CHAPTER FOUR

RESULTS

4.1

Analysis of clinical data

4.1.1

Patient population

Breast fine needle aspirates (FNAs) were obtained over a period of 5 years from 61 patients with confirmed breast carcinoma. Patient control samples were obtained from 13 patients undergoing breast reduction mammoplasty. The characteristics with respect to age (Figure 20), menopausal status, clinical stage (Figure 21), tumour size, nodal involvement and presence of metastases (TNM) (Figure 22), hormone treatment and ER status of the breast carcinoma patients are listed in Table 5. In 58 cases (95%) patients presented with infiltrated duct carcinoma while 3 (5%) were diagnosed with lobular carcinoma of the breast. Information on tamoxifen treatment was available for 18 patients of which 72% (13/18) responded favourably. At the end of the study only two patients (3%) had died from the disease.

	Characteristic	Frequency (%)
Patients	Total number	61
	Age in years	
	Mean	52.9
	Range	25-87
	Menopausal status	
	Premenopausal	27
	Postmenopausal	34
	•	
Tumours	Size (cm)	
	T1 (<=2)	4(6)
	T2 (2-5)	18(30)
	T3 (>5)	14(23)
	T4	23(38)
	Unknown	2(3)
	Nodal status	
	NO	20(33)
	N1	19(32)
	N2	18(31)
	Unknowm	3(4)
	Metastases	
	MO	43(71)
	M1	17(28)
	Unknown	1(1)
	Histology	
	Infiltrating duct	58(95)
	Lobular	3(5)
	Stage	
		4(7)
	II	16(26)
	III	22(36)
	IV	18(30)
	Unknown	1(1)
Hormone treatment	Yes	22(36)
	No	39(64)
ERICA	Positive	26(43)
	Negative	24(39)
	Unknown	11(18)

Table 5. Characteristics of Patients, Tumours and Treatment.



Figure 20. Distribution of Age of sample breast carcinoma patients.



Figure 21. Histogram of distribution of clinical stage of disease in sample breast carcinoma patients.



Figure 22. Histograms of distribution of (A) tumour size, (B) nodal involvement and (C) presence or absence of metastases in sample breast carcinoma patients.

All samples underwent RT-PCR and sequencing for the presence of both ER α and ER β variants and the data were analyzed statistically using Chi-square (X²) analyses and Fisher's exact tests. Kaplan-Meier survival analysis was performed on 33 patients.

4.1.2

Nested PCR and sequence analysis of ERα exon variants in clinical samples

Breast tissue FNAs were reverse transcribed and PCRed for the HPRT housekeeping gene in order to assess the integrity of the RNA extracted prior to further investigation. A total of 61 patients with breast carcinoma and 13 patient control samples were further analyzed using RT-PCR for each of the ER α exons and sequencing of each PCR product was performed as described in Chapter 3.

4.1.2.1

Nested PCR and sequence analysis of ERα exon 2

PCR of ER α exon 2 revealed two bands on agarose gel electrophoresis (Figure 23). Each of these bands was excised, purified and sequenced using automatic sequencing (Appendix IV-Spectrographs A1and A2). Sequence analysis revealed the presence of the complete exon (Figure 24; Appendix VI – A1) as well as the deletion of the entire exon 2 (exon Δ 2) (Figure 24; Appendix VI – A2). 11(18%) of breast carcinoma patients exhibited only the deletion variant, exon Δ 2, 7 (12%)

exhibited only the complete exon 2 and 43 (70%) exhibited both the presence and absence of exon 2 within the sample cells.

In the control samples 3 (23%) samples exhibited only the presence of the complete exon 2, 2 (15%) lacked exon 2 (ie. exhibited only exon Δ 2) and 8 (62%) showed both the presence and absence of exon 2 within their cells.

 X^2 analysis was performed on the results comparing control and diseased tissue. ER α 2 showed no significance with regard to tumour size (T) (Table 8), nodal involvement (N) (Table 9), presence or absence of metastases (M) (Table 10), stage of disease (Table 11) or menstrual status (Table 12). The presence of the complete ER α 2, however, showed significance (p<0.05) with respect to ERICA status (Table 13). Fisher's exact tests were also performed between exon and exon deletion results of diseased tissue. ER α 2 was significantly associated with the presence of ER α 4 (Fisher exact p=0.005) and the deletion variant ER α Δ5 (Fisher exact p=0.042) (Table 6). ER α Δ2 was only significantly associated with ER α Δ6 (Fisher exact p=0.007) (Table 7).

4.1.2.2

Nested PCR and sequence analysis of ERα exon 3

Only one patient (2%) exhibited two bands on agarose gel electrophoresis (Figure 25). The 205bp band includes the deletion of ER α exon 3 (ER $\alpha\Delta3$) as the expected size of exon 3 is 117bp.

108



Figure 23. ER α exon 2 agarose electrophoresis gel.

MWM XIII (Roche, SA) is a 50bp (base pair) molecular weight marker (lane 1). ER α 2 PCR product is represented by the 427bp band (lanes 3,5,6 and 7); ER α Δ 2 PCR product is represented by the 236bp band (lanes 3,5,6 and 7).

ERα	CAGACCGGCCTCCCCTACGGCCCCGGG <mark>TCTGAGGCTGCGGC</mark>	495
ERa ERa2-427bp ERa2-236bp	GTTCGGCTCCAACGGCCTGGGGGGGTTTCCCCCCACTCAACAG ACGGCCTGGGGGGGTTTCCCCCCACTCAACAG ACGGCCTGGGGGGGTTTCCCCCCACTCAACAG	537
ERα ERα2-427bp ERα2-236bp	CGTGTCTCCGAGCCCGCTGATGCTACTGCACCCGCCGCCGCA CGTGTCTCCGAGCCCGCTGATGCTACTGCACCCGCCGCCGCA CGTGTCTCCGAGCCCGCTGATGCTACTGCACCCGCCGCCGCA	579
ERα ERα2-427bp ERα2-236bp	GCTGTCGCCTTTCCTGCAGCCCACGGCCAGCAGGTGCCCTA GCTGTCGCCTTTCCTGCAGCCCCACGGCCAGCAGGTGCCCTA GCTGTCGCCTTTCCTGCAGCCCCACGGCCAGCAGGTGCCCTA	621
ERα ERα2-427bp ERα2-236bp	CTACCTGGAGAACGAGCCCAGCGGCTACACGGTGCGCGAGG CTACCTGGAGAACGAGCCCAGCGGCTACACGGTGCGCGAGG CTACCTGGAGAACGAGCCCAGCGGCTACACGGTGCGCGAGG	662
ERα ERα2-427bp ERα2-236bp	CCGGCCCGCCGGCATTCTACAG <mark>GCCAAATTCAGATAATCGAC</mark> CCGGCCCGCCGGCATTCTACAG <mark>GCCAAATTCAGATAATCGAC</mark> CCGGCCCGCCGGCATTCTACAG	704
ERα ERα2-427bp ERα2-236bp	GCCAGGGTGGCAGAGAAAGATTGGCCAGTACCAATGACAAG GCCAGGGTGGCAGAGAAAGATTGGCCAGTACCAATGACAAG	745
ERα ERα2-427bp ERα2-236bp	GGAAGTATGGCTATGGAATCTGCCAAGGAGACTCGCTACTGT GGAAGTATGGCTATGGAATCTGCCAAGGAGACTCGCTACTGT	787
ERα ERα2-427bp ERα2-236bp	GCAGTGTGCAATGACTATGCTTCAGGCTACCATTATGGAGTCT GCAGTGTGCAATGACTATGCTTCAGGCTACCATTATGGAGTCT	830
ERα ERα2-427bp ERα2-236bp	GGTCCTGTGAGGGCTGCAAGGCCTTCTTCAAGAGAAGTATTCA GGTCCTGTGAGGGCTGCAAGGCCTTCTTCAAGAGAAGTATTCA	873
ERα ERα2-427bp ERα2-236bp	AGGACATAACGACTATA <mark>TGTGTCCAGCCACCAACC</mark> AGTGCACC AGGACATAACGACTATA <mark>TGTGTCCAGCCA </mark>	916
Primer ERA4	82 Primer ERA908R ERα exon 2 deletion	

Figure 24. The gene alignment of ERα2 427bp and 236bp second round PCR products with the ERα gene.

The numbers on the right refer to the nucleotide number according to Green et

al, 1986. Exon boundaries according to Ponglikitmongkol *et al*, 1988.

Table 6. Statistical analyses of ER α 2 and significant ER exon

associations.

		ER	α2	% patients	Fisher exact p
		+	-		
	+	34	2	59.0	0.005
ERU4	-	16	9	41.0	0.005
FRaA5	+	32	3	57.4	0.042
	-	18	8	42.6	0.012
% patients +/- exon/exon deletion		82.0	18.0		

(+/- refers to the presence or absence of the exon or exon deletion).

Table 7. Statistical analysis of ER $\alpha\Delta 2$ and ER $\alpha\Delta 6$.

	ER	α∆2	% patients	Fisher exact p
	+	-		
	5	4	14.8	
+ ΕRαΔ6 -	49	3	85.2	0.007
% patients +/- exon/exon deletion	88.5	11.5		

(+/- refers to the presence or absence of the exon or exon deletion).

All patients and controls revealed a band of 322bp (Figure 26) which was excised, purified and sequenced using an automated sequencer. Sequencing revealed the presence of the complete ER α exon 3 (Appendix IV-Spectrograph B; Appendix VI – B1; Figure 26) within the sample tissues. The presence or rare absence of ER α 3 thus does not seem to play a role in breast tumourigenesis of the patients studied.

4.1.2.3

Nested PCR and sequence analysis of ERa exon 4

Nested PCR of ER α exon 4 revealed up to three bands on agarose gel electrophoresis (Figure 27). Sequence analysis of the excised and purified bands revealed that the 555bp band represented the entire ER α exon 4 (Appendix IV – Spectrograph C1; Appendix VI – C1; Figure 28). Sequencing of the 219bp band revealed the entire deletion of exon 4 (ER $\alpha\Delta4$) (Appendix IV- Spectrograph C2; Appendix VI – C2; Figure 28). A third band of approximately 425bp was observed, usually in conjunction with the 555bp band and was identified as an artifact (a random mixture of partial sequences of exon 4) by sequencing.



Figure 25. ERα exon 3 agarose electrophoresis gel.

MWMXIII (Roche, SA) is a 50bp molecular weight marker (Lane 7). ER α 3 PCR product is included in the 322bp band (Lane 5); ER $\alpha\Delta$ 3 PCR product is represented by the 205bp band (Lanes 2, 3 and 5).

ERα ERα3-322bp	GGGCTGCAAGGCCTTCTTCAAGAGAAGT <mark>ATTCAAG</mark> GACA	879			
ERα ERα3-322bp	TAACGACTATATGTGTCCAGCCACCAACCAGTGCACCAT ATATGTGTCCAGCCACCAACCAGTGCACCAT	918			
ERa ERa3-322bp	TGATAAAAACAGGAGGAAGAGCTGCCAGGCCTGCCGGC TGATAAAAACAGGAGGAAGAGCTGCCAGGCCTGCCGGC	956			
ERα ERα3-322bp	TCCGCAAATGCTACGAAGTGGGAATGATGAAAGGTGGGA TCCGCAAATGCTACGAAGTGGGAATGATGAAAGGTGGGA	995			
ERα ERα3-322bp	TACGAAAAGACCGAAGAGGAGGAGGAGAATGTTGAAACACA TACGAAAAGACCGAAGAGGAGGAGGAGAATGTTGAAACACA	1034			
ERα ERα3-322bp	AGCGCCAGAGAGATGATGGGGGAGGGCAGGGGTGAAGTG AGCGCCAGAGAGATGATGGGGGAGGGCAGGGGTGAAGTG	1072			
ERα ERα3-322bp	GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCC GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCC	1110			
ERα ERα3-322bp	AAGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCT AAGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCT	1149			
ERα ERα3-322bp	GGCCTTGTCCCTGACGGCCGACC <mark>AGATGGTCAGTGCCT</mark> GGCCTT	1187			
ERα	TGT TGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGA	1227			
Primer ERA	<mark>\869 Primer ERA1190R ERα exon 3</mark>				
Overlap of Primer ERA869 and ERa exon 3 position					

Figure 26. The gene alignment of ER α 3 322bp second round PCR product with the ER α gene.

The numbers on the right indicate the nucleotide numbers according to Green *et al*, 1986. Exon boundaries according to Ponglikitmongkol *et al*, 1988.

EXON		TUMOUR SIZE (T)					% EXON (+/-)	X ² P
		Т0	T1	T2	T3	T4		
ERα2	+	11	4	15	11	18	80.6	0.860
	-	2	0	3	3	5	19.4	
ERαΔ2	+	10	3	14	14	21	86.1	0.272
	-	3	1	4	0	2	13.9	
ERα3	+	13	4	18	14	23	100	
	-	0	0	0	0	0	0	
ERαΔ3	+	0	0	1	0	0	1.4	0.55
	-	13	4	17	14	23	98.6	
ERα4	+	3	4	10	9	12	52.8	0.062
	-	10	0	8	5	11	47.2	
ERα∆4	+	13	4	15	14	20	91.7	0.275
	-	0	0	3	0	3	8.3	
ERα5	+	13	4	18	14	23	100	
	-	0	0	0	0	0	0	
ERαΔ5	+	7	4	10	8	13	58.3	0.547
	-	6	0	8	6	10	41.7	
ERα∆5pl	us+	0	1	2	2	4	12.5	0.557
	-	13	3	16	12	19	87.5	
ERα6	+	13	3	18	14	23	98.6	0.002*
	-	0	1	0	0	0	1.4	0.40
ΕRαΔ6	+	0	0	3	1	4	11.1	0.43
	-	13	4	15	13	19	88.9	0.000
ERα/	+	12	4	10	11	21	80.6	0.026*
	-	1	0	8	3	2	19.4	0 507
ΕκάΔη	+		4	0			00.9	0.507
0/ potion	- to T	2	U 5.6	2	う 10.4	1	11.1	
% patien	ເຮັ	10.1	0.C	25.0	19.4	31.9		

Table 8. Associations between ER α exons and tumour size (T).

(T0 refers to normal breast tissue samples; +/- refers to the presence or absence of the exon or exon deletion; *indicates significance).

EXON		NODES			% EXON (+/-)	X ²
		0	1	2		,
ERα2	+	15	17	15	81.0	0.50
	-	5	2	4	19.0	0.50
ERαΔ2	+	18	15	18	87.9	
	-	2	4	1	12.1	0.308
ERα3	+	20	19	19	100	
	_	0	0	0	0	
ERαΔ3	+	1	0	0	1.7	
		10	10	10	08.3	0.380
FRα4	-+	19	19	19	60.3	
			10	10		0.609
	-	8	6	9	39.7	
ERαΔ4	+	19	17	16	89.7	0.542
	-	1	2	3	10.3	
ERα5	+	20	19	19	100	
	-	0	0	0	0	
ERαΔ5	+	12	10	12	58.6	0.705
	_	8	9	7	41.4	0.795
ERαΔ5pl	us+	2	3	4	15.5	
		10	16	15	84.5	0.635
ERα6	+	10	10	19	98.3	
_		4	0	0	4 7	0.380
EDave	-	<u> </u>	0	0	1./	
ЕКИДО	т	Z	4	2	13.0	0.534
	-	18	15	17	86.2	
ERα7	+	15	13	17	77.6	0.201
	-	5	6	2	22.4	0.201
ERαΔ7	+	19	15	18	89.7	
	-	1	4	1	10.3	0.174
% patient with node	ts es	34.5	32.8	32.8		

Table 9. Associations between ER α exons and Nodal involvement (N).

(+/- refers to the presence or absence of the exon or exon deletion).

EXC	DN	Δ	Λ	% EXON	\mathbf{v}^2
			1	(+/-)	× P
		MO	M1		
ERα2	+	38	11	81.7	0.070
	-	5	6	18.3	0.078
ERαΔ2	+	37	16	88.3	0.666
	-	6	1	11.7	
ERa3	+	43	17	100	
	-	0	0		
ERαΔ3	+	0	1	1.7	0.628
	-	43	16	98.3	
ERα4	+	31	4	58.3	0.002*
	-	12	13	41.7	
ΕΚαΔ4	+	37	17	90.0	0.252
	-	6	0	10.0	
ERα5	+	43	17	100	
EDa/6	-	0	0	50.2	
ERUDS	т	16	0	00.0 /1 7	0.410
EBa/5nl	-	6	9	41.7	
	-	37	13	83.3	0.608
ERα6	+	42	16	96.7	0.915
	-	1	1	3.3	
ERαΔ6	+	3	6	15.0	0.018*
	-	40	11	85.0	
ERα7	+	32	15	78.3	0.411
	-	11	2	21.7	
ΕΚαΔ/	+	39 4	15 2	90.0	0.849
% patier metast	- nts +/- ases	71.7	28.3	10.0	

Table 10. Associations between ERα exons and presence or absence of Metastases (M).

(+/- refers to the presence or absence of the exon or exon deletion;*indicates significance).

EXON			STA	% EXON	X ²		
		1	2	3	4	(+/-)	P
ERα2	+	4	14	19	12	81.7	0 232
	-	0	2	3	6	18.3	0.202
ERαΔ2	+	3	12	21	17	88.3	0 159
	-	1	4	1	1	11.7	0.100
ERα3	+	4	16	22	18	100	
	-	0	0	0	0	0	
ERαΔ3	+	0	0	0	1	1.7	
	-	4	16	22	17	98.3	0.5
ERα4	+	4	12	14	5	58.3	
	-	0	4	8	13	41.7	0.008*
ERαΔ4	+	4	14	19	17	90.0	0 735
	-	0	2	3	1	10.0	0.735
ERα5	+	4	16	22	18	100	
	-	0	0	0	0	0	
ERαΔ5	+	4	10	13	8	58.3	0.221
	-	0	6	9	10	41.7	•
ERα∆5pl	us+	3	15	18	14	83.3	0.596
	-	1	1	4	4	16.7	
ERα6	+	3	16	22	17	96.7	0.06
	-	1	0	0	1	3.3	
ERαΔ6	+	0	3	0	6	15.0	0.023*
	-	4	13	22	12	85.0	0.020
ERα7	+	4	11	16	16	78.3	
	-	0	5	2	2	21.7	0.313
ERαΔ7	+	4	14	20	16	90.0	
	-	0	2	2	2	10.0	0.896
% patient Stage	ts at	6.7	26.7	36.7	30.0		

Table 11. Associations between $\text{ER}\alpha$ exons and Stage of disease.

(+/- refers to the presence or absence of the exon or exon deletion; *indicates significance).

EXON		MENSTRU	AL STATUS	% EXON (+/-)	X ² P	
		Pre	Post	(17-)	,	
ERα2	+	22	28	82.0	0.805	
	-	5	6	18.0		
ERαΔ2	+	23	31	88.5	0.745	
	-	4	3	11.5		
ERa3	+	27	34	100		
	-	0	0			
ΕRαΔ3	+	1	0	1.6	0.907	
ERa/	- +	15	21	90.4 50.0		
		12	13	41.0	0.820	
FRαΛ4	+	25	30	90.2		
	-	20	00	00.2	0.893	
	-	2	4	9.8		
ERα5	+	27	34	100		
	-	0	0			
ERαΔ5	+	14	21	57.4	0.605	
	-	13	13	42.6		
ΕRαΔ5ρι	us+	3	/ 27	16.4	0.519	
EDa6	-	24	27	06.7		
	-	1	1	3.3	0.577	
FRαΛ6	+	6	3	14.8		
	-	21	31	85.2	0.270	
ERα7	+	20	28	78.7		
	-	7	6	21.3	0.639	
ERαΔ7	+	24	31	90.2		
	-	3	3	9.8	0.893	
% patie with sta	ents atus	44.3	55.7			

Table 12. Associations between ER α exons and menstrual status.

(+/- refers to the presence or absence of the exon or exon deletion).

EXC	EXON ER STATUS		% EXON (+/-)	X² P	
		Positive	Negative		
ERα2	+	26	17	86.0	0.01*
	-	0	7	14.0	
ERαΔ2	+	25	18	86.0	0.081
	-	1	6	14.0	
ERα3	+	26	24	100	
FD A O	-	0	0	-	
ΕRαΔ3	+	0	0		
	-	26	24	100	
ERα4	+	21	12	66.0	0.046*
	-	5	12	34.0	
ERα∆4	+	22	22	88.0	0.741
	-	4	2	12.0	
ERα5	+	26	24	100	
	-	0	0		
ERαΔ5	+	20	9	58.0	0.011*
	-	6	15	42.0	
ERα∆5pl	us+	3	3	12.0	0.741
	-	23	21	88.0	
ERα6	+	25	24	98.0	0.968
A	-	1	0	2.0	
ΕRαΔ6	+	1	5	12.0	0.158
ED~7	-	25 10	19	δδ.U 79.0	
	+	19	20	78.0	0.594
ED~^7	-	/ 24	4	22.0	
	Ŧ	24	20	00.U 12 0	0.589
% patien ERICA s	ts with status	52.0	48.0	12.0	

Table 13. Associations between ER α exons and ER status.

(+/- refers to the presence or absence of the exon or exon deletion; *indicates significance).

It was observed that 30 (49%) of breast carcinoma patients exhibited both the presence and absence of ER α exon 4. Only 6 (10%) showed the entire exon 4 alone and 25 (41%) exhibited ER $\alpha\Delta4$ alone. Among the control patients 3 (23%) had the complete ER α exon 4 combined with ER $\alpha\Delta4$ within their cells and 10 (77%) exhibited ER $\alpha\Delta4$ alone. None of the control patients exhibited the entire exon 4 alone and the presence of the 555bp band in all patients was always accompanied by the artifact band (425bp) on PCR.

 X^2 analyses performed on results comparing normal and diseased tissue indicate that ER α 4 is not significant with respect to tumour size (T) (Table 8), nodal involvement (N) (Table 9) and menstrual status (Table 12).

The presence of the complete ER α 4 exon, however, has significant associations with the presence of metastases (M) (*p*=0.002) (Table 10), stage of disease (*p*=0.008) (Table 11) and ERICA status (*p*=0.046) (Table 13). As already described, ER α 4 has a significant association with ER α 2 (Fisher exact *p*=0.005) (Table 6). Statistical significance was also observed between ER α 4 and ER α Δ 6 (Fisher exact *p*=0.025) (Table 14).



Figure 27. ER α exon 4 agarose electrophoresis gel.

100bp ladder (Promega, SA) (lane 1); ER α 4 PCR product is included in the 555bp band (lanes 2 and 5); ER α \Delta4 PCR product is represented by the 219bp band (lanes 2-5); the 425bp band (lanes 2 and 5) is a PCR artifact.

ERα ERα4-555bp ERα4-219bp	TATATGTGTCCAGCCACCAACCAGTG <mark>CACCATTGATAAAAACAGG</mark> AGG CCATTGATAAAAACAGGAGG CCATTGATAAAAACAGGAGG	934
ERα ERα4-555bp ERα4-219bp	AAGAGCTGCCAGGCCTGCCGGCTCCGCAAATGCTACGAAGTGGGAA AAGAGCTGCCAGGCCTGCCGGCTCCGCAAATGCTACGAAGTGGGAA AAGAGCTGCCAGGCCTGCCGGCTCCGCAAATGCTACGAAGTGGGAA	980
ERα ERα4-555bp ERα4-219bp	TGATGAAAGGTG <mark>GGATACGAAAAGACCGAAGAGGAGGAGGAGAATGTT</mark> TGATGAAAGGTG <mark>GGATACGAAAAGACCGAAGAGGAGGAGGAGAATGTT</mark> TGATGAAAGGTG	1026
ERα ERα4-555bp ERα4-219bp	GAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGT GAAACACAAGCGCCAGAGAGATGATGGGGGAGGGCAGGGGTGAAGT	1071
ERα ERα4-555bp ERα4-219bp	GGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCG GGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCG	1117
ERα ERα4-555bp ERα4-219bp	CTCATGATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGAC CTCATGATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGAC	1164
ERα ERα4-555bp ERα4-219bp	GGCCGACCAGATGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCATAC GGCCGACCAGATGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCATAC	1211
ERα ERα4-555bp ERα4-219bp	TCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGA TCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGA	1259
ERα ERα4-555bp ERα4-219bp	TGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATC TGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATC	1306
ERα ERα4-555bp ERα4-219bp	AACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATTTGACCCTCCATGA AACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATTTGACCCTCCATGA GCTTTGTGGATTTGACCCTCCATGA	1353
ERα ERα4-555bp ERα4-219bp	TCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGG TCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGG TCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGG	1401
ERα ERα4-555bp ERα4-219bp	TCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTC <mark>C</mark> TCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGT TCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCC	1449
ERα ERα4-555bp ERα4-219bp	TAACTTGCTCTTGGACAGGAAACCAGGGAAAATGTGTAGAGGGCATGGT	1497
Primer ERA	913 Primer ERA1467R ERα exon 4 deletion	

Figure 28. The gene alignment of ER α 4 555bp and 219bp second round PCR products with the ER α gene.

The numbers on the right refer to the nucleotide numbers (according to Green *et al*, 1986). Exon boundaries according to Ponglikitmongkol *et al*, 1988.

	ER	8α4	% patients	Fisher exact p	
	+ -				
	2	7	14.8		
+ ΕRαΔ6 -	34	18	85.2	0.025	
% patients +/- exon/exon deletion	59.0	41.0			

Table 14. Statistical analysis of ER α 4 and ER α Δ 6.

(+/- refers to the presence or absence of the exon or exon deletion).

4.1.2.4

Nested PCR and sequence analysis of ERα exon 5

Nested PCR of ER α exon 5 revealed up to three bands on agarose gel electrophoresis (Figure 29). Sequencing analysis of the excised and purified bands indicated that the 544bp band included the complete ER α exon 5 (Figure 30; Appendix IV – Spectrographs D1a and D1b; Appendix VI – D1). Sequencing of the 405bp band revealed the absence of the entire exon 5 (ER $\alpha\Delta5$) (Figure 30; Appendix IV – Spectrographs D2a and D2b; Appendix VI – D2) and sequencing of the 80bp band (further referred to as ER $\alpha\Delta5$ plus) revealed a small segment PCR product which may be a PCR artifact or a truncated ER α variant of exon 5 (Figure 30; Appendix IV – Spectrograph D3; Appendix VI – D3).

The entire ER α exon 5 was expressed in all breast cancer patient samples (544bp band). ER $\alpha\Delta$ 5 (405bp band) was expressed in 35 (57%) of the patients and 10

(16%) exhibited the 80bp band. All the control patients also showed the presence of the entire sequence of ER α 5 and 7 (54%) exhibited ER α Δ 5 in their cells.

X² analyses of ERα5 and ERαΔ5 and clinical parameters indicated no significance with respect to tumour size (T) (Table 8), nodal involvement (N) (Table 9), presence or absence of metastases (M) (Table 10), stage of disease (Table 11) or menstrual status (Table 12). The deletion variant ERαΔ5, however, is significant with regard to ERICA status (p=0.011) (Table 13). Fisher's exact tests performed on ERα5 and its variants revealed that ERαΔ5plus is significant with regard to ERαΔ5 (Fisher exact p=0.034) and ERα6 (Fisher exact p= 0.025) (Table 15).

4.1.2.5

Nested PCR and sequence analysis of ERα exon 6

Nested PCR of ER α exon 6 showed two bands (Figure 31). The 388bp band includes the complete ER α exon 6 and this band was observed in all patients and controls (Figure 32; Appendix IV – Spectrograph E1a and E1b; Appendix VI – E1). A faint second band of 254bp was observed simultaneously in 9 (15%) patients. There was not sufficient PCR product to sequence this band but the size indicates an absence of ER α exon 6 (ER $\alpha\Delta6$).



Figure 29. ERα exon 5 agarose electrophoresis gel.

MWM XIII (Roche, SA) is a 50bp molecular weight marker (lane 1). ERa5 PCR product is represented by the 544bp band (lane 2); ERa Δ 5 PCR product is represented by the 405bp band (lanes 2 and 3); the 80bp band represents a PCR artifact (lanes 2 and 3).

ERa G ERa5-544bp ERa5-405bp ERa5-80bp	GAGGAGGGAGAATGTT <mark>GAAACACAAGCGCCAGAGAG</mark> ATGATGGGGAGGGCAGGGGTG GGGGAGGGCAGGGGTG GGGGAGGGCAGGGGTG 	1067
ERa A ERa5-544bp A ERa5-405bp A ERa5-80bp	AAGTGGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGAT AAGTGGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGAT AAGTGGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGAT	1125
ERa (ERa5-544bp (ERa5-405bp (ERa5-80bp	CAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGT CAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGT CAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGT	1183
ERa (ERa5-544bp (ERa5-405bp (ERa5-80bp	GCCTTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTT GCCTTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTT GCCTTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTT	1242
ERa (ERa5-544bp (ERa5-405bp (ERa5-80bp	CAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCAC CAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCAC CAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCAC	1300
ERa A ERa5-544bp A ERa5-405bp A ERa5-80bp	ATGATCAACTGGGCGAAGAGGGTGCCAG <mark>GCTTTGTGGATTTGACCCTCCATGATCAGG</mark> ATGATCAACTGGGCGAAGAGGGTGCCAG <mark>GCTTTGTGGATTTGACCCTCCATGATCAGG</mark> ATGATCAACTGGGCGAAGAGGGGTGCCAG <mark>G</mark>	1358
ERa ERa5-544bp ERa5-405bp ERa5-80bp	TCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGGTCTCGTCTGGCGCTCC TCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGGTCTCGTCTGGCGCTCC	1417
ERa ERa5-544bp ERa5-405bp ERa5-80bp	ATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTAACTTGCTCTTGGACAGGAACCAGG ATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTAACTTGCTCTTGGACAGGAACCAGG AACCAGG CTCTTGGACAG	1475
ERa ERa5-544bp ERa5-405bp ERa5-80bp	GAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTC GAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTC GAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTC GAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTC	1532
ERa ERa5-544bp ERa5-405bp ERa5-80bp	GGTTCCGCATGATGAATC <mark>TGCAGGGAGAGGAGTTTGTG</mark> TGCCTCAAATCTATTATTTT GGTTCCGCATGAT GGTTCCGCATGATGAATC <mark>TGCAGGGAGAGGAGTTTGTG</mark> T GGTTCCGCATGATGAATC <mark>TGCAGGGAGAGGAGTTTGTG</mark>	1590
Primer ERA	1027 Primer ERA1570R ERα exon 5 deletion	

Figure 30. The gene alignment of ERα5 544bp, 405bp and 80bp second round PCR products with the ERα gene.

The numbers on the right refer to the nucleotide number according to Green *et al*, 1986. Exon boundaries according to Ponglikitmongkol *et al*, 1988.

		ERαΔ	5plus	% patients	Fisher exact p
		+	-		
	+	9	26	57.4	0.004
ΕRαΔ5	-	1	25	42.6	0.034
50.0	+	8	51	96.7	0.005
ERa6	-	2	0	3.3	0.025
% patients +/- exon/exon deletion		16.4	83.6		

Table 15. Statistical analyses of ER $\alpha\Delta$ 5plus compared with ER $\alpha\Delta$ 5 and ER α 6.

(+/- refers to the presence or absence of the exon or exon deletion).

X² analyses performed on ERα6 results and clinical parameters indicated no significance with regard to nodal involvement (N) (Table 9), menstrual status (Table 12) or ERICA status (Table 13). ERα6 is, however, significant in terms of tumour size (T) (p=0.002) (Table 8) and ERαΔ6 is significant with regard to M (Table 10) (p=0.018) and stage of disease (p=0.023) (Table 11). Fisher's exact tests performed on ERα6 indicated significance with regard to the 80bp ERα5 variant, ERαΔ5plus, (Fisher exact p=0.025) as previously described (Table 15). The ERα6 deletion variant, ERαΔ6, also showed significance with respect to ERαΔ2 (Fisher exact p=0.007) (Table 7) and ERα4 (Fisher exact p=0.025) (Table 14) as previously described.



Figure 31. ER α exon 6 agarose electrophoresis gel.

MWM 100bp ladder (Promega, SA) (lane 1); ER α 6 PCR product is included in the 388bp band (lanes 2-4); ER α \Delta6 PCR product is represented by the 254bp band.

ERα ERα6-388bp	GCCTGGCTAGAGA <mark>TCCTGATGATTGGTCTCGTCT</mark> GGCG <mark>TCT</mark> GGCG	1413
ERa ERa6-388bp	CTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTAA CTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTAA	1452
ERa ERa6-388bp	CTTGCTCTTGGACAG <mark>GAACCAGGGAAAATGTGTAGAGG</mark> CTTGCTCTTGGACAG <mark>GAACCAGGGAAAATGTGTAGAGG</mark>	1490
ERa ERa6-388bp	GCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCAT GCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCAT	1529
ERa ERa6-388bp	CTCGGTTCCGCATGATGAATCTGCAGGGAGAGGAGTTTG CTCGGTTCCGCATGATGAATCTGCAGGGAGAGGAGGAGTTTG	1568
ERa ERa6-388bp	TGTGCCTCAAATCTATTATTTTGCTTAATTCTG TGTGCCTCAAATCTATTATTTTGCTTAATTCTG GAGTGTA	1608
ERa ERa6-388bp	CACATTTCTGTCCAGCACCCTGAAGTCTCTGGAGAGGAA CACATTTCTGTCCAGCACCCTGAAGTCTCTGGAGAGGAA	1647
ERa ERa6-388bp	GGACCATATCCACCGAGTCCTGGACAAGATCACAGACA GGACCATATCCACCGAGTCCTGGACAAGATCACAGACA	1685
ERa ERa6-388bp	CTTTGATCCACCTGATGGCCAAGGCAGGCCTGACCCTG CTTTGATCCACCTGATGGCCAAGGCAGGCCTGACCCTG	1723
ERa ERa6-388bp	CAGCAGCAGCACCAGCGGCTGGCCCAGCTCCTCCT CAGCAGCAGCACCAGCGGCTGGCCCAGCTCCTCCT <mark>CAT</mark>	1761
ERa ERa6-388bp	CCTCTCCCACATCAGGCACATGAGTAACAAAGGCATGGA CC	1800
Primer ERA	<mark>1389</mark> Primer ERA1776R ERα exon 6	

Figure 32. The gene alignment of ER α 6 388bp second round PCR product with the ER α gene.

The numbers on the right indicate the nucleotide numbers according to Green *et al*, 1986. Exon boundaries according to Ponglikitmongkol *et al*, 1988.

4.1.2.6

Nested PCR and sequence analysis of ER α exon 7

Nested PCR of ER α exon 7 revealed up to three bands on agarose gel electrophoresis (Figure 33). The size of the band and sequence analysis of the excised and purified bands revealed that the 345bp band represented the presence of the ER α exon 7 (Figure 34; Appendix IV – Spectrograph F1a; Appendix VI – F1). This band was expressed in 48 (79%) of the patients and in 12 (92%) of the controls. In only 6 (9%) patients and 2 (15%) controls was this band present without the 161bp band. Sequencing of the 310bp band represents the presence of ER α 7 with random base pairs missing. This may be an incomplete PCR or artifact as it was usually associated with the expression of the 345bp band (Figure 34; Appendix IV – Spectrograph F2a; Appendix VI – F2).

Sequencing of the 161bp band indicated the complete absence of ER α exon 7 (ER $\alpha\Delta$ 7) (Figure 34; Appendix IV – Spectrograph F3a; Appendix VI – F3). This band was expressed in 42 (69%) of patients together with the 345bp band representing the complete ER α exon 7. In the control samples 10 (77%) expressed both bands. In 13 (22%) of patients and 1 control (8%) the ER $\alpha\Delta$ 7 was expressed alone.

 X^2 analyses of ER α 7 and its variant ER α Δ 7 indicated that ER α 7 is significant with respect to tumour size (T) (*p*=0.026) (Table 8) but none of the other clinical parameters such as N, M, stage of disease, menstrual status or ERICA status (Tables 9, 10, 11, 12 and 13).

131



Figure 33. ER α exon 7 agarose electrophoresis gel.

MWM 100bp ladder (Promega, SA) (lane 1); ER α 7 PCR product is included in the 345bp band (lanes 3 and 7); ER α Δ 7 PCR product is represented by the 161bp band (lanes 3, 5 and 6); the 310bp band represents ER α 7 with random base pairs missing (PCR artifact) (lane 3).

ERa ERa7-345bp ERa7-310bp ERa7-161bp	GGGCATGGTGGAGATCTTCGACATGC <mark>TGCTGGCTACATCATCT</mark>	1531
ERα ERα7-345bp ERα7-310bp ERα7-161bp	CG GTTCCGCATGATGAATCTGCAGGGAGAGGAGTTTGTGTGC CATGATGAATCTGCAGGGAGAGGAGTTTGTGTGC ATCTGCAGGGAGAGGAGTTTGTGTGC GTTCCGCATGATGAATCTGCAGGGAGAGGAGGTTTGTGTGC	1573
ERα ERα7-345bp ERα7-310bp ERα7-161bp	CTCAAATCTATTATTTTGCTTAATTCTG <mark>GAGTGTACACATTTCTG</mark> CTCAAATCTATTATTTTGCTTAATTCTG <mark>GAGTGTACACATTTCTG</mark> CTCAAATCTATTATTTTGCTTAATTCTG <mark>GAGTGTACACATTTCTG</mark> CTCAAATCTATTATTTTGCTTAATTCTG	1618
ERα ERα7-345bp ERα7-310bp ERα7-161bp	TCCAGCACCCTGAAGTCTCTGGAGAGGAAGGACCATATCCAC TCCAGCACCCTGAAGTCTCTGGAGAGGAAGGACCATATCCAC TCCAGCACCCTGAAGTCTCTGGAGAGGAAGGACCATATCCAC	1660
ERa ERa7-345bp ERa7-310bp ERa7-161bp	CGAGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGG CGAGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGG CGAGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGG	1703
ERα ERα7-345bp ERα7-310bp ERα7-161bp	CCAAGGCAGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTG CCAAGGCAGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTG CCAAGGCAGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTG	1744
ERα ERα7-345bp ERα7-310bp ERα7-161bp	GCCCAGCTCCTCCTCATCCTCTCCCACATCAGGCACATGAGTA GCCCAGCTCCTCCTCATCCTCCCCACATCAGGCACATGAGTA GCCCAGCTCCTCCTCATCCTCCC TA	1787
ERa ERa7-345bp ERa7-310bp	ACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGT ACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAA	1830
ERα ERα7-345bp	GGTGCCCCTCTATGACCTGCTGCTGGAGATGCAAGAACGT	1872
ERα7-3100p ERα7-161bp	GGTGCCCCTC <mark>TATGACCTGCTGCTGGAGA</mark> TGCTGGACGCCCA	
Primer ERA1	1515 Primer ERA1859R ERα exon 7 deletion	

Figure 34. The gene alignment of ER α 7 345bp, 310bp and 161bp second round PCR products with the ER α gene.

The numbers on the right indicate the nucleotide numbers according to Green *et al*, 1986. Exon boundaries according to Ponglikitmongkol *et al*, 1988.

ER $\alpha\Delta$ 7 was not significant with respect to any of the parameters described. ER α 7 and ER $\alpha\Delta$ 7 were not observed as significant with regard to the other ER α exons or ER β .

4.1.3

Nested PCR and sequence analysis of ERβ in clinical samples

Nested PCR and sequence analysis of ER β was performed on fewer samples than ER α variants due to the lack of sample material (FNA) after the examination of the ER α exons.

4.1.3.1

Nested PCR and sequence analysis of ER β DBD

The number of clinical patients with sufficient FNA material to assess ER β DBD was 28 out of the original 61 (46%) and 5 control samples out of 13 (38.5%) had sufficient material for PCR analysis and therefore individual ER β exons could not be analyzed as with the ER α exons.

As described in Chapter 3 the first round of nested PCR of the ER β gene involved RT-PCR of a fragment of ER β located between exon 2 and exon 7. A second round of nested PCR utilized primers designed within exons 2 and 4 in order to amplify the segment of the gene incorporating the DNA binding domain of ER β .



Figure 35. ERβ DNA Binding Domain agarose electrophoresis gel.

MWM 100bp ladder (Promega, SA) (lane 1);ER β DBD PCR product is included in the 214bp band (lanes 3,4,6 and 7); lanes 2 and 5 are negative for ER β DBD.

ERβ ERβ-DBD	CGGGATATCACTATGGAGTCTGGT <mark>CGTGTG</mark>	597
ERβ ERβ-DBD	AAGGATGTAAGGCCTTTTTTAAAAGAAGCA GTAAGGCCTTTTTTAAAAGAAGCA	627
ERβ ERβ-DBD	TTCAAG ²¹³ GACATAATGATTATATTTGTCCAG TTCAAG ²¹³ GACATAATGATTATATTTGTCCAG	658
ERβ ERβ-DBD	CTACAAATCAGTGTACAATCGATAAAAACC CTACAAATCAGTGTACAATCGATAAAAACC	688
ERβ ERβ-DBD	GGCGCAAGAGCTGCCAGGCCTGCCGACTT GGCGCAAGAGCTGCCAGGCCTGCCGACTT	717
ERβ ERβ-DBD	CGGAAGTGTTACGAAGTGGGAATGGTGAA CGGAAGTGTTACGAAGTGGGAATGGTGAA	746
ERβ ERβ-DBD	TGTG ³⁴⁴ GCTCCCGGAGAGAGAGATGTGGGT TGTG ³⁴⁴ GCTCCCGGAGAGAGAGATGTGGGT	774
ERβ ERβ-DBD	ACCGCCTTGTGCG <mark>GAGACAGAGAAGTGCC</mark> ACCGCCTTGTGCG <mark>GAGA</mark>	803
ERβ ERβ-DBD	GACGAGCAGCTGCACTGTGCCGGCAAGGC	832
Primer ERB	<mark>592 Primer ERB805R</mark> <mark>ERβ DBD</mark> ^{exon boundary}	

Figure 36. The gene alignment of ER β DBD 214bp second round PCR product with the ER β gene.

The numbers on the right refer to the nucleotide numbers according to Ogawa *et al*, 1998a. Exon boundaries and position of LBD according to Herynk and Fuqua, 2004.

In 24 (86%) of clinical patients a band of the expected size of 214bp was observed on agarose gel electrophoresis (Figure 35). In the control samples 2 (40%) revealed the presence of this fragment. The sequence of this fragment was confirmed using automated sequencing (Figure 36; Appendix V – Spectrographs A1a and A1b; Appendix VII – A1).

 X^2 analysis of these results showed that ER β DBD expression was not significant with respect to clinical parameters such as tumour size (T) (Table 16), nodal involvement (N), presence or absence of metastases (M), stage of disease or menstrual status of the patients studied.

EXON		TUM	OUR SIZ		% EXON (+/-)	X² P	
	Т0	T1	T2	T3	T4		
ERβDBD +	2	2	7	6	8	78.1	0.145
-	3	0	0	2	2	21.9	
% patients T for ERβDBD	15.6	6.2	21.9	25.0	31.2		
ERβLBD +	5	0	9	4	9	90	0.159
-	0	0	0	2	1	10	
% patients T for ERβLBD	16.7	0	30.0	20.0	33.3		

Table 16. Associations betwe	en ER β DBD and ER β LBD and
Tumour size (T).	

(T0 refers to normal breast tissue samples; +/- refers to the presence or absence of the exon).

4.1.3.2

Nested PCR and sequence analysis of ER_β LBD

The number of clinical patients with sufficient RNA to assess ER β LBD was 26 out of the original 61 (43%) and 5 control samples out of 13 (39%) had sufficient material for analysis.

First round RT-PCR of the ER β gene was performed as described in Chapter 3. In order to amplify the segment of the ER β gene incorporating the ligand binding domain a second round of PCR utilizing a primer set designed within ER β exons 6 and 7 was performed. The resulting amplified fragment of 222bp (Figure 37) was observed in 23 (89%) of the clinical patients analyzed and in all 5 (100%) of the control samples. The position of this fragment within the ER β gene was confirmed by automated sequencing (Figure 38; Appendix V – Spectrographs B1a and B1b; Appendix VII – B1).

 X^2 analysis of the ER β LBD results showed that ER β LBD expression was not significant with respect to clinical parameters such as tumour size (T) (Table 16), nodal involvement (N), presence or absence of metastases (M), stage of disease or menstrual status of the patients studied.



Figure 37. ERβ Ligand Binding Domain agarose electrophoresis gel.

MWM 100bp ladder (Promega, SA) (lane 1); ER β LBD PCR product is included in the 222bp band (lanes 2 to 5).

ERβ ERβ-LBD	TCTTGTTCTGGACAG ^{5/6} GGATGAGGGGGAAATGCGTA GGATGAGGGGAAATGCGTA	1208
ERβ ERβ-LBD	GAAGGAATTCTGGAAATCTTTGACATGCTCCTGG GAAGGAATTCTGGAAATCTTTGACATGCTCCTGG	1242
ERβ ERβ-LBD	CAACTACTTCAAGGTTTCGAGAGTTAAAACTCCAA CAACTACTTCAAGGTTTCGAGAGTTAAAACTCCAA	1277
ERβ ERβ-LBD	CACAAAGAATATCTCTGTGTCAAGGCCATGATCC CACAAAGAATATCTCTGTGTCAAGGCCATGATCC	1311
ERβ ERβ-LBD	TGCTCAATTCCA ^{9//} GTATGTACCCTCTGGTCACAGC TGCTCAATTCCA ^{9/7} GTATGTACCCTCTGGTCACAGC	1345
ERβ ERβ-LBD	GACCCAGGATGCTGACAGCAGCCGGAAGCTGGC GACCCAGGATGCTGACAGCAGCCGGAAGCTGGC	1378
ERβ ERβ-LBD	TCACTTGCTGAACGCCGTGAC <mark>CGATGCTTTGGTTT</mark> TCACTTGCTGAACGCCGTGAC <mark>C</mark>	1413
ERβ ERβ-LBD	GGGTGATTGCCAAGAGCGGCATCTCCTCCCAGCA	1447
ERβ ERβ-LBD	GCAATCCATGCGCCTGGCTAACCTCCTGATGCTCC	1482
ERβ ERβ-LBD	TGTCCCACGTCAGGCATGCGAG <mark>⁷¹⁸TAACAAGGGCAT</mark>	1516
Primer ERE	<mark>81196</mark> Primer ERB1417R ERβ LBD ^{exon boundary}	

Figure 38. The gene alignment of ER β LBD 222bp second round PCR product with the ER β gene.

The numbers on the right refer to the nucleotide numbers according to Ogawa *et al*, 1998a. Exon boundaries and position of LBD according to Herynk and Fuqua, 2004.

4.1.4

Response to Hormone Treatment and Survival Analysis

Fisher's exact tests performed comparing each of the ER α and ER β exons with patient responses to tamoxifen treatment revealed that no particular exon or exon variant played a significant role in response. Similarly Kaplan-Meier survival analysis in each case also showed no significance with respect to any exon or exon variant.

4.2

The Cloning of T-47D cells

In a pilot study MCF-7 cells were cloned as described in Chapter 3 for T-47D cells. These MCF-7 cells, however, showed no response to oestrogen during oestrogen response testing and therefore T-47D clones were adopted as the model for these studies.

T-47D cells were cloned as described in Chapter 3.9. Initial oestrogen response testing indicated that T-47D cells are oestrogen responsive. Five clones were selected for evaluation using oestrogen response testing (Chapter 3.10) and RT-PCRs of ER α and ER β exons. Two clones were selected, namely, TCA3, which was observed as oestrogen and tamoxifen responsive (Figure 40A), and TCC1, which showed resistance to tamoxifen (Figure 40B).



Figure 39. Slides of parent cells of TCA3 and TCC1 T-47D clones (40 X magnification).



Figure 40. Graphs of oestrogen response tests of parent lines of T-47D clones TCA3 and TCC1.

(red arrow indicates time of oestradiol supplementation as described in Chapter 3.10).

Slides of the parent cells of each clone were stained and viewed at 40 X magnification as described in Chapter 3.11 (Figure 39).

Both clones expressed the complete exons of ER α 2,- α 3, - α 4, - α 5, - α 6 and - α 7. The TCA3 parent clone also expressed complete deletions of ER α exon 2 (ER Δ 2), ER Δ 4, ER Δ 5 and ER Δ 7 (Table 17). The TCA3 parent clone also expressed the entire PCR regions of both ER β DBD and ER β LBD as described in Chapter 3.

4.3

Induction of ER mutation by environmental manipulation

T-47D parent clones TCA3 and TCC1 were each plated out into small petri dishes and exposed to seven different conditions separately for a further 10 passages. Each clone was grown in plates containing medium with FCS only (control plates) as well as petri dishes containing one of each of the following respectively: 10^{-8} M 17- β -oestradiol, 10^{-8} M 4-hydroxytamoxifen, 10^{-8} M aminoglutethimide, 10^{-8} M androstenedione, 10^{-8} M oestriol and 10^{-8} M cholesterol. Hormone response tests were performed after the tenth passage and RNA was extracted from the cells for RT-PCR analysis of ER α and ER β .

4.3.1

TCA3 clone responses

The TCA3 clone was observed to grow faster than the TCC1 clone. Both clones grew very slowly in medium containing 10⁻⁸M tamoxifen. RNA was extracted from TCA3 cells grown in 10⁻⁸M tamoxifen for 10 passages but the cells started to round up and detach during the oestrogen response test and during the preparation of the slides. Similarly, TCA3 cells grown in 10⁻⁸M cholesterol survived for 10 passages and although RNA was extracted from them they did not attach to the substrate during the oestrogen response test. Hormone response profiles for these two conditions were therefore not available.

The TCA3 control cells grown for 10 passages (TCA3 [10]) maintained the same ER α and ER β exon profiles and the same hormone responsiveness as the parent clone (Figure 41; Tables 17 and 18). The hormone response profiles for the other conditions also remained the same as the parent clone (Figures 42, 43 and 44). The ER α 3, ER α 6 (Tables 17 and 18), ER β DNA and ER β LBD profiles in all cases were observed as complete and unchanged from the parent clone. ER α 2, ER α 5 and ER α 7 had both their complete exons and their deleted exons expressed simultaneously as observed in the parent clone. In all conditions, except for growth in 10⁻⁸M aminoglutethimide, the TCA3 clone expressed both the complete ER α 4 alone thus deviating from the parent clone profile (Table 18).

145

Table 17. Table of hormone responses and ER α and ER β exon profiles of T-47D parent clones TCA3 and TCC1.

Clone	Hormone Response Test	ERαΔ2	ERαΔ3	ERα∆4	ERαΔ5	ERαΔ6	ERαΔ7	ERβ DBD	ERβ LBD
TCA3	Strongly oestrogen responsive								
	Tamoxifen responsive	+	-	+	+	-	+	+	+
TCC1	Slightly oestrogen responsive Tamoxifen resistant	-	-	+	-	-	-	+	+

(+ or – refers to the presence or absence of the exon deletion with the

simultaneous presence of the complete exon).



Figure 41. TCA3 clone at passage 10 (Control).

A. Graph of oestrogen response test of TCA3 (10) control cells.

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCA3 (10) control cells.



- Figure 42. A. Graph of oestrogen response test of TCA3 clone at passage 10 (grown in 10⁻⁸M oestradiol).
 - B. Graph of oestrogen response test of TCA3 clone at passage 10 (grown in 10⁻⁸M oestriol).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

Figure 43. TCA3 clone at passage 10 (grown in 10⁻⁸M aminoglutethimide).

 A. Graph of oestrogen response test of TCA3 clone at passage 10 (+ aminoglutethimide).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCA3(10) cells (+ aminoglutethimide).

Figure 44. TCA3 clone at passage 10 (grown in 10⁻⁸M androstenedione).

A. Graph of oestrogen response test of TCA3 clone at passage 10 (+ androstenedione).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCA3(10) cells (+androstenedione).

Table 18. Table of hormone responses and ER α exon profiles o	f T-47D
clone TCA3 under various growth conditions.	

Condition (10 ⁻⁸ M)	Hormone Response Test	ERαΔ2	ERαΔ3	ERαΔ4	ERα∆5	ERαΔ6	ERαΔ7
Control	Oestrogen responsive	+	-	+	+	-	+
	responsive						
Oestradiol	Oestrogen responsive Tamoxifen	+	-	+	+	-	+
Tamoxifen		+	-	+	+	-	+
Aminoglutethimide	Oestrogen responsive Tamoxifen responsive	+	-	+*	+	-	+
Androstenedione	Oestrogen responsive Tamoxifen responsive	+	-	+	+	-	+
Oestriol	Oestrogen responsive Tamoxifen responsive	+	-	+	+	-	+
Cholesterol		+	-	+	+	-	+

(+ or – refers to presence or absence of the exon deletion simultaneous with the presence of the complete exon; * refers to the presence of ER $\alpha\Delta4$ alone).

4.3.2

TCC1 clone responses

The clone TCC1 cells grown in 10⁻⁸M tamoxifen and 10⁻⁸M cholesterol for 10 passages did not attach during oestrogen response tests thus hampering further investigation and therefore hormone response profiles were not available for these two conditions. RNA was extracted after the tenth passage in each case and RT-PCR was performed on these extracts as described.

The TCC1 (10) control cells showed greater tamoxifen sensitivity than the parent clone (Figure 45) with ER α 2, ER α 5 and ER α 7 deletions also becoming apparent after the tenth passage under control conditions (Table 19). ER β DBD and ER β LBD were present under all conditions of growth. The ER α exon profiles under all growth conditions mirrored the control sample at the tenth passage with complete exon deletions in exons ER α 2, ER α 4 and ER α 5 simultaneous with the presence of the exons. ER α 7, however, although co-expressed with ER $\alpha\Delta$ 7 in the control showed no deletions in the other growth conditions (Table 19).

Hormone responsiveness varied between growth conditions with growth in 10⁻⁸M oestradiol showing slight tamoxifen responsiveness (Figure 46). Growth in 10⁻⁸M aminoglutethimide (Figure 48), in 10⁻⁸M androstenedione (Figure 49), in 10⁻⁸M oestriol (Figure 47) and in 10⁻⁸M cholesterol (Figure 49) all indicated tamoxifen resistance.

152

Figure 45. TCC1 clone at passage 10 (Control).

- A. Graph of oestrogen response test of TCC1 (10) control cells. (red arrow indicates time of oestradiol/tamoxifen supplementation as
- described in Chapter 3.10).B. Slide photograph of TCC1 (10) control cells.

Figure 46. TCC1 clone at passage 10 (grown in 10-8M oestradiol).

A. Graph of oestrogen response test of TCC1 clone at passage 10 (+ oestradiol).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCC1 (10) cells (+ oestradiol).

Figure 47. TCC1 clone at passage 10 (grown in 10⁻⁸M oestriol).

A. Graph of oestrogen response test of TCC1 clone at passage 10 (+ oestriol).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCC1 (10) cells (+ oestriol).

Figure 48. TCC1 clone at passage 10 (grown in 10⁻⁸M aminoglutethimide).

A. Graph of oestrogen response test of TCC1 clone at passage 10 (+ aminoglutethimide).

(red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

B. Slide photograph of TCC1 (10) cells (+ aminoglutethimide).

Figure 49. A. Graph of oestrogen response test of TCC1 clone at passage 10 (grown in 10⁻⁸M androstenedione).

B. Graph of oestrogen response test of TCC1 clone at passage 10 (grown in 10⁻⁸M cholesterol) (red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10).

Table 19. Table of hormone responses and ERα exon profiles of T-47D clone TCC1 under various growth conditions.

Condition (10 ⁻⁸ M)	Hormone Response	ERαΔ2	ERαΔ3	ERαΔ4	ERαΔ5	ERαΔ6	ERαΔ7
Control	Oestrogen						
	responsive						
	-	+	-	+	+	-	+
	Tamoxifen						
	responsive						
Oestradiol	Oestrogen						
	Responsive	+	_	+	+	_	
	Tamoxifen		-	•	•	-	-
	responsive						
	(slightly)						
Tamoxifen							
		+	-	+	+	-	-
Aminoglutethimide	Oestrogen						
	responsive			+	+		
	Tamoxifen		-	•	•	-	-
	resistant						
Androstenedione	Oestrogen						
	responsive						
		+	-	+	+	-	-
	Tamoxifen						
Ocatrial	resistant						
Cestrioi	responsive						
	responsive	+	_	+	+	_	_
	Tamoxifen						
	resistant						
Cholesterol	Oestrogen						
	responsive						
	_	+	-	+	+	-	-
	Lamoxiten						
	resistant						

(+ or – refers to presence or absence of the exon deletion simultaneous with the presence of the complete exon).