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Oestrogen receptor mutations and their influence on breast cancer growth

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CHAPTER THREE

MATERIALS AND METHODS

3.1

Sources of Tissue and Cell Lines

Samples were taken from 61 patients undergoing surgery at the Chris Hani Baragwanath Academic Hospital, Johannesburg, with primary breast cancer. The breast fine needle aspirates (FNAs) were taken from patients by routine clinical procedure and placed immediately into lysis buffer containing β-mercaptoethanol (as provided by the RNeasy mini kit from Qiagen) and stored at -70°C. An aliquot of each FNA was also used for routine cytology to confirm the presence of tumour cells within each sample. Normal breast tissue samples were obtained from 10 patients undergoing breast reduction surgery and were stored in the same way. Ethical approval was sought and obtained and all patients gave informed consent (see Appendix 1).

The MCF-7 breast carcinoma cell line from the American Type Culture Collection (ATCC 22) was used as a positive control and was maintained in Earle's Minimal Essential Medium (EMEM) (Gibco BRL, Life Technologies, UK) with 10% foetal calf serum (Delta Biologicals, Kempton Park, South Africa). The breast carcinoma cell line T47D (ATCC HTB 133) was maintained in RPMI 1640 medium (Gibco BRL, Life Technologies,

FCS supplemented with 2 IU insulin/ml (1 mg/ml Humulin N, Eli Lilly, Isando, South Africa) and 1mM Sodium Pyruvate (Gibco BRL).

3.2 Ribonucleic acid extraction

3.2.1 Human breast tissue

Ribonucleic acid (RNA) was extracted from human breast tissue samples using RNeasy Mini Kit (Qiagen, Germany). The samples were collected in a lysis buffer containing guanidinium thiocyanate and β-mercaptoethanol as provided in the kit in order to protect the RNA. The tissue samples were manually homogenized using scalpel blades and the lysates were pipetted directly onto Qiashredder (Qiagen, Germany) spin columns and centrifuged in a microcentrifuge (Eppendorf centrifuge 5402, Eppendorf, Hamburg, Germany) for 2 minutes at room temperature (14000 rpm). One volume of 70% ethanol was added to the lysate and mixed well by pipetting to create conditions that promote selective binding to the RNeasy silica-gel membrane. Each sample was then applied to an RNeasy mini column (Qiagen, Germany), contaminants were washed away and RNA was eluted from the columns with RNase-free water according to the manufacturer's instructions. The RNA was stored at -70°C until required.

3.2.2 Tissue culture cells

Medium was decanted from the flasks and the cells were w
buffered saline (PBS) (Appendix 3). 1ml lysis buffer containing gua
thiocyanate and β-mercaptoethanol (Buffer RLT, Qiagen, Germany)
each flask and the lysed cells were poured into Eppendorf t
pipetted onto Qiashredder (Qiagen, Germany) spin columns and centrifuge
microcentrifuge for 2 minutes at maximum speed (14000 r
70% ethanol was added to each homogenized lysate to adju
Each sample was applied to an RNeasy minidspin RNA column, w
eluted from the columns with water and treated with RNase according to manufacturer s
instructions. The RNA was stored at -70°C until required.

3.3 Reverse transcription

The extracted RNA was converted into complementary DNA
to allow for the DNA polymerase, used in the PCR, to function
avian myeloblastosis virus (AMV) reverse transcriptase was

A 20µl reverse transcription reaction was performed for e
culture sample prior to first round nested PCR. 10µl of 2x PCR buffer
[500 mM KCl; 100 mM Tris-HCl (pH 8.4); 0.1% gelatin] and 3mM $MgCl_2$ added to

(25 mM) (2 µl), 10 mM dNTP mix (free acid) (1 µl), 40 U/µl RNasin (Promega, Madison, USA) was made up to 20 µl with DEPC-treated water and 1 µl (15 U) of AMV reverse transcriptase (Promega). As the RNA is small and thus the concentration of RNA extracts would be too low to quantitate the RNA spectrophotometrically for fear of losing a random amount of RNA was therefore added to the reverse transcription reaction and mixed well. Initially, the RNA samples were heated with oligo(dT_{15}) primer at 70°C for 5 min and cooled slowly to room temperature to facilitate primer annealing. After the addition of the remaining components, reactions were incubated for 1 hour at 37°C. The reverse transcriptase activity was inactivated by heating at 95°C for 5 min and cooling on ice. The cDNA was allowed to cool for 1 hour before amplification by PCR was performed.

A reverse transcription control reaction was performed simultaneously with addition of DEPC-treated water in place of RNA solution prior to reverse transcribing in all cases.

3.4 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is extremely sensitive and stringent precautions were taken to avoid contamination of extraneous DNA. PCR reagent preparation and amplification of samples were assigned to

areas. Pipettes were designated for PCR preparation, sepa
general use and never used for pipetting PCR product
contaminating DNA source. Surfaces were regularly wiped dow
1 M HCl and gloves were changed frequently. All primers used were synthes
by Invitrogen Life Technologies (Paisley, UK).

3.4.1 PCR of HPRT housekeeping gene

Hypoxanthine phosphoribosyltransferase (HPRT) is a soluble
cytoplasmic enzyme that catalyses the conversion of prefo
and hypoxanthine to guanylic and inosinic acids in the pres
pyrophosphate (PRPP) (Patterson, 1986). The promoter of the human gene res
those of other characterized housekeeping genes and the
is constitutively expressed (Patterson, 1986). The entire gene is 42 kilobase
in length and is organized into 9 exons and 8 introns (Kim et al, 1988).

In order to assess the integrity of the RNA extracted from the
and the tissue culture cells each sample was amplified by reverse trans
the HPRT housekeeping gene. Samples positive for HPRT w
for *Ha-ras* and *hprt* mutation.

3.4.1.1

HPRT primers

The forward primer, HPRT-8, 5'-GCTTTCCTTGGTCAGGCAGTATAATCCAAAGATGGTCAA-3', located in exon 7, begins at map position 604 (Jolly et al., 1983) of the HPRT gene reverse primer, HPRT-4, 5'-CAAGGGCATATCCTACAACAAACTTGTCTGGTTATATTCCA-3', begins at map position 103 on the reverse cDNA strand and is located in e

3.4.1.2

HPRT PCR amplification conditions

20ml of cDNA product was amplified in a 50ml volume containing 1 Taq polymerase (Gibco BRL, Life Technologies, Inc.); 1mM primers, HPRT-8 and primer HPRT-4; 5ml of 10 x PCR buffer [200mM Tris-HCl (pH 8.4); 500mM KCl and 1.5mM final concentration of $MgCl_2$]. Nucleotides were added as reaction was performed for 30 cycles in a programmable (Hybaid, Middlesex, UK). Initial heat denaturation at 96°C for 10 min, a program of 30 cycles of 94°C for 1 min, 56°C for 30 seconds, 72°C for 1 min 30 seconds and a final extension period was for 10 min at 72°C.

3.4.1.3 Visualization of HPRT PCR product

The PCR product was visualized by running on a 2% agaros
ethidium bromide (10mg/ml) in TAE buffer at pH 8.0. Volta
hour. 50bp molecular weight ladder (Promega, Madison,
simultaneously and the gel was viewed on an ultraviolet light box and
photographed. The HPRT PCR product was viewed as a ban
(Figure 1.6).

3.4.2 Nested and Touchdown PCR

In situations where there are problems with the quantity an
to be amplified one approach to amplifying a product reliably is
PCR. Generally, the sample is first amplified using 20-30 cycles of PCR
primer set. A very small aliquot of this reaction is then a
using an inner primer set. The inner set of primers is targeted within the
DNA so that the complementary sequence for the inner pri
the PCR product obtained in the first PCR reaction.

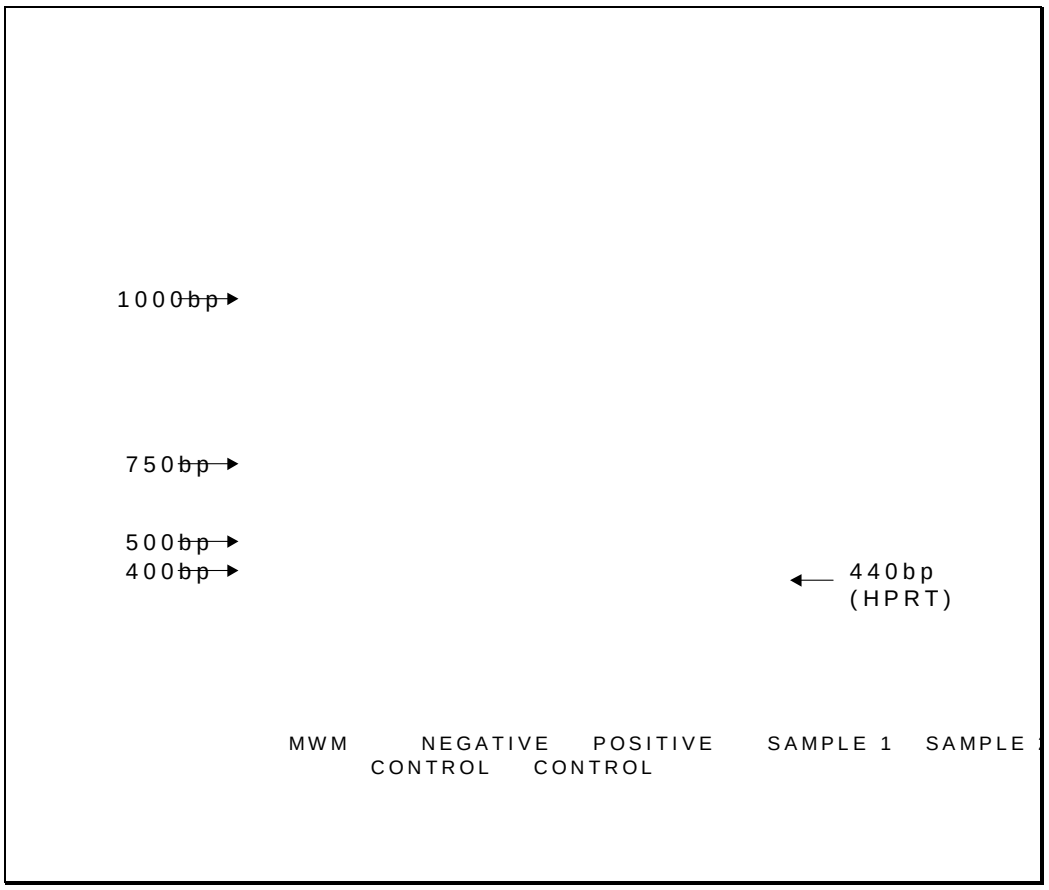


Figure 6 Visualization of HPRT products on an agarose gel

50bp ladder (Promega, SA) (lane 1); negative control (lan
HPRT 440bp PCR product 3 (lane 5)

Nested PCR is extremely sensitive and as the oocyte micro sam
small, and thus the RNA obtained from them also minimal,
ERα and ERβ were designed in order to be able to PCR the var
less material.

Artefacts may occur where incompletely extended produc
rehybridize to an adjacent segment of DNA to prime an uni
sequence internal to one or both primers will still be prese
will differ. The appearance of spurious smaller bands in th
to mispriming by one or both of the oligonucleotide amplimers f
the reaction products and may be due to an advantage that
products enjoy over the longer correct product during re
solution, which takes advantage of the exponential nature of PCR re
touchdown PCR. Using this technique the annealing tempe
is decreased 2°C every second cycle from 65°C to touchdown at a rele
annealing temperature. Any difference in the incorrect and incor
annealing will give an advantage to the correct product
al, 1991).

3.4.2.1 First Round Nested PCR Page of the ER

The first round of nested PCR of the ER was divided into two ar
namely the DNA binding domain (DBD) and the ligand binding domain
(LBD). The DBD incorporated exons 2 to 6 and the LBD incorporate

exons 3 to 8 (Figure 17). In addition, touchdown PCR and an Enhancer s
(Gibco BRL, Life Technologies, Paisley, UK) was used for efficient amplific
the problematic template.

PCR parameters for first round nested PCR of the ER in Tab
1.

3.4.2.1.1 ERα DNA Binding Domain PCR

The sense primer, ERA1373 (5'-TGAGCCTTTGC-3'), corresponds to bases 1301-1313 in exon 1 of the ER (Green et al 1986). ERA1484R (5'-CATTTTCCCTGGGTTC-3') represents the antisense strand of the ER DNA bases 1484-1467 in exon 6 of the ER (Figure 17).

20 µl of cDNA was amplified in a volume containing 1 µl primer ERA1373 (3 µM), 1 µl primer ERA1484R (3 µM), 5 µl 10x PCR buffer (200 mM Tris 8.4]; 500 mM KCl); 0.2 mM dNTPs; 1.5 mM $MgCl_2$; 1x PCR Enhancer Solution (G BRL). No additional dNTPs were added and touchdown PCR p programmable DNA Thermal Cycler (Hybaid, Middlesex, UK).

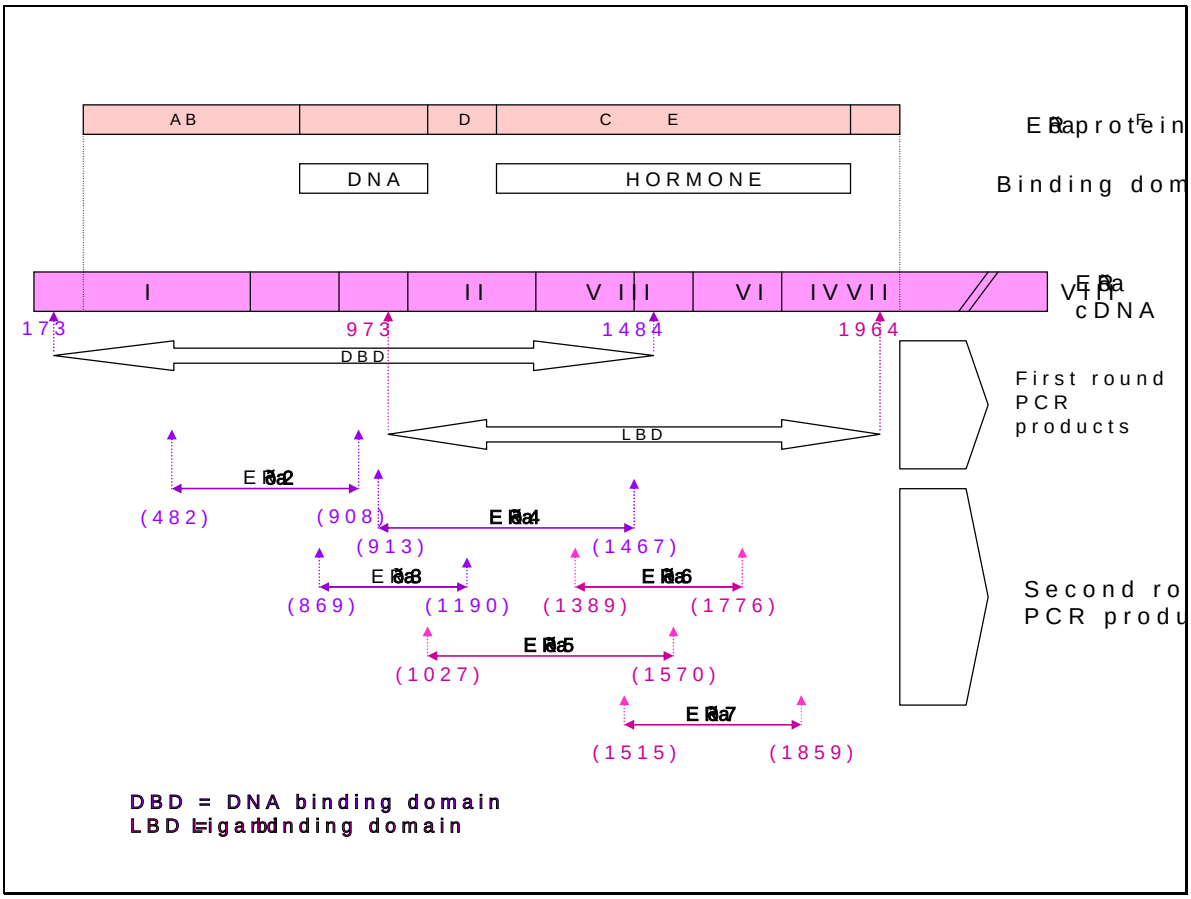


Figure 1. Positions of nested PCR primers (according to Green 1986).

After an initial heat denaturation (94°C for 10 min) the annealing temperatu
of 65°C for 1 min 1 Taq polymerase (Gibco BRL, Life Technolo
was added and the reaction allowed to extend (72°C for 2 min). Cycles consis
of denaturation (94°C for 1 min, annealing for 1 min (where th
temperature was decreased by 2°C every 2 cycles starting from 6
extension (72°C for 2 min were carried out until the anneali
reached 55°C. A further 17 cycles were carried out with an anne
of 55°C and a final extension period of 10 min was performed.

The resulting PCR product of 1312bp was then used in seco
of exons 2, 3, 4, 5, 6, 7, 8 and 9 of ERα as in 3.4.2.2.1. to 3.4.2.2.3.

3.4.2.1.2

ERα Ligand Binding Domain PCR

The sense primer ERA973 (5'-ATGGGAATGATGAAAGG
corresponds to bases 973-990 in exon 3 of the ER (Figure 17). ERA1964
(5'-AATGCGATGAAGTAAGC) represents the antisense strand of the ER
cDNA bases 1964-1947 in exon 8 of the ER (Figure 17).

20ng of cDNA was amplified in a 50µl reaction volume containing 1
primer ERA973 and primer ERA1964 and 3µl 10 x PCR buffer (200mM
HCl [pH 8.4]; 500 mM KCl). Next 1.5mM $MgCl_2$ and 1 unit Taq DNA
was added and the reaction was performed in a DNA Thermal Cycler (Hyba

Middlesex, UK). After an initial heaC dfenrat1.0 a min in the 9
temperature was reduced to an annealiCng fotemmpierabtuerfeo fth4e7
addition of Taq polymerase (Gibco BRL, UK). An extension ti
72C was performed and reaction was allowed toC rfuonr for 3
1 min, 94C for 1 min and 72C for 2 min. A final extension of 72 was
then performed.

The resulting 922bp PCR product was used in the second round PCR of
exons 5, 6 and 7 as in 3.4.2.2.4. to 3.4.2.2.6.

3.4.2.2.

Second Round Nested PCR of the ER

ER exons 2, 3 and 4 utilized the first round DBD PCR produ
second round PCR (Table 2). The first round DBD PCR pr
template for second round PCR of ER exons 5, 6 and 7 (Table 2, Figure
second round PCR products were as described in 3.4.2.2.5. The
viewed were excised and purified using the freeze method (3.6) to
quantitation (3.7) and sequenced in order to confirm the nature of the pr
observed (3.8).

Table 1. PCR parameters for nested RT-PCR and EMSA.

Primer Name	Primer seq 5 >>3	Primer positio	$MgCl_2$ conc (mM)*	Ann. Temp (°C)	ER detecte	area regio detecte	Produc size (v (bp)
ERA173	GGTTTCTG -CCTTCT	173-190	0.78	TD 65-55	ERα	DBD	1312
ERA148	CACATTTT -CTGGTTC	146-84					
ERA973	AGTGGGAA -ATGAAAG	973-990	-	47	ERα	LBD	922
ERA196	AATGCGAT -AGTAGA	194-64					

TD = Touchdown

* $MgCl_2$ concentration in addition to that already present from

In each case, if the original sample size was too small and
product was observed after electrophoresis, the second
repeated as described replacing the first round product wit
product.

3.4.2.2.1

PCR of the ERα exon 2

The sense primer, ERA-748F (5' GAGGCTGCCGGCTC 3'), corresponds to bases -448 to -432 in exon 1 of the ERα gene. The ERα908-R (5' GGTTGGTGGGGTACAC 3') represents the antisense strand of the ERα cDNA bases 908 to 891 in exon 3 of the ERα gene (Figure 17; Table 2).

5 µl of first round DNA ERα PCR product was amplified in a 50 µl reaction volume containing 10 mM primer ERA-748F, 10 mM primer ERA908R, 5 x PCR buffer (Tris-HCL, KCL_2, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7]; Qiagen, Germany); 200 µM dNTP mix; 10 x solution Q (Qiagen, Germany) and 1 Unit Taq DNA polymerase (Qiagen, Germany). The reaction was performed in a DNA Thermocycler (Hybaid, Middlesex, UK) with an initial heat denaturation temperature of 95°C for 15 min followed by 30 cycles of 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension time of 10 min at 72°C was then performed.

The expected PCR product size was 427 base pairs (bp). A
would reveal a PCR product of 236 bp.

3.4.2.2.2

PCR of the ERα Exon 3

The sense ERα primer, ERA8-69F (5'-AAGGACATAA-3'), corresponds to bases 869 in exon 2 of the ERα gene. ERA119-0R (5'-ACAAGGCACTGAC-3') represents the antisense strand of the DNA bases 1-1190 in exon 4 of the ERα gene (Figure 17; Table 2).

2 µl of first round DNA PCR product was amplified in a 50 µl reaction volume containing 1 µM primer ERA869F, 1 µM primer ERA1190R, 5.0 x PCR buffer (Qiagen, Germany); 2.5 mM MgCl2; 5.0 x solution Q (Qiagen, Germany) and 1 Unit HotStar DNA polymerase (Qiagen, Germany). The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 10 min was then performed.

The expected PCR product size was 322 bp. (ERα ±Δ3) Absence would produce a band 205 bp in size.

3.4.2.2.3

PCR of ERα exon 4

The sense ERα primer, ERA9-13AC (5' ATTGATAAAA-3'), corresponds to bases 913-932 of the ERα gene. ERA146-7R (5' CTGTCCAAGAGCAA-3') represents the antisense strand of the ERα cDNA bases 1146-79 in exon 5 of the ER (Figure 17; Table 2).

2µl of first round ERα PCR product was amplified in a 50µl reaction volume containing 10µM primer ERA913-32, primer ERA146-7R, 5µl 10 x PCR buffer (Qiagen, Germany); 2.5mM MgCl, 200µM dNTP mix, 5µl 10x solution Q (Qiagen, Germany) and 1 Unit Taq DNA polymerase (Qiagen, Germany). The reaction was performed in a programmable cycler (Hybaid, Middlesex, UK) with an initial denaturation at 95°C for 15 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. The PCR program was finished with an extension time of 10 min at 72°C.

The size of the expected PCR product was 555bp and a c
exon 4 (ER+4) was expected at 219bp.

Table 2. PCR parameters for second round nested ER domain-RT-PCR analysis.

Primer Name	Primer seq 5 >>3	Primer Positio	Ann. Temp (°C)	ERα region detect	Produ size (wt) (bp)	Product size (variant (bp)
ERA482	TCTGAGGC -CGGCGT	482-498	55	ERα2	427	236
ERA908	GGTTGGTG -TGGAACCA	891-908				
ERA869	ATTCAAGG -ATAACGA	869-887	51	ERα3	322	205
ERA119	ACAAGGCA -GACCATC	117-1319				
ERA913	CACCATTG -AAAAACAG	913-931	51	ERα4	555	219
ERA146	CTGTCCAA -GCAAGTTA	144-1467				

3.4.2.2.4

PCR of the ERα exon 5

The sense ERα 5 primer, ERA1027F (5'-CACAAGCGCCAGGAGAG-3'), is located between bases 1027 and 1046 in the gene. The three ERα exon 4 primer ERA1570R (5'-AACTCCTCTCCCTGCCA-3') corresponds to bases 1551 to 1570 in exon 6 on the strand of the ERα gene (Figure 17; Table 3).

3 µl of first round ERα PCR product was amplified in a 50 µl reaction volume containing 1 µM primer ERA1027F, 1 µM primer ERA1570R, 1 x PCR buffer (Qiagen, Germany), 1.25 mM MgCl$_2$, 200 µM dNTP mix, 1 x solution Q (Qiagen, Germany) and 1 Unit HotStar Taq DNA polymerase. The reaction was performed in a programmable DNA thermal Cycler (Hybaid, Middlesex). A denaturation at 95°C for 15 min. This was followed by 30 cycles of 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 10 min completed the program.

The ERα exon 5 PCR product size was 554 bp and would reveal a product of 405 bp.

3.4.2.2.5

PCR of the ERα exon 6

The sense ERα6 primer, ERA1389 (5'-ATGATTGGTCTCTCGTCT-3'), is located between bases 1389 and 1406 in exon 6 of the ERα gene (EMBL accession number X03635). The antisense primer ERA1776 (5'-CTCATGTGGGAGAGGTA-3') corresponds to bases 1759 to 1776 in the antisense strand of the DNA (Figure 17; Table 3).

3 µl of first round RT-PCR product was amplified in a total volume of 50 µl containing 1 µM primer ERA1389, 1 µM primer ERA1776, 5 µl of a 10 x PCR buffer (Qiagen, Germany), 2.5 mM $MgCl_2$, 5 µl of 10 x solution Q (Qiagen, Germany) and 1 U HotStarTaq DNA polymerase (Qiagen, Germany). The PCR was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min. A final extension time of 10 min at 72°C was subsequently performed.

The size of the expected PCR product was 388 bp. An exon 6 deleted a PCR product of 254 bp.

3.4.2.2.6

PCR of ðaER exon 7

The senseðaERprimer, ERA1-5'-TTCCT(G5'-GCTACATCATCTCG-3'), is located between bases 1515 to 1533ðaigene exon ERA1 of the ER (ER TCTCCAGCAGCAGG3'). ACTOR corresponds to the cDNA bases 1841 exon 8 of the same strand of the ER (Figure 17; Table 3).

3ðm of first round PCR product was amplified in a volume containing 1ðM primer ERA15, 1ðM primer ERA18, 5ðl of a 10 x PCR buffer (Qiagen, Germany), 2.5ðM MgCl2, 5ðl 10x solution Q (Qiagen, Germany) and 1ðU HotStar Taq DNA polymerase (Qiagen, Germa The PCR reaction was performed on a programmable DN (Hybaid, Middlesex, UK) with an initial denaturation at 95ðC for 15 min, was followed by 30 cycles of denaturation at 94ðC for 1 min, annealing at 64ðC for 1 min and extension at 72ðC for 2 min. A final extension time of 10 was then performed.

The size of the expected PCR product was 345 bp, A comp ER±"7, would yield a band 161 bp in size.

Table 3. PCR parameters for secondary oligonucleotides targeting ER domain-RT-PCR analysis.

Primer Name	Primer seq. 5 >>3	Primer Position	Ann. Temp (°C)	ERα region detect	Product size (wt) (bp)	Product size (variant) (bp)
ERA102	GAAACACAAG -CAGAG	102-1046	61	ERα5	543	405
ERA157	CACAAACTCC -CCTGC	155-1570				
ERA138	TCCTGATGAT -CTCGTC	138-1409	59	ERα6	388	254
ERA177	CTGATGTGGG -GGAT	175-1776				
ERA1515	TGCTGGCTAC -ATCTC	151-1533	57	ERα7	345	161
ERA185	TCTCCAGCAG -GTCAT	184-1859				

3.4.2.3

First Round Nested PCR of ER

The first round of nested PCR involved the PCR of a fragment of the ER gene located between exon 2 and exon 7. Great care was taken in designing primers for this PCR in such a way as to exclude co-amplification of ER pseudogene. The first round PCR was used to isolate ER.

The sense primer, ERB5-428 (5'-GCATGCTCACTTTCTGC-3'), is located between bases 528 and 544 (Wang et al., 1990) within exon 2 of the ER gene (Figure 18). The anti-sense primer ERB1-481R (5'-GAGCATCAGGAGGTTAGC-3') corresponds to bases 1464 to 1481 in the reverse strand of the ER.

20ng cDNA was amplified in a reaction volume containing 1 ERB528 primer, 1 primer ERB1481R; 3.5 PCR buffer (200mM Tris 8.4]; 500mM KCl) and 0.75mM MgCl. Additional dNTPs were added reaction. PCR was performed in a programmable DNA Ther Middlesex, UK). After an initial heat denaturation at 96 annealing temperature of 1 min 1.5 Units of polymerase (Invitrogen, UK) were added and the reaction allowed to ex This was followed by 25 cycles of denaturation at annealing 5 for 1 min extension at 72 min. A final extension time o 72 completed the reaction.

The resulting PCR product of 954 bp was used in second ro

DNA and ligand binding domains of ER

3.4.2.4

Second Round Nested PCR of ER gene

The second round of nested PCR of the ER gene utilized the first round

product as a template. One primer set was designed in orde

the ER DNA binding domain between bases 592 and 650 of th

exons 2 and 4 respectively) and one primer set was designed in o

segment of the ER binding domain between bases 1196 an

ER gene (ER exons 6 and 7 respectively) (Figure 18).

3.4.2.4.1

PCR of ER DNA binding domain

The sense ER DNA primer, ERB592 -(5' GTGTGAAGGATGTAAGG

corresponds to bases 592 to 609 in the ER gene. 2 ER DNA antisense primer (ER

TCGGCACTTCTCTG3') corresponds to the complementary strand of the DNA ER

bases 788-805 in exon 4 of the ER gene (Figure 18).

Table 4. PCR parameters for first and second round ERβ RT-PCR analysis.

Primer N	Primer seq. 5 >>3	Primer Positio	Ann. Temp (°C)	Area detected	Produc size (wt) (bp)
ERB528 ERB1481	AGGGATGCTCA -TCTGC GAGCATCAGGA -TTAGC	528-544 1464-1481	51	ERβ1 First rou	954
ERB592 ERB805R	CGTGTGAAGGA -TAAG TCGGCACTTCT -TCT	592-609 788-805	52	ERβ DBD	214
ERB1196 ERB1417	GGGGAAATGC -GAAG ACCCAAACCAA -CATC	1196-1211 1401-1417	51	ERβ LBD	222

5µl of first round PCR product was amplified in a 50µl volume containing 1µM primer ERBB2; primer ERBB5; 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl), 2mM MgCl2 and 200µM dNTP mix. The reaction was performed in a programmable DNA Therma Middlesex, UK). After an initial heat denaturation at 94°C for 1.0 min the temperature was reduced to an annealing temperature of 52°C for 1 min before the addition of 1µl Taq polymerase (Invitrogen, UK). An extension at 72°C was performed and the reaction was allowed to run for 1 min at 52°C, 1 min at 72°C and 1 min at 94°C. A final extension of 5 min at 72°C was then performed.

As the ERBB2 signal was expected to be very low and due to the s ERBB2 DBD PCR was repeated as described using 5µl of the first round PCR product as template in a 50µl reaction. 5µl of the second round product was run on a 2% agarose gel in 1 x TAE buffer as described in 3.5. The expected PCR product was 219bp. The bands were excised, purified (3.6), quantitated (3.7) and sequenced

3.4.2.4.2 PCR of ERBB2 ligand binding domain

The sense ERBB2 primer, ERB1196 (5'-AATGCGTAGAAGG corresponds to bases 1196 to 1213 of the human ERBB2 gene (Fig 1.17). ERB1417 (5'-ACCCAAACCAAAGCATCC) represents bases 1400 to 1417 in ex antisense strand of the ERBB2 gene (Figure 18).

5ng of surrounding PCR product was amplified in a 50µl volume containing 1µM primer ERBB1M96F, 1µM primer ERB1417R, 1 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl), and 200µM dNTP mix. The reaction was performed in a ProFlex Thermocycler (Hybaid, Middlesex, UK). After an initial denaturation at 96°C for 10 min the temperature was reduced to 80°C and 1 Unit of Taq DNA polymerase (Invitrogen, UK) added to the mixture. After an extension time of 2 min at 72°C the reaction was run for 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min. A final extension at 72°C for 5 min was then performed.

Due to the small sample sizes and the low yield of ERBB1 PCR product, a second round of PCR was repeated as described for the first round using 5µl of the first round PCR product. The expected size of the final PCR product was 172bp. The PCR product was visualized by running on a 2% agarose gel in 1 x TAE buffer. The bands were excised and purified (3.6) quantitated (3.7) and sequenced (3.8).

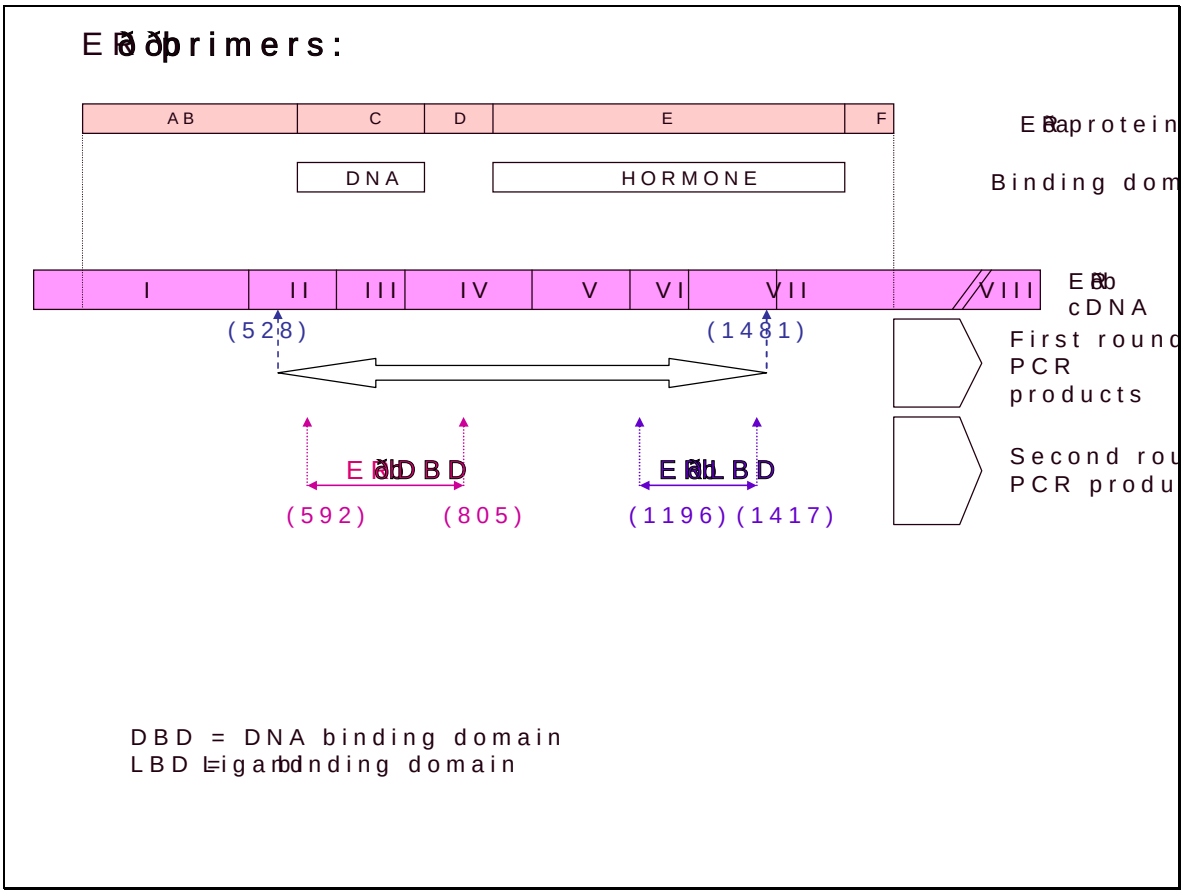


Figure 18. Positions of the ER PCR primers (according to Ogawa 1998a and Eltom 1997).

3.5 Agarose gel electrophoresis

Molecular Grade Agarose was obtained from Pronadisa (Madrid bromophenol blue and other chemicals from Merck (Dar unless otherwise stated.

In order to visualize the PCR products, loading buffer (5g sucrose; 5 EDTA; 10g Bromophenol Blue; 1g Ficoll Type 400) was added to each PCR product and PCR products were run on 2% agarose gels in 1 (Appendix II) containing 1µg/ml ethidium bromide (Sigma, Germany) a hours, viewed on a UV transilluminator (Spectroline, New York, USA photographed using a polaroid CU5 Land Camera with a 5 weight markers run simultaneously were either the 100bp m (Promega, Madison, USA) or MWM XIII (Roche, SA).

3.6 PCR product purification: Phenol-freeze method

The gel band was cut out of the gel and sliced finely wit agarose slices were placed into an Eppendorf tube and 0 phenol (ICN Biomedicals Inc., Ohio, USA) was added. The all the agarose was in solution (occasionally this also req assist the melting of the agarose). The mixture was frozen ra

in an ethanol/ice bath for 2 min and centrifuged at 14000 rp
(Eppendorf, Hamburg, Germany) for 5 min. The freezing a
repeated twice after which and aqueous phase was collecte
fresh Eppendorf tube. An equal volume of chloroform: is
(Sigma Chemical Corporation, St Louis, USA) was added to the
mixed and centrifuged at 14000 rpm for 5 min. The resulta
transferred into a fresh Eppendorf tube and one tenth of t
acetate [pH 5.2] (Appendix 1) and two volumes of absolute ethanol were ad
mixed. This mixture was stored overnight at -20°C to precipitate the cDN
mixture was centrifuged at 14000 rpm in a microfuge for 15 min at 4°C. The
resulting precipitate was washed in 70% ethanol, centrifuged for 5 min. The
pellet was dried and resuspended in 12 µl sterile water. The cDNA was qua
as described in 3.7 prior to sequencing (3.8).

3.7 Ethidium Bromide Dot Quantitation of cDNA

This method was adapted from Lin and Wu (1991).

DNA standards were prepared using DNA (Roche Diagnostics, Randl
South Africa) in sterile water at the following concentrations: 1ng/µl, 2ng/µl,
5ng/µl, 10ng/µl, 20ng/µl, 50ng/µl and 100ng/µl.

2µl of a 10ng/µl solution of ethidium bromide (Sigma, USA) was added to 2
each standard DNA solution and of each cDNA sample of unkn

concentrations respectively, mixed well. The standard and
DNA/ethidium bromide solutions were spotted onto plastic
transilluminator and photographed (Figure 19). The unknown c
were estimated by comparison to the fluorescence of the st

3.8 Sequencing of PCR products

50 to 100ng of each cDNA sample was added to 3.3pmol/µl primer w
provided by the Division of Human Genetics at the South African
Laboratory Services and the samples were sequenced with a B
Terminator Sequencing Kit (Applied Biosystems, Foster C
programmable DNA Thermal Cycler with a hot start of 96°C for 1
min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min with a
final holding temperature of 4°C. The sequencing reactions were performe
plate and were subsequently cleaned by ethanol precipita
sequencing precipitates were resuspended in 8µl of loa
deionized formamide to 1 part 25mM EDTA pH 8.0 with 50 mg/ml blue de
The samples were sealed, heated at 92°C for 2 min and placed on ice
electrophoresis on an ABI377 automated sequencer (Ap
Results were provided as spectrographs using GeneScan® Analysis software
(Appendices IV and V)

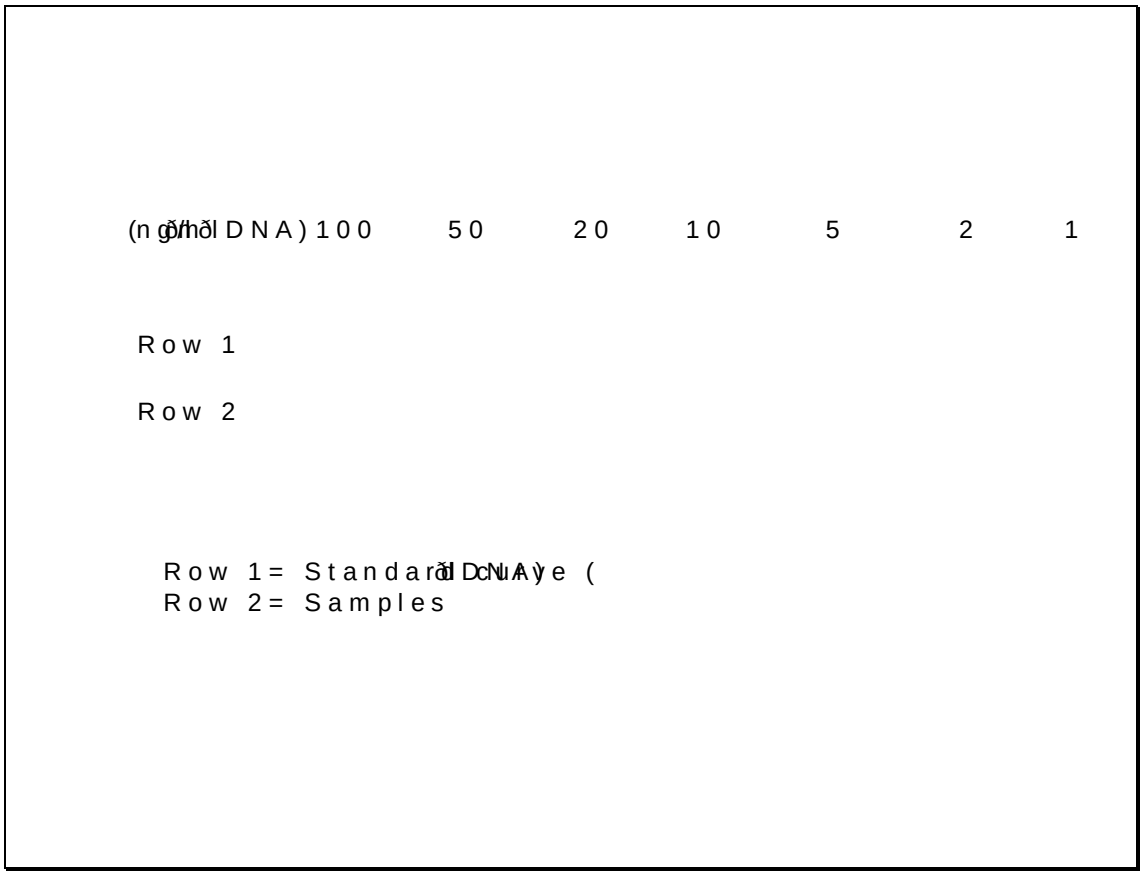


Figure 19. Ethidium Bromide dot quantitation of cleaned PC

The concentration (ng/ to the standard curve (Row 1

3.9

Cloning of T-47D cell line

T-47D breast duct carcinoma cells were obtained from the European Culture Collection (ECACC) and were grown in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, Life Technologies, UK) supplemented with 2mM-L-glutamine, 0.2 I.U (1.0ng/ml) Humulin N, Eli Lilly, Isando, South Africa), 1mM Sodium Pyruvate (Gibco BRL), 100 I.U Penicillin/100µg/ml Streptomycin (Gibco BRL) and FCS 10%. All media were phenol red free due to the oestrogenic effects of phenol red (Berthois et al., 1986).

Clones of the T-47D line were derived by using a modified limiting dilution technique. Exponentially growing cultures were harvested, counted, diluted, and plated into Nunclon (Nunc Intermediate, Denmark) 96 well plates at 1 cell/well/100µl. Each well was examined under phase contrast microscopy and any well not containing 1 cell was excluded. After 3 weeks, colonies were harvested and transferred into Nunclon 24 well plates and after 12 weeks 13 clones were established and managed to produce sufficient cells for cryopreservation of stock vials. After 20 passages 5 clones were retained for further study, the remainder discarded on the basis of poor proliferation, fungal or bacterial contamination.

PCR for ERα and ERβ were performed as previously described and proliferation response tests were performed on the 5 clones as described

results of these investigations two clones were selected fo
TCA3 and TCC1.

3.10

Hormone response test

Hormone response tests were performed every 24 hours (up
the parent clones and on each clone, 10 passages later, u
and after exposure to 10^{-12} - 10^{-8} M 17β-oestradiol, 10^{-8} M tamoxifen, 10^{-8}
aminoglutethimide and 10^{-8} M oestenedione, 10^{-8} M oestriol and 10^{-8} M cholesterol
separately. 17β-oestradiol and 4-hydroxytamoxifen were obtained from S
Chemical Co. (St Louis, MO, USA) and were of tissue culture grade. The solvent in
concentrations of methanol in the cultures were less than 0

Cells were harvested by trypsinization and washed in RPMI
phenol red, FCS and 1×10^4 cells were seeded into each well of a 96
plate containing 0.1 ml indicator free RPMI 1640 medium and 10%
(SFCS) (Appendix III) for each time frame (ie. 0h, 24h, 48h,
after plating (time 0h) medium was decanted off from each w
indicator free medium containing 10% SFCS was added to each well. Th
control wells/clone/plate received only 0.1% of the ve
wells/clone/plate were supplemented with 10^{-6} M 4-hydroxytamoxifen (for 48
hours) after which 3 of these wells were replaced with med
10^{-8} M 17β-oestradiol for a further 48 hours; 6 wells were suppl

17β-oestradiol (for 48 hours) after which 3 of these wells we

supplemented with 10^{-6}M 4-hydroxytamoxifen for a further 48 hours.

At each time frame number was determined using the MTT

described in 3.10.1. A standard curve for each clone was r

the MTT assay performed at time 0h.

3.10.1 MTT assay

This assay involves the ability of viable cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a soluble tetrazolium salt, into a purple formazan precipitate (Mosmann, 1983). The resulting purple formazan crystals are dissolved in an organic solvent such as dimethyl sulfoxide and the optical density of the solution may be measured using a spectrophotometer (ELISA plate reader) (Twentyman and Luxton, 1990).

The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the dimethyl sulfoxide (DMSO) were both obtained from Sigma (Poole, Dorset). Optical density measurements were carried out using a Titertek multiscan reader (ELISA reader) (Flow Laboratories, Rickmansworth, Herts) with a 570nm filter.

Cells were grown in 96-well plates (Nunc, Roskilde, Denmark) in volumes of 200µl of medium per well as described previously in 3.10. The

in PBS (Appendix II) at a concentration of 5 mg/ml and filter
(Millipore, Bedford, MA, USA) was added to each well and incubated at
37°C in a CO_2 incubator for 4 hours. The medium was then car
from the wells so as not to disturb the formazan crystals. 2
to each well and the plates were agitated on a plate sha
reading the optical density at 540nm on a Titertek Multi
standard curve was prepared for each cell clone and cell
as a comparison between the sample and standard optical
cases 3 replicates were used to determine each point.

3.11 Staining of slides

Cells were grown on slides by plating into Nunc slide flask
relevant medium. The slide flasks were incubated at 37°C in a CO_2
cell growth was stopped. The slide flasks were removed from the
media discarded and the slides were gently prised off the
by placing into absolute methanol for 5 min. Slides were
Grunwald's stain (BDH Chemicals, Poole, England) (Appendix III) for
Giemsa stain (diluted 1:10) (BDH Chemicals Ltd) (Appendix
in distilled water twice and mounted using solubilized
Chemicals Ltd). Slides were viewed and photographed using a 40x
2 MOT microscope (Carl Zeiss, Jena, Germany).