of expression is calculated. In addition, the range of Tm values in ˚C is given for each PCR product. The NTC samples and Tm values were all below the Tm values taken for variant expression (Tm values not shown).

The percentage expression of wild-type ERα varied with different primer sets, the ERαΔ3 primer set being most efficient in detecting the wild-type ERα (43%; Figure 5A). However, from Table V it can be seen that two samples were not positive for the wild-type with this primer set but were positive when a primer set for ERαΔ5 (sample #40), and ERαΔ6, 7, 6+7 (sample #23) was used.

A high percentage of samples expressed the ERαΔ3, Δ5, and Δ7 variants (30, 40, and 33%, respectively) with ERαΔ6 and ERαΔ6+7 being least expressed. Moreover, ERβ variant expression was higher than that of wild-type ERβ1; ERβ2 was detected in 20% and ERβ5 in 23% of the samples (Figure 5B).

By comparing ReT-PCR results with tumour grade (only possible for 20 samples), we found that the wild-type ERα was the most expressed ER isoform in grade II tumours, with ERαΔ5 being the most expressed splice variant of ERα in both grades II and III tumours, showing even more expression with higher tumour grade (Table VI). ERα Δ3 and ERα Δ7 were expressed more frequently in higher grade tumours, while ERα Δ6 expression was the converse of this. ERα Δ6+7 was not expressed in any of the grade II and III tumour samples. ERβ1 expression was seen only in grade II tumours. ERβ2 had a similar expression in both tumour grades, while ERβ5 was expressed in 4 out of 8 of grade II tumours and 2 out of 12 grade III tumours (Table VI).

Table V. *Expression of wild-type ER isoforms and variants as determined by ReT-PCR.*

Sample #	Actin 201 bp	ERαΔ3 primer set		ERαΔ5 primer set		ERαΔ6, 7, 6+7 primer set				ERβ primer set		
		wt 281 bp	ERα Δ3 165 bp	wt 466 bp	ERα Δ5 328 bp	wt 452 bp	ERα Δ6 318 bp	ERα Δ7 268 bp	ERα Δ6+7 134 bp	ERβ1 268 bp	ERβ2 214 bp	ERβ5 295 bp
1	*										*	
2	*											
3	*	*					*				*	
4	*	*						*				*
5	*									*		
6	*	*										*
7	*	*										
8	*	*		*								
9	*	*							*			
10	*											*
11	*	*								*		*
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13	*				*							*
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15	*				*			*				
16	*		*		*			*			*	
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22	*	*	*		*			*				
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37	*				*							
38	*	*	*					*		*		
39	*										*	
40	*		*	*				*				
# Samples studied	40	40	40	40	40	40	40	40	40	40	40	40
Positive results	40	17	12	3	16	2	4	13	4	6	8	9
% positive	100	43	30	8	40	5	10	33	10	15	20	23
Tm range °C	80.2-83.5	79.2-81.9	75.1-78.8	81.6-83.6	75-79	84-85.4	82.1-83.2	78.9-81.6	77-77.9	77.1-84.3	77.8-80.6	82-83

Blanks in the table indicate no result.

Discussion

Studies on ER-negative breast cancer have shown that they are negative for the wild-type isoform but may express ER variants (37). Evaluation of ERs by IHC may give misleading results as IHC cannot detect ER isoforms/variants using a single antibody. ReT-PCR, on the other hand, could give more sensitive results revealing both the presence and quantity of expression of each variant in question.

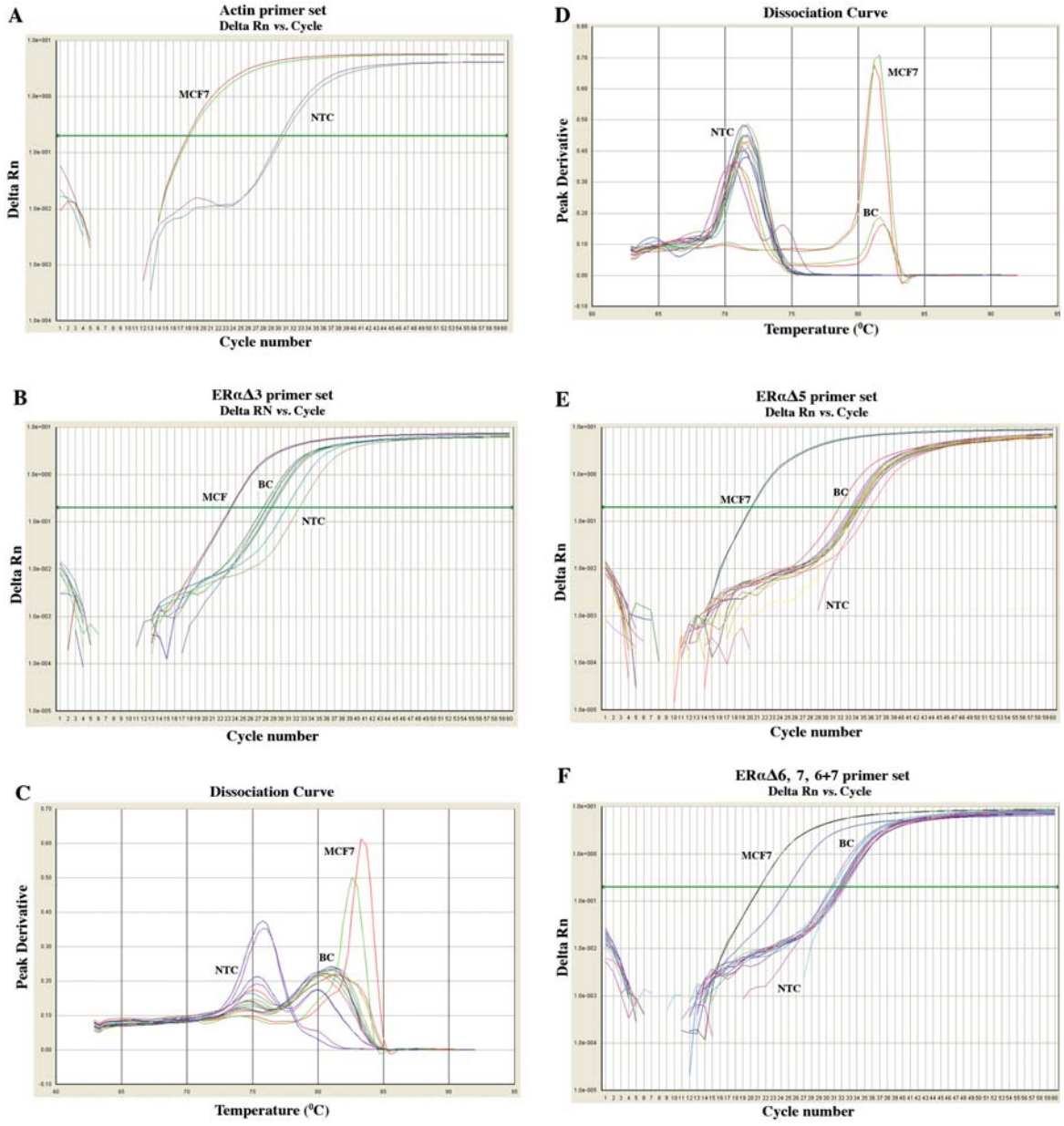
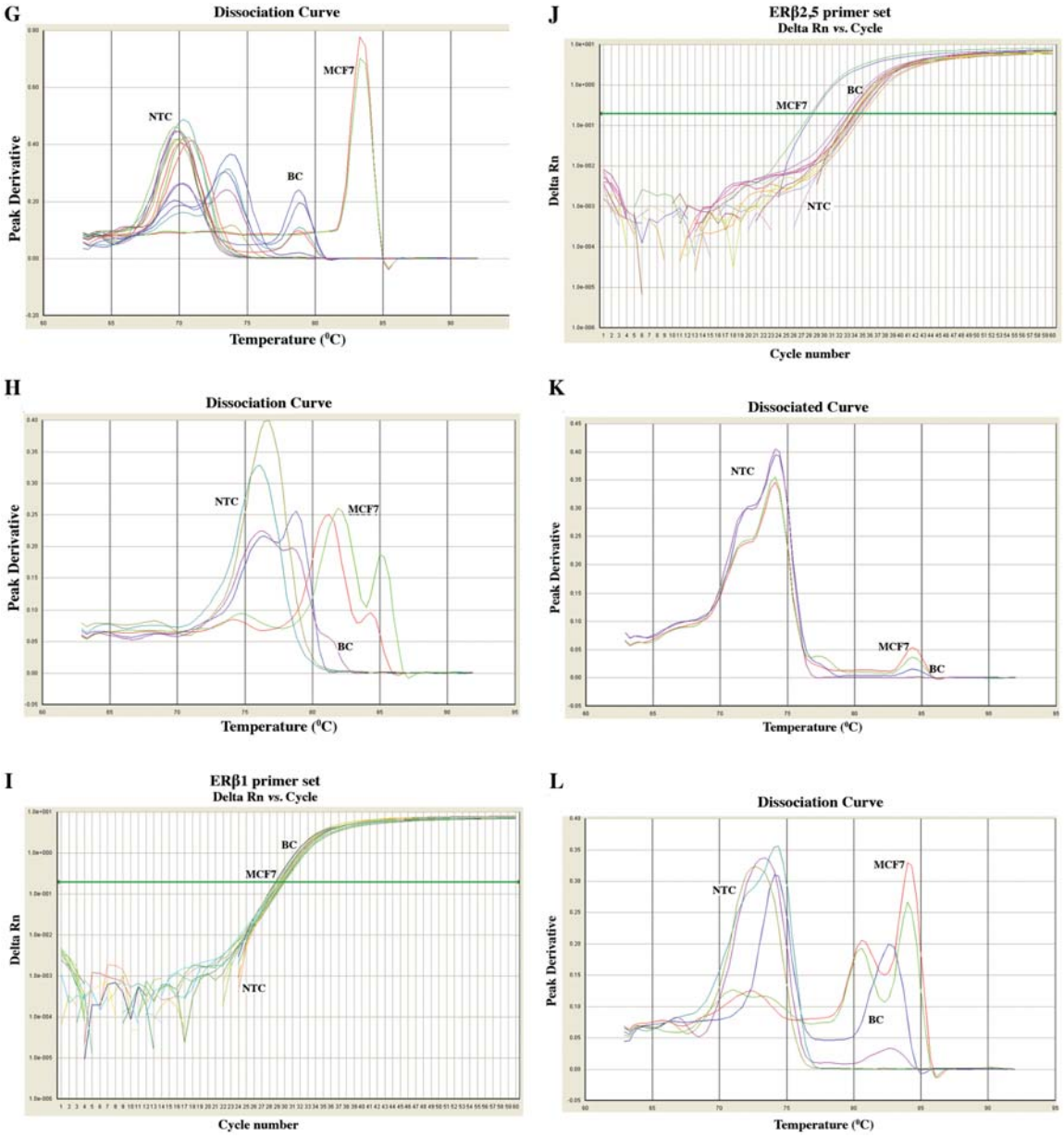


Figure 3. *Graphs showing delta Rn versus cycle number for the amplification plots (panels A, B, E, F, I, J) and the peak derivative versus the Tm for the dissociation curves (panels C, D, G, H, K, L) for representative samples studied for actin, ERα and ERβ primer sets. The green horizontal line in panels A, B, E, F, I and J refers to the cycle threshold value. In panels C, D, G, H, K and L, if more than one peak appears then it is indicative of the presence of variants; the higher the peak, the greater the gene expression, as the starting material was the same for all samples. BC: Breast cancer sample, MCF7: breast cancer cell line used as positive control, NTC: no template control used to exclude reagent contamination.*

ER variants may play a crucial role in the development of breast cancer as indicated by their high level of expression in malignant tissues. Wild-type ERs are present at significantly lower levels in breast tumours than in normal tissues, unlike ER variants which are expressed at higher levels (38, 39). Our findings of tumours that only expressed the variants and not the wild-type isoforms are in agreement with such studies.

Currently, hormone therapy depends on the presence of ERα. There is growing evidence suggesting that detection of ERα variants is equally important since ERα-negative tumours were found to express only splice variants (39). With studies showing that ERβ overexpression is associated with responsiveness to endocrine therapy (40), the significance of the ERβ isoform, and its variants, also has to be considered.

Figure 3. *continued*



Our results reveal that the ERαΔ3 variant was expressed in 4 out of 12 of grade III tumours compared to 1 out of 8 of grade II tumours. This suggests a higher expression of this variant with tumour progression. Patients with ERαΔ3 may not benefit from endocrine therapy, such as tamoxifen (TAM), as this variant is reported to have an inhibitory effect on wild-type ERα activity and is involved in resistance (2, 20).

Our findings are consistent with the fact that the ERαΔ5 variant has been reported to be expressed at significantly higher levels in breast tumour when compared with matched adjacent normal breast tissue (41). We have shown that this variant is frequently expressed in breast tumours, especially in those with a higher grade (7 out of 12 in grade III *versus* 2 out of 8 in grade II tumours). This is in agreement with studies which reported that ERαΔ5 is involved in the progression of breast tumours (7, 20, 39, 41, 42, 43). The ERαΔ5 variant has been shown to be expressed at high levels in ER-positive pS2-positive TAM-resistant tumours in comparison (41). Moreover, in cell lines, TAM treatment had no significant effect in the presence of this variant (44). Such reports suggest that

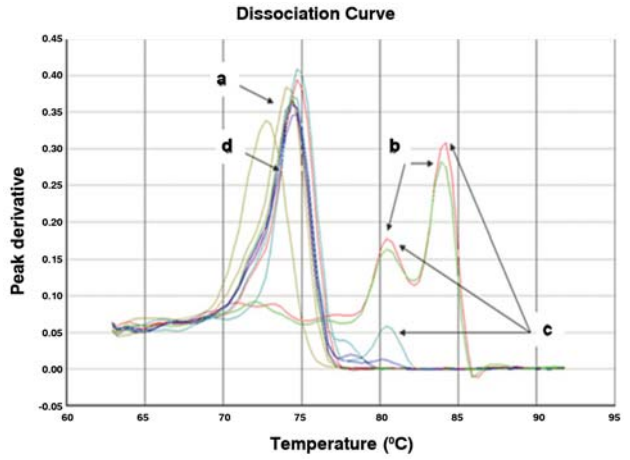


Figure 4. *Dissociation curve showing peak derivatives indicative of variants having different Tm. From the dissociation curves, it was possible to determine the peak derivative for each variant relative to the melting temperature (Tm).*

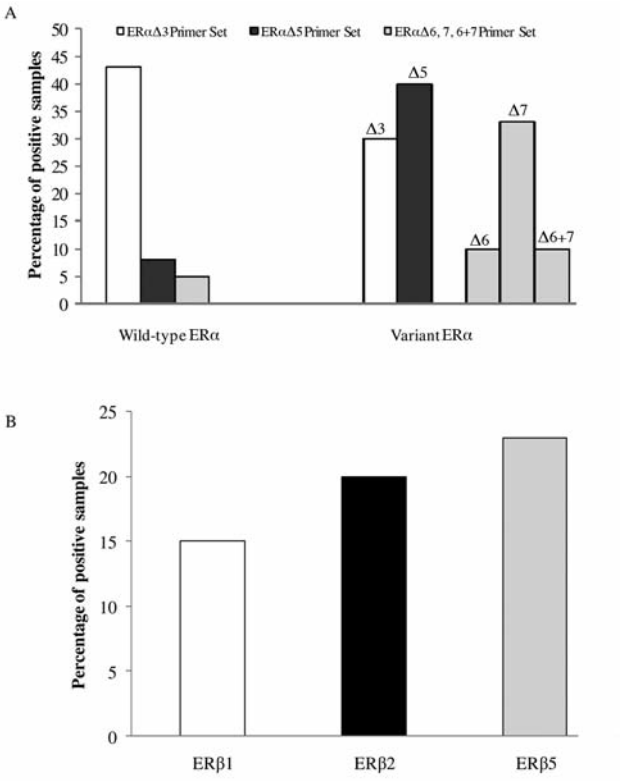


Figure 5. *Percentage of samples expressing wild-type ERα isoform and variants (A) and wild-type ERβ isoform and variants (B). A: ERαΔ3, Δ5 and (Δ6, 7, 6+7) primer sets all showed wild-type ERα expression in 43%, 8% and 5%, respectively. ERαΔ3, Δ5 and Δ6, Δ7, Δ6+7 were seen in 30%, 40%, 10%, 33% and 10% of samples, respectively. B: Wild-type ERβ1 was expressed in 15% of samples and the variants ERβ2 and ERβ5 in 20% and 23% of samples, respectively.*

evaluating ERαΔ5 expression in breast cancer can help in deciding the appropriate treatment modality.

ERα variants Δ6, Δ7 and Δ6+7 were also expressed in some of our samples. Very few samples expressed the ERαΔ6+7 variant as compared to the ERαΔ7 variant, which has been reported to be less often expressed when compared to the ERαΔ7 variant in breast cancer patients (11, 13, 18). Our data showed 1 out of 8 tumour grade II samples and 1 out of 12 tumour grade III samples expressed ERαΔ6, while 1 out of 8 of tumour grade II samples and 3 out of 12 of tumour grade III samples expressed ERαΔ7 (Table VI). Despite the absence of association between expression and tumour grade, the expression of these variants may suggest a possible effect on response to endocrine therapy. ERβ variant expression also plays a crucial role in prognosis and therapy outcome. Thus proper identification of ERβ isoform and variant expression is clinically important.

ERβ5 mRNA has been associated with favourable tumour differentiation and slower tumour growth, whilst ERβ2 mRNA expression shows no correlation with tumour size, grade, nodal status or systemic recurrence (33). Our modest number of cases showed that ERβ1 was expressed only in grade II tumours; ERβ2 in both grade II and grade III tumours (1 out of 8 *versus* 2 out of 12, respectively); and ERβ5 more often in grade II than in grade III tumours (4 out of 8 *versus* 2 out of 12, respectively). This is contrary to published data showing that ERβ5 is related to higher proliferative activity and that ERβ1 and ERβ2 are the most commonly expressed variants in invasive tumours (45). The reason for this could be that our samples may have contained some non-tumour cells. Moreover, the wild-type ERβ1 isoform has been shown to play a role as a tumour suppressor, with its anti-invasiveness property and its ability in maintaining a benign phenotype

Table VI. *Summary of frequency of ER expression using ReT-PCR in relation to tumour grade.*

Tumour grade	Samples n	ERα						ERβ		
		wt	Δ3	Δ5	Δ6	Δ7	Δ6+7	β1	β2	β5
II	8	5	1	2	1	1	–	2	1	4
III	12	4	4	7	1	3	–	–	2	2

(46). This inverse relationship between expression of ERβ1 mRNA and tumour grade has been suggested as a useful marker of tumour progression (22).

A significant number of ERα–negative breast tumours have been shown to express ERβ1 and ERβ2 (47). Gruvberger-Saal *et al.* have suggested that those ERα–negative tumours that express ERβ respond positively to TAM therapy (48). Therefore, routine testing of ERβ, alongside ERα, might be justified in ERα–negative tumours.

As indicated above, the levels of ERβ2 and ERβ5 mRNAs have been reported as being higher than that of ERβ1 in breast tumours. The loss of ERβ, and in particular ERβ2, was reported to result in a more aggressive cancer growth and an increased risk of metastasis (28). Our results are consistent with the previous literature, ER variant is more frequent with higher tumour grade.

Previous RNA studies have been carried out on tumour tissues extracted from sections that contained a mixture of neoplastic and benign epithelial and stromal cells, which have been shown to express ER isoforms (49). Any RNA extract would, as a result, reflect expression from the pool of heterogeneous cell types. ReT-PCR does not account for tumour heterogeneity and therefore contributions from different elements other than invasive tumour cells, such as normal and/or preneoplastic breast cells, in addition to vascular and lymphoid cells, cannot be ruled out (40). Immunohistochemistry, in contrast, allows for a more selective evaluation and localization of ER expression in tumour cells.

As shown above, detection of ER isoforms could provide additional parameters regarding prognosis and response to therapy in breast cancer. ReT-PCR evaluation of these isoforms on FFPE tissue often yields data representing both normal and tumour tissue. Selective evaluation reflecting tumour status therefore requires careful tissue sample selection.

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