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TITLE: The Estrogen Receptor and Its Variants as Risk Factors in
Breast Cancer

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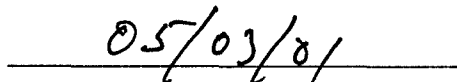
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13. ABSTRACT (Maximum 200 Words) The overall goal of this research is to understand how the estrogen receptor (ER) signal transduction pathway is altered during breast tumorigenesis and if altered ER signal transduction increases the risk of developing breast cancer. Our previous data suggest that altered expression of ER α , ER β and their variants occurs during breast tumorigenesis. Our current data suggest that at least two co-activators of ER, i.e. SRA and AIB1, as well as activated MAP kinase, that can activate ER in a ligand independent fashion, are significantly increased during breast tumorigenesis. In contrast, a repressor of ER activity (REA) is not significantly altered during breast tumorigenesis. Our previous results together with our current results suggest that multiple factors involved in estrogen receptor mediated signal transduction, are altered during human breast tumorigenesis and may have a role in the development of breast cancer. These data are consistent with the hypothesis that alterations of ER signal transduction occurring during the early stages of pre-neoplastic progression may effect the risk of developing breast cancer.				
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4. INTRODUCTION: The overall goal of this research is to understand how the estrogen receptor signal transduction pathway is altered during breast tumorigenesis and if altered ER action increases the risk of developing breast cancer. Currently, it is thought that the steroid hormone, estrogen and its cellular mechanism of action have a major role in both the development and progression of human breast cancer. The estrogen receptor (ER) is a key component that undergoes an increase in its level of expression during tumorigenesis. We and others have evidence to suggest that the expression of the ER, its variants and other isoforms are altered during breast tumorigenesis (1-6). While the expression of specific ER variants and/or isoforms and their overall prevalence relative to wild type ER is different between normal and neoplastic tissue, the pathophysiological significance of these ER variants/isoforms and their potential influence in modulation of the ER pathway in early progression of human breast cancer is not known. We proposed to test the hypothesis that increased ER activity and altered ER variant and/or isoform expression cause altered ER signal transduction in breast epithelial cells. We have now shown that several factors which can impact on ER action are altered during breast tumorigenesis as defined by the comparison of human breast tumors and their matched adjacent normal breast tissues (6, 7, 8). We now are at the stage where we can begin to determine when these changes occur during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and if they are associated with an increased risk of developing breast cancer. **Our specific aim is to determine if alteration of ER signal transduction and ER variant/isoform expression occurs during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer.** A case/control retrospective study is being undertaken in which we will examine ER signaling in comparable breast epithelium and lesions {normal small ducts, benign non-proliferative lesions (adenosis), benign proliferative ductal hyperplasia (PDWA), and ductal carcinoma in situ (DCIS)} in women with or without invasive cancer. ER level will be assessed immunohistochemically, ER-beta mRNA expression will be measured by *in situ* hybridization and/or immunohistochemistry, ER function will be assessed by measurement of progesterone receptor (PR) and pS2 expression and ER influence on proliferation rate will be assessed by immunohistochemical measurement of Ki-67 in serial paraffin sections. Other factors that have been recently identified to modify ER activity include cyclin D (9), MAP kinase (10), several coregulators such as SRA (11), REA (12), and AIB1 (13) will also be measured. They will be assessed in parallel using antibodies, if available, and *in situ* hybridization. We will also study variant ER mRNA expression in parallel sections from frozen tissue blocks, where possible, by specific RT-PCR assays to detect deleted and truncated ER variants, to determine their relative expression with respect to the wild-type ER mRNAs and assess their potential role in altered ER signaling activity.

5. BODY:

1. We previously identified blocks within pathology department archives that contain specific lesions for study. Details of these cases were shown on attached spread-sheets in appendix 1 of the previous annual report. These tissues have now all been assayed immunohistochemically for ER, PR and Ki-67. The raw data has been collected but the analysis of the data is not yet complete. The currently available analysis, where only the invasive and DCIS lesions have been so far scored for ER and PR, is attached in **appendix 1**. ER and PR immunohistochemical staining on this series was assessed by Chaukley Point Count Method. Briefly, 5 high power fields are selected first at low power magnification as representative of the strongest staining areas in the section. These are then assessed using a 25 point grid. Every grid point that coincides with a tumor cell is assessed and scored as positive or negative nucleus. The sum of positives and negatives in 5 fields is calculated and the ratio of positive to negative is used to determine the ER or PR score of the lesion. The attached spreadsheets show the mean, standard deviation and ratio for invasive and DCIS areas in each biopsy where these could be assessed. The Wilcoxon test shows that ER ratio is higher in the invasive than matched DCIS lesions ($P = 0.03$) but although PR is also higher this is not statistically significant.

Immunohistochemical analyses to be done include: i) active MAP kinase (the antibody has been validated and controls established, see 2 below); ii) AIB1: an antibody to undertake this study has recently been developed by Dr J Torchia (University of Western Ontario, London, Ontario). He has kindly provided us with an aliquot to test and the experiments are in progress. Previously no antibodies suitable for AIB1 immunohistochemistry were available; iii) cyclin D1; antibodies are available commercially and will be assessed in the next year; iv) several antibodies for ER β are commercially available, but most, in our hands, have not been suitable for immunohistochemistry. We are currently evaluating another antibody PAI-313 (Affinity Bioreagents) used in breast tissues recently by another group (14).

In situ hybridization analyses have been developed for SRA and REA RNA (see reference 8 and **appendix 2**) and will be used this year to analyze the tissue blocks collected. We have recently obtained a Ventana instrument with both autostaining and *in situ* hybridization modules, for our research laboratory only. This will increase our through-put capacity, and decrease assay times. Further, due to staff turn-over reading and scoring of all immunohistochemical and *in situ* hybridization assays has been slow. Dr Ladislav Tomes, a pathologist from the Czech Republic, has recently joined our group. Dr Tomes will be devoting most of his time to this project.

2. We have validated a commercially available antibody (polyclonal, Phospho-p44/42 MAP Kinase [Thr202/Tyr204], NEB #9101S) which detects only dually-phosphorylated (active) forms of the MAP kinase isoforms, ERK1 and 2 (p44/42), as appropriate for immunohistochemistry. Estrogen depleted MCF7 breast cancer cells were treated for 3 hours with 50 μ M of the MEK1 inhibitor, PD98059, or vehicle (DMSO) alone. Half of the cells from each group was extracted and analyzed by Western blotting. The

remainder was embedded in 3% agar, formalin fixed, paraffin embedded and processed for immunohistochemistry using the polyclonal NEB #9101S antibodies as well as the monoclonal NEB9106L antibodies. The cell pellet sections were assessed by semiquantitative scoring using an H-score system as previously described (15). Western blot analysis (Fig. 1, **appendix 3**) showed a significant decrease in the 44 and 42 kDa ERK1 and 2 MAP kinase bands of the PD treated cell extracts compared to the vehicle alone treated cells using an antibody which recognized only the dually phosphorylated (active) MAP kinase isoforms, ERK1 and ERK 2. No change in total MAP kinase levels was seen when the blot was stripped and reprobed with an antibody recognizing total MAP kinase (SantaCruz ERK-1[C-16] #sc93-G), supporting the conclusion that inhibition of MEK 1 the kinase that activates ERK 1 and ERK 2, led to decreased detection of active MAP kinase with no effect on total MAP kinase levels, which were equivalent between the two treatment groups. Immunohistochemistry using two different antibodies to active MAP kinase showed the presence of nuclear and some cytoplasmic staining in some but not all cells. Importantly the intensity and the % of cells staining was significantly reduced in the PD treated cells compared to the vehicle alone treated cells (Fig. 2, **appendix 3**). The immunohistochemistry results were therefore consistent with the Western blot analysis. The polyclonal antibody (Fig. 2. panels B, E) gave a better signal immunohistochemically and was used on randomly selected human breast tumor sections. The results showed little if any background staining in these tissue sections and positive nuclear staining was seen in some of the epithelial tumor cells (Fig. 2, panel A and D). Therefore this antibody was used to undertake a study to determine the expression of active MAP kinase during human breast tumorigenesis, i.e. comparison of human breast tumors and their matched adjacent normal breast tissues. We used 17 ER+ breast tumor samples with matched adjacent normal breast tissues containing normal ductal epithelium. Interestingly, of the 17 cases, only 8 had detectable staining in the tumor epithelium. These cases were subjected to semi-quantitative H-score analysis, which was then analyzed by a Wilcoxon matched pairs statistical test. The expression of active MAP kinase seen in the tumor cells was significantly increased compared to their adjacent matched normal epithelial cells ($P = 0.03$). These preliminary data are encouraging and provide evidence that increased active MAP kinase expression occurs during breast tumorigenesis, may have some role in the process and could influence ER activity (12). This approach will be used to address the question of at which stage during breast tumorigenesis is active MAP kinase likely to have a role using the cases, tissues and blocks already identified and collected as described in 1 above.

3. 100 breast samples from Poland have been received and stored in the NCIC/Manitoba Breast Tissue Bank. The histopathological analysis and cataloguing of these samples will begin in the near future, and then these samples will be analyzed for ER variant RNA by RT-PCR. However, for unknown reasons this source of fresh frozen tissues has terminated during the last year. Dr Watson has recently traveled to China and is in the process of developing future research collaborations with 2 groups there, to collect and analysis fresh frozen breast biopsy samples, containing a continuum of breast lesions, that could also be used for ER variant RNA analysis using RT-PCR.

4. Preliminary studies to determine if some relevant estrogen receptor coregulators (16), that can modulate ER transcriptional activity, are altered in expression during breast tumorigenesis and/or breast cancer progression *in vivo* have been completed and published (8,17,18 see **appendix 4**). We have examined the expression of a novel recently described steroid receptor RNA activator (SRA, 11) and another coactivator with a different mechanism of action, AIB1 (13), as well as a specific repressor of ER activity (REA, 12) in human breast tissues. These data suggest that alteration of factors, that can modulate ER signal transduction, occurs during breast tumorigenesis. Thus providing a very strong rationale to determine in parallel their expression during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer, in order to assess a potential role in increasing the risk of invasive breast cancer.

6. KEY RESEARCH ACCOMPLISHMENTS.

- * ER, PR and Ki67 immunohistochemical assays completed on tissue sections of blocks of initial patient cohort previously collected and reviewed. Scoring and data analysis has begun.

- * validation of an appropriate antibody to measure activated MAP kinase in formalin fixed, paraffin embedded tissues sections is completed.

- * analysis of activate MAP kinase expression during human breast tumorigenesis as defined by comparison of breast tumors and matched adjacent normal breast tissue. Manuscript in preparation.

- * establishment of SRA and REA *in situ* hybridization assays.

- * analysis of SRA, AIB1 and REA RNA expression during human breast tumorigenesis as defined by comparison of ER+ breast tumors and matched adjacent normal breast tissue, completed and manuscript in press.

- * analysis of REA expression in breast tumors correlates with markers of good prognosis i.e. estrogen receptor expression and low grade, manuscript published.

7. REPORTABLE OUTCOMES.

- * Simon S, Parkes A, Leygue E, Dotzlaw H, Snell L, Troup S, Adeyinka A, Watson P, Murphy LC (2000) Expression of REA in human breast tumors: relationship to some known prognostic markers. Cancer Res 60: 2796-9. **Appendix 4**

- * Murphy LC, Simon S, Parkes A, Leygue E, Dotzlaw H, Snell L, Troup S, Adeyinka A, Watson P. (2000) Altered expression of estrogen receptor coregulators during human breast tumorigenesis. Cancer Res: in press. **Appendix 4**

- * Murphy LC, Simon S, Parkes A, Leygue E, Dotzlaw H, Snell L, Troup S, Adeyinka A, Watson P. (2000) Altered expression of estrogen receptor coregulators during human breast tumorigenesis. Abstract OR200 presented at the 11th International Congress of Endocrinology, 29th October - 2 November, 2000, Sydney, Australia. **Appendix 4**

* Murphy LC. Oestrogen receptors (ERs) in human breast tumorigenesis and breast cancer progression. Invited seminar at the Hormones and Cancer 2000 Symposium, November 3-7, 2000 Port Douglas, Australia. **Appendix 4**

* Murphy LC, Leygue E, Dotzlaw H, Coutts A, Lu B, Huang A, Watson PH (2000) Multiple facets of the estrogen receptor in human breast cancer. In, Endocrine Oncology. Chapter 2, pp.17-34. S Ethier (ed). Humana Press, Totowa, New Jersey. **Appendix 4**

8. CONCLUSIONS.

Our previous results together with our current results suggest that multiple factors involved in estrogen receptor mediated signal transduction, are altered during human breast tumorigenesis, (see Figure 3 for summary, **appendix 3**) and may have a role in the development of breast. These data are consistent with the hypothesis that alterations of ER signal transduction occurring during the early stages of pre-neoplastic progression may effect the risk of developing breast cancer.

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Appendix 1

[illegible]

61	1701-98	58	3	11.6	0.6	1.81659	0.547723	19.33333	75	13	15	2.6	4.062019	1.67332	5.769231
62	2714-98														
63	2849-98														
64	3033-98														
65	3319-98														
66	3982-98														
67	5152-98	39	16	7.8	3.2	1.923538	2.167948	2.4375	45	30	9	6	2.738613	1.732051	1.5
68	5401-98	66	14	13.2	2.8	1.643168	0.83666	4.714286	74	13	14.8	2.6	5.674504	0.894427	5.692308
69	5417-98														
	c1	2	53	0.4	13.25	0.894427	1.258306	0.037736							
	c2	46	31	9.2	7.75	1.30384	1.892969	1.483871							
	c3	65	9	13	2.25	2	1.258306	7.222222							

Value
Data Table-1 Columns A and B

Wilcoxon signed rank test
P value
0.0317
Gaussian Approximation
P value summary
Yes
Are medians signif. different? (t
Two-tailed
One- or two-tailed P value
193.0 , -60.00
Sum of positive, negative n
Sum of signed ranks (W
133

How effective was the pairing?
rs (Spearman, Approximat
P Value (one tailed)
0.6166
P value summary
0.0009
Was the pairing significantly eff

Yes

WILCOXON T TEST P=0.1

Number of values
Minimum
25% Percentile
Median
75% Percentile
Maximum

Mean
Std. Deviation
Std. Error

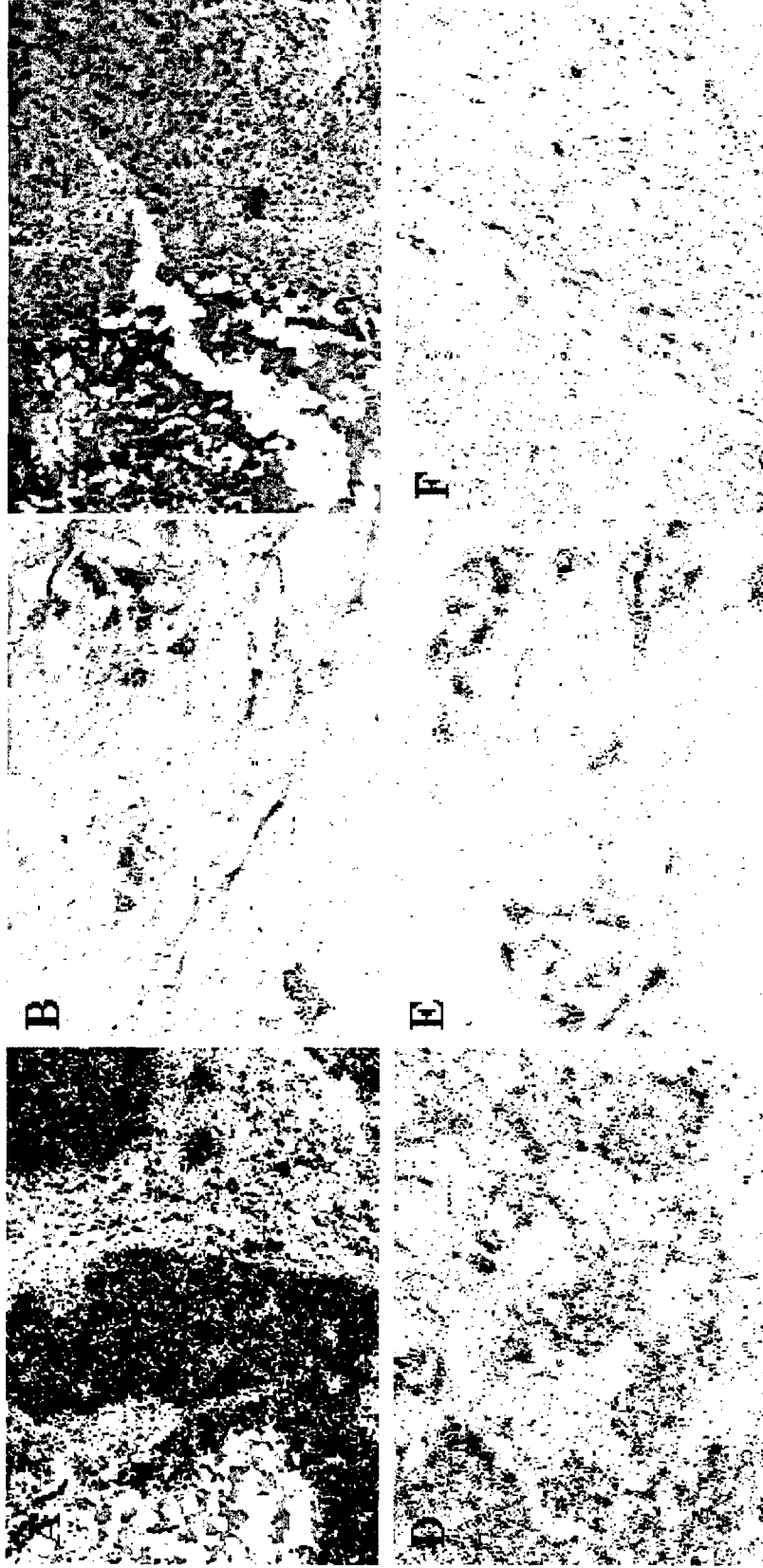
Lower 95% CI
Upper 95% CI

DCIS
ratio
26
0
1.31
1.73
4.113
7
2.598
2.032
0.40
1.777
3.42

INV
ratio
27
0
1.23
3.00
5.227
19.33
4.509
4.814
0.93
2.605
6.41

no	patl_id	INV sum+ve	INV sum-ve	INV mean+ve	INV mean-ve	INV sd +ve	INV sd -ve	INV ratio%	DCIS sum+ve	DCIS sum-ve	DCIS mean+ve	DCIS mean-ve	DCIS sd +ve	DCIS sd -ve	DCIS ratio%	WILCOXON T TEST P=NS									
																Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Mean	Std. Deviation	Std. Error	Lower 95% CI
1	125-97	34	13	4.25	1.625	3.862194	1.767787	2.815385	42	18	5.25	2.25	4.464143	2.659216	2.333333	INV 35	DCIS 21								
2	127-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
3	129-97	26	17	3.25	2.125	2.915476	2.295181	1.529412	67	9	8.375	1.125	7.347254	1.125982	7.444444	0.03957	0.03278								
4	1303-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.591	2.556								
5	1400-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.294	17.33								
6	1434-97	82	13	7.75	1.625	6.870932	1.59799	4.769231	0	0	0	0	0	0	0	5.081	2.586								
7	1459-97	14	33	1.75	4.125	1.832251	3.681518	0.424242	0	0	0	0	0	0	0	1.982	0.8688								
8	1594-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.054	4.398								
9	1681-97	65	10	8.125	1.25	6.812541	1.581139	6.5	0	0	0	0	0	0	0	5.081	2.586								
10	1855-97	1	31	0.125	3.875	0.353553	3.482097	0.032258	57	14	7.125	1.75	6.379375	1.908627	4.071429	1.982	0.8688								
11	1856-97	57	22	7.125	2.75	6.33443	2.659216	2.590909	0	0	0	0	0	0	0	1.054	0.7735								
12	1856-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.108	4.398								
13	2342-97	5	71	0.625	8.875	0.744024	7.529703	0.070423	0	0	0	0	0	0	0	0	0								
14	2622-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
15	2846-97	0	51	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
16	3756-97	12	36	1.5	4.5	1.772811	4.105745	0.333333	18	30	2.25	3.75	2.764572	4.097037	0.6	0	0								
17	4591-97	0	0	0	0	0	0	0	1	56	0.125	7	0.353553	6.071008	0.017857	0	0								
18	5039-97	1	49	0.125	6.125	0.353553	5.33017	0.020408	0	0	0	0	0	0	0	0	0								
19	6262-97	0	70	0	8.75	0	9.513149	0	48	18	5.75	2.25	5.522681	2.251983	2.555558	0	0								
20	6303-97	49	9	6.125	1.125	5.43632	1.359203	5.444444	51	11	6.375	1.375	7.229651	1.767767	4.696364	0	0								
21	6325-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
22	6381-97	54	3	6.75	0.375	5.675763	0.744024	18	24	25	3	3.125	3.70328	3.97986	0.98	0	0								
23	6611-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
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25	6639-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
26	7008-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
27	7476-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
28	7560-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
29	7633-97	0	77	0	9.625	0	8.087699	0	0	0	0	0	0	0	0	0	0								
30	7633-97	9	57	1.125	7.125	0.991031	6.424068	0.157895	0	59	0	7.375	0	8.122939	0	0	0								
31	7679-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
32	7771-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
33	8209-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
34	8657-97	72	14	9	1.75	7.653197	1.752549	5.142857	36	20	4.5	2.5	3.891382	2.618615	1.8	0	0								
35	8692-97	0	0	0	0	0	0	0	46	9	5.75	1.125	5.522681	1.246423	5.111111	0	0								
36	8743-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
37	8756-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
38	8296-97	61	8	7.625	1	6.586681	1.069045	7.625	0	0	0	0	0	0	0	0	0								
39	8417-97	59	7	7.375	0.875	6.501373	1.125982	8.428571	0	0	0	0	0	0	0	0	0								
40	9726-97	6	78	0.75	9.75	0.707107	8.259194	0.076923	0	0	0	0	0	0	0	0	0								
41	10476-97	0	0	0	0	0	0	0	36	20	4.5	2.5	3.891382	2.618615	1.8	0	0								
42	10625-97	0	0	0	0	0	0	0	46	9	5.75	1.125	5.522681	1.246423	5.111111	0	0								
43	10898-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
44	11006-97	1	55	0.125	6.875	0.353553	6.33443	0.018182	0	0	0	0	0	0	0	0	0								
45	11112-97	0	44	0	0	0	0	0	52	3	6.5	0.375	5.903984	0.517549	17.33333	0	0								
46	11361-97	83	11	10.375	1.375	8.700369	1.59799	7.545455	0	0	0	0	0	0	0	0	0								
47	11398-97	1	48	0.125	6	0.353553	5.12696	0.020833	2	61	0.25	7.625	0.707107	8.331309	0.032787	0	0								
48	11411-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
49	11703-97	38	13	4.75	1.625	4.026697	2.065679	2.923077	26	28	3.25	3.5	3.011881	3.891382	0.928571	0	0								
50	11708-97	0	50	0	0	0	0	0	0	46	0	5.75	0	5.750776	0	0	0								
51	11870-97	38	4	4.75	0.5	3.955105	0.755929	9.5	0	0	0	0	0	0	0	0	0								
52	12384-97	0	52	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
53	12732-97	68	1	8.5	0	7.425824	0	68	0	0	0	0	0	0	0	0	0								
54	12739-97	0	61	0	7.625	0	7.160023	0	0	0	0	0	0	0	0	0	0								
55	12815-97	46	11	5.75	1.375	5.311712	1.407886	4.181818	0	55	0	6.875	0	6.289163	0	0	0								
56	361-98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
57	523-98	3	64	0.375	8	0.517549	7.4642	0.046875	5	55	0.625	6.875	1.407886	6.356942	0.090909	0	0								
58	653-98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
59	769-98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
60	1676-98	39	14	4.875	1.75	4.12094	2.12132	2.785714	0	0	0	0	0	0	0	0	0								
61	1701-98	0	0	0	0	0	0	0	59	40	7.375	5	6.30051	4.568962	1.475	0	0								
62	2714-98	0	0	0	0	0	0	0	54	13	6.75	1.625	5.984045	1.767767	4.153946	0	0								
63	2848-98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
64	3033-98	64	3	4.75	0.375	4.234214	0.744024	12.66667	0	0	0	0	0	0	0	0	0								
65	3319-98	38	3	4.75	0.375	4.234214	0.744024	12.66667	0	0	0	0	0	0	0	0	0								
66	3962-98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
67	5152-98	0	67	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
68	5401-98	0	64	0.875	8	1.457738	6.697548	0.109375	0	54	0	6.75	3.285018	4.627171	0.756757	0	0								
69	5417-98	37	14	4.625	1.75	3.925648	1.581139	2.842857	28	37	3.5	4.625	3.285018	4.627171	0.756757	0	0								

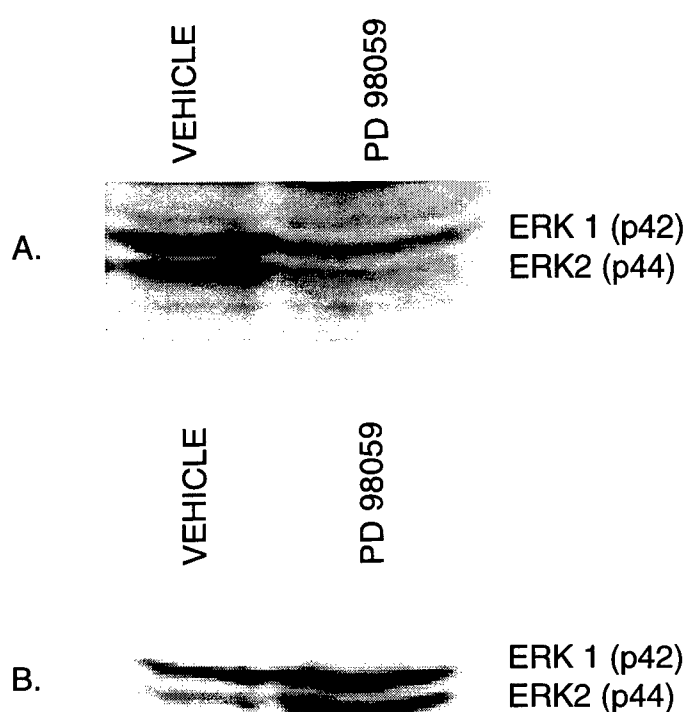
Appendix 2



***In situ* hybridization analyses of SRA and REA RNA expression in human breast tissue sections.** Antisense SRA riboprobes (A, B) were used to detect SRA RNA expression in a section from an ER+ human breast tumor (A) and the matched normal breast tissue from the same patient (B). Sense SRA riboprobes were used as a specificity control and the results from the adjacent section of the tumor shown in A are shown (C). Antisense REA riboprobes (D, E) were used to detect REA mRNA expression in a section from an ER+ human breast tumor (D) and the matched normal breast tissue from the same patient (E). Sense REA riboprobes were used as a specificity control and the results from the adjacent section of the tumor shown in D are shown (F). Magnification 100X. Black dots/grains represent hybridization signals, the coloured background represents the counterstaining.

Leigh Murphy

Appendix 3



T5 human breast cancer cells were exposed to 50 μ M PD 98059 for 3 hours and proteins extracted (1). Proteins were resolved by 10% PAGE/SDS under reducing conditions with a 4% stacking SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane for 1 hour at 120V. Membranes were baked for 30 minutes at 65°C and blocked in 0.2% I-block for 1 hour.

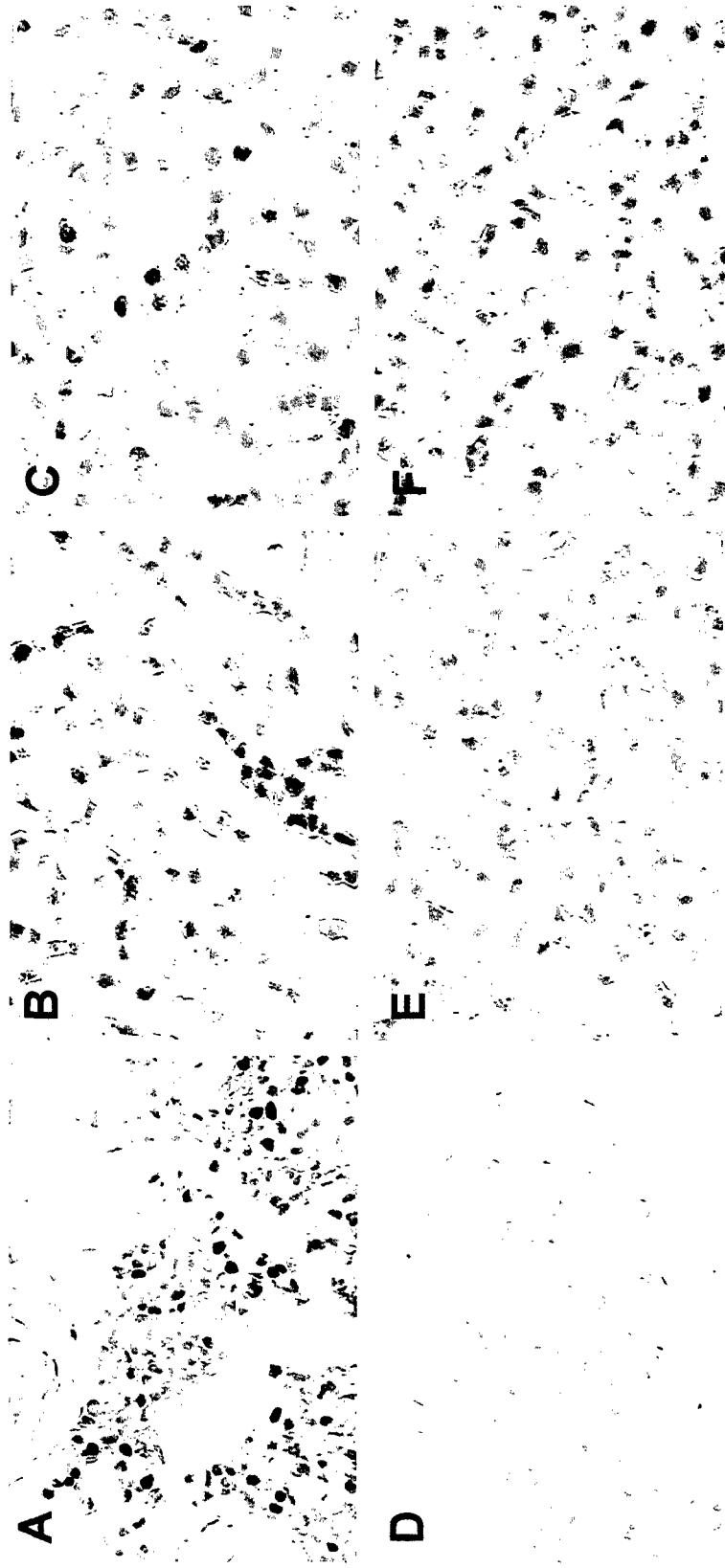
A) Blots were incubated with rabbit anti-active MAPK (1:1000 dilution, 0.2% I-block containing 0.1% Tween-20) (Promega) for 3 hours at room temperature and incubated with horseradish peroxidase-conjugated goat anti-rabbit (Bio-rad) antibody and detected by chemiluminescence (Pierce).

B) Blots from A were stripped with a glycine stripping buffer, blocked in 0.2% I-block for 1 hour and probed with goat anti-total MAPK (1:1000 dilution, 0.2% I-block containing 0.1% Tween-20) (Santa Cruz Biotechnology) for 3 hours at room temperature. Membranes were incubated with an anti-goat HRP-conjugated antibody (Santa Cruz Biotechnology) and visualized via chemiluminescence (Pierce).

Reference 1. Joel PB, Traish AM, Lannigan DA.

Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase.

J Biol Chem. 1998 May 22;273(21):13317-23

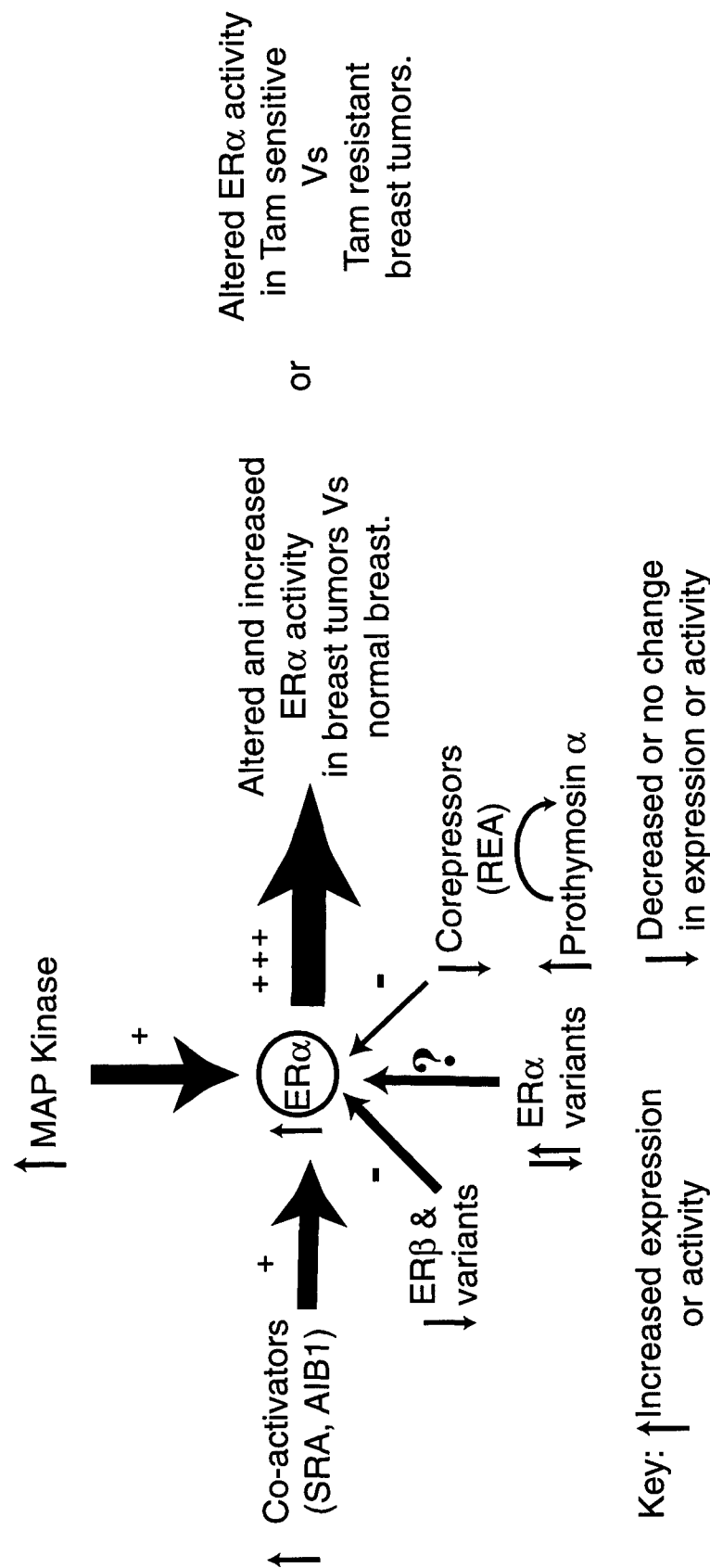


A= invasive tumour; D=matched normal; B = untreated cells polyclonal antibody; E= PD treated polyclonal antibody

C= untreated cells Monoclonal antibody; F= PD treated monoclonal antibody

Magnification at microscopy x200

Figure 3. Summary of changes in factors that can directly effect ER α activity in breast tumors versus matched normal breast tissues.



Appendix 4

Expression of a Repressor of Estrogen Receptor Activity in Human Breast Tumors: Relationship to Some Known Prognostic Markers¹

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Abstract

The expression of a specific repressor of estrogen receptor activity (REA) was investigated by a semiquantitative reverse transcription-PCR assay in 40 human breast tumor biopsy samples with respect to steroid hormone receptor status and other known prognostic variables. The data showed that REA expression was positively correlated with estrogen receptor (ER) levels as defined by ligand-binding assays (Spearman $r = 0.3231$; $P = 0.042$) and that the median level of REA mRNA was significantly (Mann-Whitney two-tailed test, $P = 0.0424$) higher in ER+ tumors (median = 94.5; $n = 30$) compared with ER- tumors (median = 64.5; $n = 10$), with no significant differences ($P = 0.4988$) associated with progesterone receptor status alone. In addition, REA expression was inversely correlated with tumor grade (Spearman $r = -0.4375$; $P = 0.0054$). When the tumors were divided into two groups based on grade, REA expression was significantly (Mann-Whitney two-tailed test, $P = 0.0024$) higher in low-grade (median = 97; $n = 16$) compared with high-grade (median = 76; $n = 23$) tumors. These results provide preliminary data suggesting that the expression of REA varies among breast tumors and is correlated with known treatment response markers and inversely correlated with a marker of breast cancer progression. REA together with ER status may be an improved marker of endocrine therapy responsiveness in human breast cancer.

Introduction

Estrogens have important roles in both normal and neoplastic mammary tissues; however, marked changes occur in estrogen action during both breast tumorigenesis and breast cancer progression (1). The mechanisms underlying altered estrogen signal transduction in target tissues is the focus of much research at present. Current concepts of estrogen action include cofactors that can either enhance or repress the transcriptional activity of the ER³ (2). Recently, a highly specific repressor of the transcriptional activity of ligand-occupied ERs (ER- α and ER- β but not other steroid hormone receptors such as PR or type II nuclear receptors) was identified and characterized using a yeast two-hybrid system (3). Furthermore, part of its mechanism of action appeared to involve functional competition with steroid hormone receptor coactivators such as SRC-1 (2). This repressor differed from previously identified corepressors such as nuclear receptor corepressor and silencing mediator for retinoid and thyroid hormone

receptor; in that it was not structurally related to either of them, it showed great selectivity for ER as opposed to other steroid hormone or non-steroid-binding members of the nuclear receptor family, and it required ER to be bound to ligand with preferential effects being seen when the ligand was an antiestrogen (3). This repressor was therefore called REA. Because REA is selective for ER, it is highly relevant to investigate the expression of this gene in human breast tissues both normal and neoplastic.

Recently we demonstrated that REA is expressed in both normal and neoplastic human breast tissues,⁴ as measured by RT-PCR. Furthermore, the expression of REA was not significantly different between ER+ breast tumors and their matched adjacent normal breast tissues.⁴ However, the tumor cohort in the previous study were all ER+ as determined by ligand-binding assays and did not address the question of whether REA expression in breast tumors was correlated with known prognostic and endocrine treatment response markers. In this study, we investigated the relationship of REA expression in breast tumors to ER and PR status and other known prognostic variables.

Materials and Methods

Human Breast Tumors. Forty invasive ductal carcinomas were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand-binding assays. The ER levels were 0–151 fmol/mg of protein, and 30 tumors were classified as ER+ (defined as >3 fmol/mg of protein). PR levels were 0–285 fmol/mg of protein, and 20 tumors were classified as PR+ (defined by >10 fmol/mg of protein). These tumors spanned a wide range of grade (grades 4–9), determined using the Nottingham grading system.

Cell Culture. T-47D human breast cancer cells were obtained from Dr. D. Edwards (Denver, CO), and MCF7 cells were obtained from the late Dr. W. McGuire (San Antonio, TX). T-47D cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin as described previously (4). Cells were plated at 1×10^6 in 100-mm dishes and 2 days later were treated with 10 nM medroxyprogesterone acetate and harvested at various times (1–48 h). MCF7 human breast cancer cells were depleted of estrogen by passaging stock cells twice in phenol red-free DMEM supplemented with 5% twice charcoal-stripped fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin (5% twice charcoal-stripped fetal bovine serum) as described previously (5). Cells were then plated as above in 5% twice charcoal-stripped fetal bovine serum and 2 days later treated with 10 nM estradiol-17 β and harvested for analysis at various times (1–48 h). The steroids were added directly from 1000 $\times$ stock solutions in ethanol to achieve the required concentrations. The cells were harvested by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at -70°C until RNA was isolated.

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³ The abbreviations used are: ER, estrogen receptor; PR progesterone receptor; REA, repressor of estrogen receptor activity; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

⁴ L. C. Murphy, S. L. R. Simon, A. Parkes, E. Leygue, H. Dotzlaw, L. Snell, S. Troup, A. Adeyinka, and P. H. Watson. Altered relative expression of estrogen receptor coregulators during human breast tumorigenesis, submitted for publication.

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20- μ m frozen tissue sections (20 sections per tumor) or cell pellets using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μ g of total RNA was reverse transcribed in a final volume of 25 μ l as described previously (6).

Primers and PCR Conditions. The primers used were primer REAU (5'-AAA ATC TCC TCC CCT ACA-3'; sense; positions, GenBank Accession No. AF150962) and primer REAL (5'-CCT GCT TTG CTT TTT CTA CCA-3'; antisense; position, GenBank Accession No. AF150962). PCR amplifications were performed and PCR products analyzed as described previously (7) with minor modifications. Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 20 μ l in the presence of 4 ng/ μ l of each primer and 0.3 units of *Taq* DNA polymerase (Life Technologies). Each PCR consisted of 27 cycles (30 s at 57°C, 30 s at 72°C, and 30 s at 94°C) for measuring REA. PCR products were then separated on 1.8% agarose gels stained with ethidium bromide as described previously (7). Amplification of ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (7). The identities of PCR products were confirmed by subcloning and sequencing, as reported previously (6).

Quantification and Statistical Analysis of REA Expression. After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst (Bio-Rad, Hercules, CA). At least three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the REA signal of one particular sample and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified, and after analysis of PCR products on stained agarose gels, signals were quantified by scanning using MultiAnalyst. Three independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the same tumor. For each sample, the average of REA signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Correlation between REA expression and tumor characteristics was tested by calculation of the Spearman coefficient, *r*. Differences between tumor subgroups were tested using the Mann-Whitney rank-sum test, two-sided.

Results

Measurement of REA mRNA Expression in Primary Human Breast Tumors with Different ER and PR Status. We previously developed a semiquantitative RT-PCR approach to measure REA mRNA in small amounts of human breast tissues.⁴ Cloning and sequencing confirmed the identity of the expected 397-bp PCR product as REA, and this PCR product was used to probe Northern blots of RNA extracted from human breast tumor biopsies as described previously (8). An ~1.5-kb transcript was detected, consistent with the previously described REA mRNA (Fig. 1). Varying levels of REA mRNA were detected in human breast tumor biopsy samples, which raised the question of whether the expression of REA in breast tumors

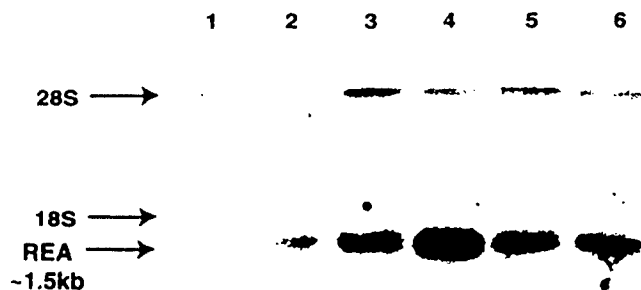


Fig. 1. Northern blot analysis of poly(A)⁺ enriched RNA (15 μ g) isolated from several human breast cancer biopsy samples. The 397-bp REA PCR product was used to probe the Northern blot as described previously (8). Residual 28S and 18S bands are shown, as is the ~1.5-kb band corresponding to REA mRNA.

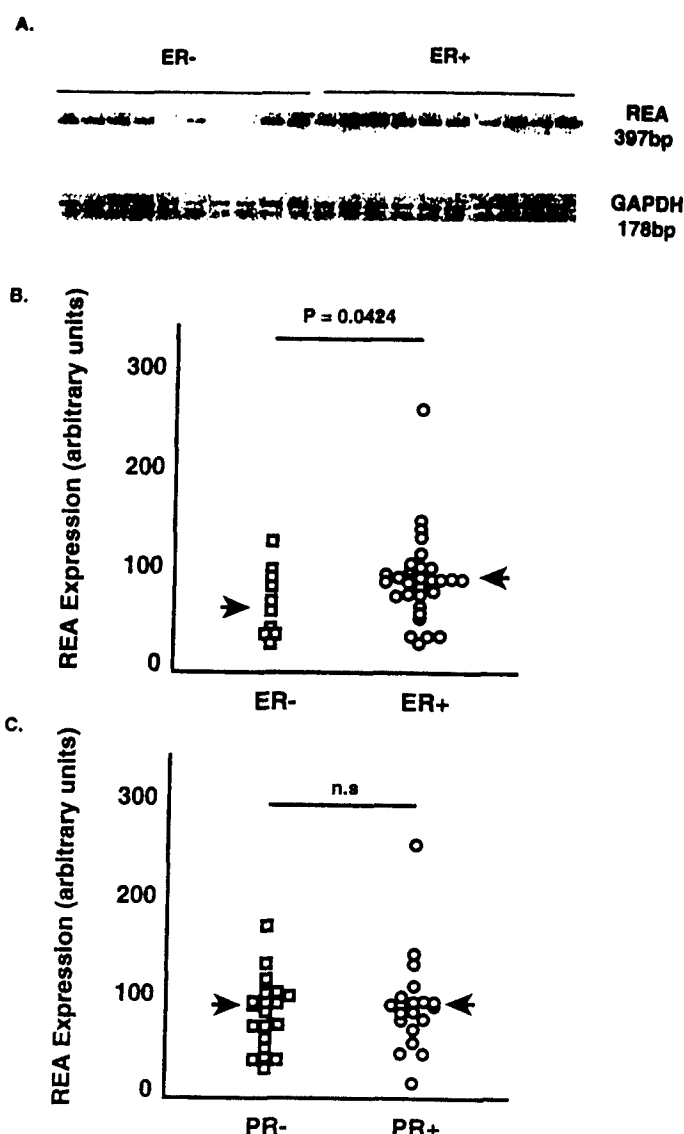


Fig. 2. A. RNA was extracted and assayed for REA expression using RT-PCR as described in "Materials and Methods." After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of some ER⁻ and ER⁺ breast tumors is shown (top). The expected 397-bp REA PCR product (confirmed by sequence analysis) is shown. Ethidium bromide-stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples is shown below the REA analysis. The expected 178-bp *GAPDH* PCR product is shown. B. for each tumor (*n* = 40), REA expression was quantified and expressed in arbitrary units corrected for *GAPDH* signal as described in "Materials and Methods." The tumors were divided into ER⁺ (>3 fmol/mg of protein; ○) and ER⁻ (≤3 fmol/mg of protein; □) as defined by ligand-binding assays. The results are presented as a scatter graph. Arrows indicate the median value in each group. REA expression is significantly less in ER⁻ tumors compared with ER⁺ tumors (Mann-Whitney two-tailed, *P* = 0.0424). C. for each tumor (*n* = 40), REA expression was quantified and expressed in arbitrary units corrected for *GAPDH* signal as described in "Materials and Methods." The tumors were divided into PR⁺ (>10 fmol/mg of protein; ○) and PR⁻ (≤10 fmol/mg of protein; □) as defined by ligand-binding assays. The results are presented as a scatter graph. Arrows indicate the median value in each group. REA expression is not significantly (*n.s.*) different between PR⁻ tumors and PR⁺ tumors.

was correlated with the known prognostic and treatment response variables, such as ER and PR status.

Tumors were identified according to their ER or PR status as defined by ligand-binding analysis (see "Materials and Methods"). REA mRNA levels were measured by RT-PCR and normalized to the *GAPDH* mRNA level as measured in parallel by RT-PCR. Examples of the results obtained are shown in Fig. 2A. The results obtained for all tumors assayed are shown as scatter graphs in Fig. 2B (arranged according to ER) and Fig. 2C (arranged according to PR status of the

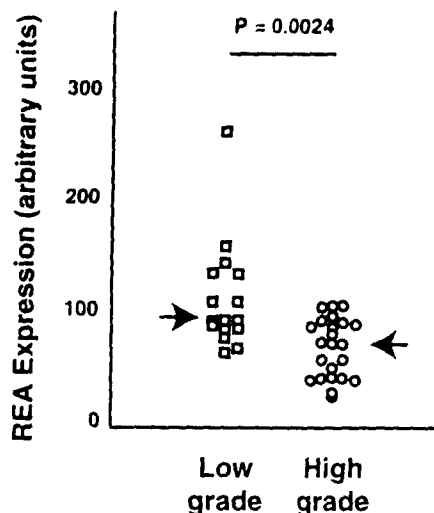


Fig. 3. For each tumor ($n = 40$), REX expression was quantified and expressed in arbitrary units corrected for GAPDH signal as described in "Materials and Methods." The tumors were divided into low-grade (Nottingham grades 3–6; □) and high-grade (Nottingham grades 7–9; ○). The results are presented as a scatter graph. Arrows indicate the median value in each group. REX expression is significantly higher in low-grade tumors compared with high-grade tumors (Mann-Whitney two-tailed, $P = 0.0024$).

tumor as measured by ligand-binding analysis). When the level of REX mRNA in tumors was assessed according to either ER status or PR status alone, as defined by ligand-binding analysis, the level of REX mRNA was significantly (Mann-Whitney two-tailed test, $P = 0.0424$) higher in ER+ tumors (median, 94.5; $n = 30$) compared with ER– tumors (median, 64.5; $n = 10$), with no significant differences ($P = 0.4988$) associated with PR status alone (PR+ median, 91.5; $n = 20$; PR– median, 87.5; $n = 20$).

The relationship of the level of REX mRNA levels with ER status in human breast tumor biopsies suggested the hypothesis that REX expression may be regulated by estrogens and/or progestins. However, no effect of estrogen (10 nM estradiol-17 β) on the steady-state REX mRNA levels in estrogen-depleted MCF7 cells was observed over a 48-h time span (data not shown). In addition, no effect of progestin (10 nM medroxyprogesterone acetate) treatment on REX mRNA in T-47D cells was observed over a similar time span (data not shown). It was concluded that the expression of REX mRNA was not regulated by estrogens or progestins in human breast cancer cell lines.

Correlation of REX Expression with Tumor Characteristics. Spearman analysis showed a significant correlation of the level of REX mRNA in the tumors with the level of ER as measured by ligand-binding assays (Spearman $r = 0.3231$; $P = 0.042$) but no significant correlation with the level of PR as measured by ligand-binding assays (Spearman $r = 0.2777$; $P = 0.0841$). These data are consistent with the data analyzed using clinically relevant cutoff values for ER (ER+ >3 fmol/mg of protein) and PR (PR+ >10 fmol/mg of protein) status as shown above. However, statistical significance of the correlation of REX mRNA and ER binding was lost when Spearman analysis was applied only to those tumors that were ER+ (>3 fmol/mg of protein). The level of REX mRNA was also found to be inversely correlated with tumor grade (Spearman $r = -0.4375$; $P = 0.0054$). When the tumors were divided into two groups based on grade (low, Nottingham grades 3–6; high, Nottingham grades 7–9), the level of REX mRNA (Fig. 3) was significantly (Mann-Whitney two-tailed test, $P = 0.0024$) higher in low-grade (median, 97; $n = 16$) compared with high-grade (median, 76; $n = 23$) tumors, which is consistent with the Spearman correlation analysis.

No significant correlations were found between the level of REX mRNA and age, nodal status, percentage of normal duct and lobular

epithelium, or percentage of stromal or fat cell content within the tumor sections analyzed.

Discussion

Our data show that the level of REX mRNA in human breast tumors is significantly correlated with ER status and inversely correlated with grade. These data are the first to identify a correlation between REX mRNA expression and known prognostic and treatment response markers in human breast cancer biopsies. The positive correlation of REX and ER expression (a good prognostic variable and a marker of response to endocrine therapies) together with inverse correlation of REX expression and grade suggests that REX expression could also be a marker of good prognosis and likelihood of response to endocrine therapies such as antiestrogens. The loss of statistical significance of the correlation between ER levels and REX mRNA when only ER+ breast tumors were analyzed may be due to the reduced numbers of observations in that analysis ($n = 30$ compared with $n = 40$ for total tumor cohort) or may indicate the existence of some threshold effect associated with expression of ER and REX. This latter suggestion together with the lack of correlation of absolute ER levels and REX mRNA in ER+ tumors would be consistent with our observation that REX expression, at least at the RNA level, was found not to be regulated by estrogen.

REX has been identified as a protein that interacts in a yeast-two hybrid system with a dominant negative mutant ER α (3). It was shown to be a selective repressor of ER (both ER α and ER β) transcriptional activity as determined in transient transfection assays using several estrogen-responsive element-containing promoters regulating a chloramphenicol acetyltransferase reporter gene. Cotransfection of a REX expression vector enhanced the potency of antiestrogens such as 4-hydroxytamoxifen and ICI 182780. Furthermore, REX competitively reversed coactivator, *i.e.*, SRC-1, transcriptional enhancement of ER activity. Together these data suggest that REX is a corepressor of ER transcriptional activity.

The current concept of the mechanism by which nuclear hormone receptors regulate gene transcription involves three main components as proposed by Katzenellenbogen *et al.* (9): the receptor, its ligands, and its coregulators. Coregulators appear to consist of at least two classes: those that enhance nuclear hormone receptor activity, referred to as coactivators, and those that repress nuclear hormone receptor activity, referred to as corepressors (2). Furthermore, it has been suggested that differences in the ratios of expression of these two different groups of coregulators may underlie altered responses to steroid hormone agonists and antagonists (10–13). More recently, we have provided the first evidence to suggest that an imbalance between factors that can enhance ER and factors that can repress ER transcriptional activity occurs during human breast tumorigenesis *in vivo*.⁴ Our data showed that the levels of expression of the two ER coactivators, steroid receptor RNA activator (14) and amplified in breast cancer-1 (15), were significantly increased in ER+ breast tumors compared with their normal adjacent breast tissues, whereas the level of REX, a repressor of ER activity, was not significantly different between the tumors and normal breast tissues in the same patient cohort. However, this investigation used only ER+ breast tumors and could not address the question of REX expression in relation to steroid receptor status and other prognostic variables in breast tumors. In addition, we and others have shown that the expression of the coactivators, steroid receptor RNA activator (16) and amplified in breast cancer-1 (17), varies among breast tumors and can be correlated with steroid receptor status in some cases.

ER status itself is associated with grade, with most ER+ breast tumors being low grade and having low tumor proliferation rates,

defined by the percentage of S-phase cells (18), and this may contribute to the inverse relationship of REA with grade observed in this study. However, REA expression is more strongly inversely correlated with grade than positively with ER status; therefore, it is possible that a repressor of ER activity that can contribute to the proliferative activity of breast tumor cells could have a significant negative effect on breast cancer progression and thus functionally influence breast cancer progression. It is speculated that the coexpression of ER and REA may therefore provide better prognostic information than either alone.

ER status is also an important treatment response marker in human breast cancer (18) where the presence of ER in breast tumors increases the likelihood of response to endocrine therapies such as antiestrogens. However, a significant portion of ER+ tumors will not respond to tamoxifen initially, and of those tumors that do respond, many eventually will develop resistance to tamoxifen and other endocrine therapies (18). It has been speculated that altered relative ratios of coactivators and corepressors of ER may in part be a mechanism underlying such endocrine resistance. Direct proof of this hypothesis *in vivo* remains to be provided by measuring expression of the relevant genes in human breast tumors that are known to be clinically sensitive or resistant to tamoxifen and/or other endocrine therapies. However, the data presented here provide preliminary information that the expression of a specific repressor of ER activity varies among breast tumors and that expression is correlated with known treatment response markers and inversely correlated with a marker of breast cancer progression.

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Advances in Brief

Altered Expression of Estrogen Receptor Coregulators during Human Breast Tumorigenesis¹Leigh C. Murphy², Sharon L. R. Simon, Alicia Parkes, Etienne Leygue, Helmut Dotzlaw, Linda Snell, Sandra Troup, Adewale Adeyinka, and Peter H. Watson

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Abstract

The hypothesis that altered expression of specific coactivators/repressors of the estrogen receptor occurs during human breast tumorigenesis *in vivo* is examined in this study. Using *in situ* hybridization and reverse transcription-PCR assays, the expression of two coactivators (SRA and AIB1) and one repressor (REA) of the estrogen receptor was compared between matched breast tumors and adjacent normal human breast tissue. The levels of SRA and AIB1 mRNA were increased in tumors compared with normal tissues ($n = 19$; Wilcoxon matched pairs test; $P < 0.01$). In contrast, the expression of REA mRNA was not different between tumors and normal tissues ($n = 19$; Wilcoxon; $P = 0.110$). The ratios of AIB1:REA and SRA:REA were higher (Wilcoxon; $P < 0.05$) in tumors compared with normal tissues. Furthermore, SRA:AIB1 was higher (Wilcoxon; $P = 0.0058$) in tumors compared with normal tissues. Although our study is small, these data are consistent with the above hypothesis and suggest that such alterations may have a role in the altered estrogen action occurring during breast tumorigenesis.

Introduction

During human breast tumorigenesis, enhanced activity of the ER α ³ signaling pathway is thought to occur and to be a major driving force in breast tumorigenesis. The assumption derives from the observations that only a minority of normal human breast epithelial cells have detectable ER α (7–17% ER α + ductal epithelial cells; Ref. 1), whereas >70% of primary breast cancers are ER α + (2). Furthermore, the majority of proliferating cells in normal human breast tissue is ER α -, and estrogen only indirectly causes proliferation in normal mammary tissues (reviewed in Ref. 3). However, estrogen can directly cause proliferation of breast cancer cells (4), and many proliferating cells in ER+ breast tumors are ER α + (5).

Factors that enhance and repress receptor activity directly, namely coactivators and corepressors, now are considered to be important in mediating steroid receptor transcriptional activity (6). As well, experimental modulation of levels of these two classes of coregulators was shown to alter steroid receptor transcriptional activity (7, 8). These data suggest that not only are ER α levels often increased during breast tumorigenesis (9), but it is likely that other factors which modulate ER α activity might also be altered during breast tumorigenesis with

an outcome of enhancement or deregulation of ER α signaling that may underlie alterations of estrogen responsiveness from indirect in normal breast epithelium to direct in ER α + breast tumor cells. We have addressed this hypothesis by investigating the expression of two known coactivators of ER α , SRA (7) and AIB1 (10), and a repressor of ER α activity, REA (8), at the mRNA level in ER+ human breast tumors and their matched adjacent normal breast tissues. The coregulators studied were chosen because they were identified as either selective for ERs and/or steroid receptors, *e.g.*, SRA (7) and REA (8), or were identified previously to be of relevance in human breast cancer *in vivo*, *e.g.*, AIB1, which is frequently amplified in breast tumors *in vivo* (10).

Materials and Methods

Human Breast Tissues. Nineteen ER+ primary human breast tumor biopsies (ER-positivity was defined as >3 fmol/mg protein in classical ligand-binding assays) were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The ER levels ranged from 3.7–83 fmol/mg protein and the PR levels ranged from 2.7–112 fmol/mg protein (PR-positivity was defined as >10 fmol/mg protein in classical ligand binding assays; 14 tumors were PR+, and 5 tumors were PR-). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block and the relative cellular composition was determined by the histopathological assessment of sections from adjacent mirror-image paraffin-embedded tissue blocks, as described previously (11). The presence of normal ducts and lobules as well as the absence of any atypical lesion were confirmed in all normal tissue specimens. The tumors spanned a wide range of grades (grade scores 5–9) as determined by the Nottingham grading system.

In Situ Hybridization. Paraffin-embedded 5- μ m breast tumor and matched adjacent normal breast tissue sections were analyzed by *in situ* hybridization according to a previously described protocol (12). The plasmid pGEM-T-SRAcore, consisting of pGEM[®]-T-easy plasmid (Promega, Madison, WI) containing a 397-bp insert of the human SRA cDNA (from nucleotide 300 to 696, numbered according to GenBank accession no. AF092038), was used as a template to generate sense and antisense riboprobes. The plasmid pGEM-T-REA, consisting of pGEM[®]-T-easy plasmid containing a 399-bp insert of the human REA cDNA (from nucleotide 385 to 783, numbered according to GenBank accession no. AF150962), was used as a template to generate sense and antisense riboprobes. UTP-³⁵S-labeled riboprobes were synthesized using Riboprobe Systems (Promega, Madison, WI) according to the manufacturer's instructions. Sense probes were used as controls. *In situ* hybridization and washing conditions were as described previously (12). Sections were developed using Kodak NTB-2 photographic emulsion and counterstained with Lee's stain after 2–6 weeks.

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20- μ m frozen tissue sections (20 sections/tumor; 35 sections for normal tissues) using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μ g of total RNA was reverse-transcribed in a final volume of 25 μ l as described previously (13).

Primers and PCR Conditions. The primers used were: (a) SRAcoreU primer (5'-AGGAACGCGGCTGGAACGA-3'; sense; positions 35–53; Gen-

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³ The abbreviations used are: SRA, steroid receptor RNA activator; AIB1, amplified in breast cancer-1; REA, repressor of estrogen receptor activity; ER, estrogen receptor; PR, progesterone receptor; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DCIS, intraductal carcinoma.

Bank accession no. AF092038) and SRAcoreL primer (5'-AGTCTGGG-GAACCGAGGAT-3'; antisense; positions 696-678; GenBank accession no. AF092038); (b) AIB1-U primer (5'-ATA CTT GCT GGA TGG TGG ACT-3'; sense; positions 110-130; GenBank accession no. AF012108) and AIB1-L primer (5'-TCC TTG CTC TTT TAT TTG ACG-3'; antisense; positions 458-438; GenBank accession no. AF012108); and (c) REA-U primer (5'-CGA AAA ATC TCC TCC CCT ACA-3'; sense; positions 385-405; GenBank accession no. AF150962) and REA-L primer (5'-CCT GCT TTG CTT TTT CTA CCA-3'; antisense; positions 781-761; GenBank accession no. AF150962).

Radioactive PCR amplifications for SRA were performed and PCR products were analyzed as described previously (14), with minor modifications. Briefly, 1 μ l of RT mixture was amplified in a final volume of 15 μ l in the presence of 1.5 μ Ci of [α - 32 P]dCTP (3000 Ci/mmol), 4 ng/ μ l of each primer, and 0.3 unit of *Taq* DNA polymerase (Life Technologies, Inc.). For SRA, each PCR consisted of 30 cycles (30 s at 60°C, 30 s at 72°C, and 30 s at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and exposed for 2 h to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA).

PCR amplifications for AIB1 and REA were performed and PCR products were analyzed as described previously (13), with minor modifications. Briefly, 1 μ l of RT mixture was amplified in a final volume of 20 μ l, in the presence of 4 ng/ μ l of each primer and 0.3 unit of *Taq* DNA polymerase (Life Technologies, Inc.).

For AIB1, each PCR consisted of 30 cycles (30 s at 55°C, 30 s at 72°C, and 30 s at 94°C). For REA, each PCR consisted of 30 cycles (30 s at 57°C, 30 s at 72°C, and 30 s at 94°C). PCR products then were separated on agarose gels stained with ethidium bromide as described previously (13). Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (13). The identity of PCR products was confirmed by subcloning and sequencing, as reported previously (15).

Quantification of SRA Expression. Exposed screens were scanned using a Molecular Imager-FX (Bio-Rad) and the intensity of the signal corresponding to SRA was measured using Quantity One software (Bio-Rad). Three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the SRA signal of one particular tumor measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified, and after analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst (Bio-Rad). Three independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the same tumor as above. For each sample, the average of the SRA signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Quantification of the Relative Expression of the Deleted SRA Variant RNA. It has been shown previously that the coamplification of a wild-type and a deleted variant SRA cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (15). For each sample, the signal corresponding to the SRA Δ el was measured using Quantity One software (Bio-Rad) and expressed as a percentage of the corresponding core SRA signal. For each case, three independent assays were performed and the mean determined.

Quantification of REA and AIB1 Expression. After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst (Bio-Rad). At least, three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the REA or AIB1 signal of one particular sample and all signals were expressed as a percentage of this signal. For each sample, the average of REA or AIB1 signals was then expressed as a percentage of the average of the *GAPDH* signal (arbitrary units), as described above.

Statistical Analysis. Differences between normal samples and their matched tumors were tested using the Wilcoxon matched pairs test, two-tailed. Differences between the relative expression of cofactors (e.g., logAIB1:REA) obtained for matched normal and tumor compartments were also tested using the Wilcoxon matched pairs test, two-tailed. Correlation between SRA, REA, or AIB1 expression and tumor characteristics was tested by calculation of the Spearman coefficient *R*.

Results

Characterization of SRA and REA RNA Expression in Human Breast Tissues by *in Situ* Hybridization. SRA is functional as an RNA molecule (7), and because no antibodies are available for the immunohistochemical detection of REA, we have therefore used an *in situ* hybridization approach to determine the cellular localization of expression of SRA and REA RNA in human breast tissues. Fig. 1 shows examples of the results obtained. Antisense RNA probes to SRA showed a strong signal over the epithelial tumor cells of an ER+ human breast tumor section (Fig. 1A), with little, if any, signal obtained when sense SRA probes were used on the adjacent section of the same tumor (Fig. 1C). Low levels of SRA expression were detected mainly over the ductal epithelial cells of normal breast tissue from the same patient. This result paralleled that for AIB1, where it was previously shown using *in situ* hybridization that AIB1 mRNA expression was significantly increased in breast cancer cells carrying increased copies of the *AIB1* gene compared with normal breast epithelial cells, although it was not stated that these samples were from the same patient in this study (10). In contrast, when the *in situ* expression of REA mRNA was examined in an ER+ tumor and its matched adjacent normal breast tissue (Fig. 1, D and E, respectively), little difference could be seen between the signal over the epithelial breast tumor cells compared with the normal breast epithelial cells. Furthermore, little if any signal was observed when REA sense probes were used (Fig. 1F). These data suggested that the expression of the steroid receptor-specific coactivator, SRA, in addition to AIB1 (10) was significantly increased in breast tumor cells compared with normal breast epithelial cells, whereas the expression of a specific ER-repressor was not altered in breast tumors compared with normal breast epithelial cells. To investigate this further, we developed a semi-quantitative RT-PCR approach to measure the expression of these coregulators in multiple samples of ER+ breast tumors and their matched adjacent normal breast tissues, as described below.

Comparison of Expression of SRA and Deleted SRA in Adjacent Normal Breast Tissue and Matched Primary Breast Tumors. Previously we have detected two SRA PCR products of 662 and 459 bp in human breast tumors (14). Cloning and sequencing revealed the identity of the 662-bp fragment with the SRA core region (7) and the 459-bp fragment as a variant form of SRA deleted in 203 bp between positions 155 and 357 (numbered according to GenBank accession no. AF092038). The current analysis identified the 662-bp product in all breast tissue samples assayed. As well, a 459-bp product corresponding to the deleted SRA transcript was detected in the majority of tumors (*n* = 18) and normal samples (*n* = 17), always together with the 662-bp product (Fig. 2A). Therefore, core SRA is expressed in all human breast tissues, and expression of the deleted SRA is not tumor-specific.

To determine whether alterations in SRA expression occur during breast tumorigenesis, SRA RNA was measured in primary breast tumors and their adjacent matched normal breast tissues from 19 different patients (examples shown in Fig. 2A). The analysis was confined to tissues from women whose breast tumor was ER+ as determined by ligand-binding assays. SRA expression corrected for the *GAPDH* signal in each sample for all matched normal and tumor pairs is shown in Fig. 3A. The level of core SRA was significantly higher (Wilcoxon matched pairs test; *P* = 0.0004) in the tumors (median = 63 arbitrary units) compared with their adjacent normal tissue (median = 7 arbitrary units). When detected, expression of the deleted SRA relative to the core SRA was not significantly different between normal breast tissue and tumors (data not shown). These data suggested that core SRA expression is up-regulated, but the relative

SRA, AIB1, AND REA EXPRESSION DURING BREAST TUMORIGENESIS

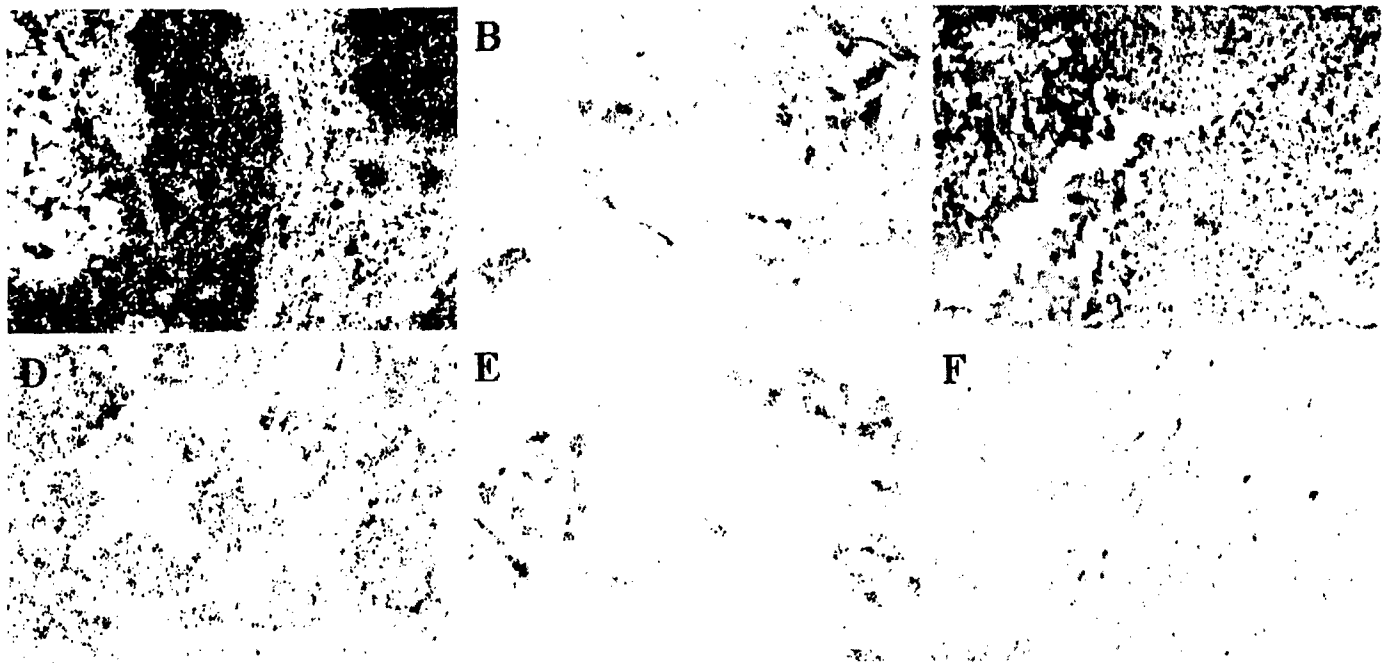


Fig. 1. *In situ* hybridization analyses of SRA and REA RNA expression in human breast tissue sections. Antisense SRA riboprobes (A and B) were used to detect SRA RNA expression in a section from an ER+ human breast tumor (A) and the matched normal breast tissue from the same patient (B). Sense SRA riboprobes were used as a specificity control, and the results from the adjacent section of the tumor shown in A are shown (C). Antisense REA riboprobes (D and E) were used to detect REA mRNA expression in a section from an ER+ human breast tumor (D) and the matched normal breast tissue from the same patient (E). Sense REA riboprobes were used as a specificity control and the results from the adjacent section of the tumor shown in D are shown (F). Magnification $\times 100$. Black dots/grains, hybridization signals; colored background, counterstaining.

expression of a deleted SRA is not altered, during breast tumorigenesis.

The level of core SRA in the tumor cohort used in this study was not correlated with PR status, grade, tumor size, or nodal status. However, the relative expression of the deleted SRA in the tumors was positively correlated with grade score (Spearman $R = 0.556$; $P = 0.0135$) and tumor size (Spearman $R = 0.655$; $P = 0.0023$), but not with PR or nodal status. These data suggested that increased relative expression of a deleted SRA is more likely to occur in those breast tumors with characteristics of a poorer prognosis, and may be associated with breast tumor progression.

Altered Expression of AIB1 mRNA between Breast Cancer and Adjacent Matched Normal Breast Tissues. To pursue further the possibility that an imbalance in expression of activators of ER action may occur during breast tumorigenesis, we investigated in the same samples the expression of another coactivator of ER activity, AIB1 (10). AIB1 is overexpressed in several human breast tumors (10, 16), although to our knowledge measurement of its RNA expression in a series of matched normal and breast tumor tissues was not reported previously. AIB1-specific primers amplified a predicted 349-bp fragment in normal breast tissues (Fig. 2B), in breast tumors (Fig. 2B), and in breast cancer cells (data not shown). Cloning and sequencing confirmed the identity of the 349-bp PCR product with AIB1 (10). Expression of AIB1 corrected for the *GAPDH* signal in each tissue sample for all of the matched pairs is shown in Fig. 3B. Expression of AIB1 mRNA was significantly higher (Wilcoxon matched pairs test; $P = 0.0058$) in tumor samples (median = 67.8 arbitrary units) compared with adjacent normal tissues (median = 36.6 arbitrary units). These data are consistent with previous data (10, 16) and suggest that expression of another ER coactivator is significantly increased during breast tumorigenesis. Expression of AIB1 in this tumor cohort was not correlated with PR status, grade, tumor size, or nodal status.

Detection of REA mRNA in Normal and Neoplastic Human Breast Tissues. To determine whether alterations in expression of a corepressor, *i.e.*, REA, also occurred during breast tumorigenesis, an RT-PCR approach was developed. The REA-specific primers amplified a predicted 397-bp fragment in normal breast tissues (Fig. 2C), in breast tumors (Fig. 2C), and in breast cancer cells (data not shown). Cloning and sequencing confirmed the identity of the 397-bp PCR product as REA (8). This product was used to probe Northern blots of RNA extracted from human breast cancer cells and breast tumor biopsies. An ~ 1.5 kb transcript was detected, consistent with the REA mRNA described previously (data not shown; Ref. 8).

To determine whether REA expression was potentially altered during breast tumorigenesis, REA mRNA levels were measured in ER+ breast tumors and their adjacent normal breast tissues (examples in Fig. 2C) from the same 19 different patients described above. REA expression corrected for the *GAPDH* signal (Fig. 2D) in each sample for all matched pairs is shown in Fig. 3C. REA expression was not significantly different (Wilcoxon matched pairs test; $P = 0.110$) in the tumors (median = 84.6 arbitrary units) compared with the adjacent normal tissues (median = 69.8 arbitrary units). REA expression in the tumors was not correlated with PR status, grade, tumor size, or nodal status.

Altered Relative Expression of Coactivators and Repressors during Human Breast Tumorigenesis. The above data suggest that alterations in the relative expression of ER activators and repressor occurred during breast tumorigenesis. To address this question, the relative expression of SRA and AIB1 mRNA to REA mRNA was compared between the breast tumors and the normal tissues. Results are shown in Fig. 4. The ratio of SRA:REA (Fig. 4A) was significantly higher (Wilcoxon matched pairs test; $P = 0.0003$) in tumors (median = 87 arbitrary units) compared with normal tissues (median = 12 arbitrary units). Similarly, the ratio of AIB1:REA (Fig. 4B) was significantly higher (Wilcoxon matched pairs test; $P = 0.0414$) in

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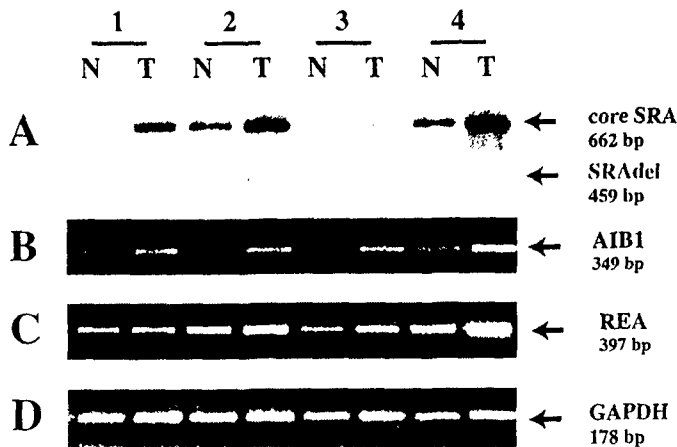
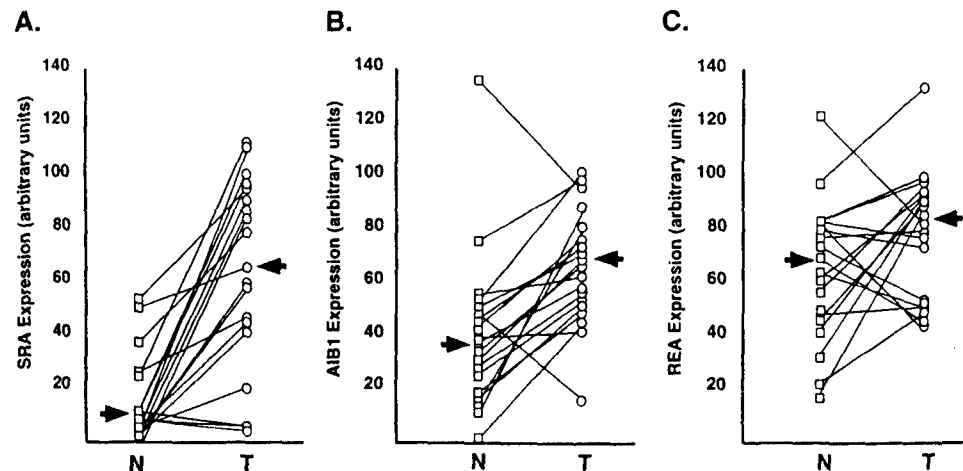


Fig. 2. A, detection of SRA and SRA Δ el in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA extracted from matched breast tumors and adjacent matched normal breast tissue was extracted from 19 different patients and assayed for SRA expression using RT-PCR as described in "Materials and Methods." PCR products were separated on 6% acrylamide gels, which were dried, exposed to phosphor-imaging screens, and scanned using a Molecular Imager-FX. A digitized image showing the results obtained from four sets of normal tissue (N) and matched tumor tissue (T) is shown. Arrows, the expected 662-bp core SRA PCR product (SRA core), confirmed by sequence analysis) and a 459-bp deleted SRA variant PCR product (SRA Δ el), which was identified by sequence analysis to correspond to an SRA variant deleted in sequences from position 155 to 357 (GenBank accession no. AF092038). B, detection of AIB1 in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA was extracted and assayed for AIB1 expression using RT-PCR as described in "Materials and Methods." After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of four sets of normal tissue (N) and matched tumor tissue (T) is shown. Arrows, the expected 349-bp AIB1 PCR product (confirmed by sequence analysis). C, detection of REA in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA was extracted and assayed for REA expression using RT-PCR as described in "Materials and Methods." After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of four sets of normal tissue (N) and matched tumor tissue (T) is shown. Arrows, the expected 397-bp REA PCR product (confirmed by sequence analysis). D, ethidium bromide-stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. Arrow, the expected 178-bp *GAPDH* PCR product.

tumors (median = 86.7 arbitrary units) compared with normal tissues (median = 61.3 arbitrary units). Furthermore, the ratio of SRA:AIB1 (Fig. 4C) was significantly higher (Wilcoxon matched pairs test; $P = 0.0058$) in tumors (median = 94.3 arbitrary units) compared with normal tissues (median = 22.8 arbitrary units), suggesting that the relative expression of ER coactivators may also change during breast tumorigenesis.

Fig. 3. Comparison of the expression of SRA, AIB1, and REA in adjacent normal breast tissue and matched primary breast tumors. For each patient ($n = 19$), SRA, AIB1, and REA expression was quantified and expressed in arbitrary units corrected for *GAPDH* signal as described in "Materials and Methods." The results are presented as a scatter graph. The normal samples are represented by $\square$ and the tumor samples by $\circ$. Each matched normal and tumor sample is joined by a line. Arrows, the median value in each group. A, the level of SRA expression in normal tissue is significantly different to the level of SRA expression in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.0004$). B, the level of AIB1 expression in normal tissue is significantly different from that in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.0058$). C, the level of REA expression in normal tissue is not significantly different from the level of REA expression in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.110$).



Discussion

In summary, although the RNA levels of two coactivators, SRA and AIB1, are significantly up-regulated in ER+ breast tumors compared with adjacent normal tissues, the RNA of a specific repressor of ER activity, REA exhibits no significant up-regulation during breast tumorigenesis in the same samples. These data are consistent with the hypothesis that factors enhancing ER activity are up-regulated in breast tumors, whereas factors repressing ER activity are not increased, providing a potential molecular basis for enhanced/altered estrogen action in human breast tumors. This is further supported by observations that the ratios of SRA:REA and AIB1:REA are increased in breast tumors compared with normal breast tissue. Interestingly, the increased relative expression of SRA:REA is greater (a 7.3-fold increase in median relative expression) than that for AIB1:REA (a 1.4-fold increase in median relative expression) between normal breast tissue and tumors, suggesting differentially altered expression of coactivators during breast tumorigenesis. This is supported by the observation that the ratio of SRA:AIB1 is also significantly increased in tumors (a 4.1-fold increase in median relative expression) compared with normal tissues.

SRA and AIB1 likely mediate their effects on ER activity via different mechanisms (7). SRA, unlike AIB1, functions as an RNA molecule (7). Also SRA requires the structurally and functionally distinct N-terminal/AF1 region of steroid receptors compared with AIB1, which requires the COOH-terminal/AF2 domain (6), possibly suggesting that estrogen target gene cascades could be differentially regulated by the relative expression of different coactivators. Therefore ER signaling could be altered during breast tumorigenesis. Such alterations during breast tumorigenesis are supported by the marked difference in breast epithelial growth responses to estrogen occurring during this process, i.e., from indirect in normal to direct in breast cancer cells (3-5).

It is the core region of SRA that is necessary and sufficient for the coactivator activity of SRA (7). Our primers for SRA (14) will detect all SRA isoforms containing core sequences, and we assume that our measurement of all intact core SRA-like RNAs correlates with total SRA activity present in any one tissue. These primers also detect a previously described isoform of SRA (GenBank accession no. AA426601) containing a deletion of sequences within the SRA core. Deletions within the core were reported previously to result in the loss of SRA activator function (7). It is likely that this deleted variant is inactive with respect to coactivator activity and could function to alter steroid signaling in breast tumors and contribute to the more aggres-

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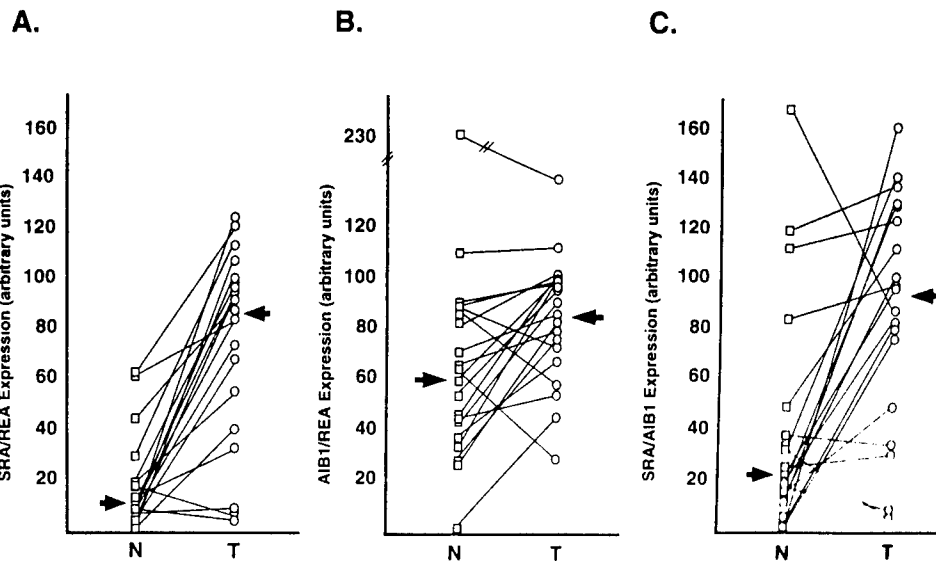


Fig. 4. Comparison of the relative expression of SRA, REA, and AIB1 in adjacent normal breast tissue and matched primary breast tumors. For each sample the expression of SRA, REA, and AIB1 has been quantified as described in "Materials and Methods," and the ratios SRA:REA (A), AIB1:REA (B), and SRA:AIB1 (C) have been calculated. The results are presented as a scatter graph. The normal samples are represented by $\square$ and the tumor samples by $\circ$. Each matched normal and tumor sample is joined by a line. Arrows, the median values in each group. A, the relative expression of SRA:REA expression in normal tissue is significantly different from that in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.0003$). B, the relative expression of AIB1:REA expression in normal tissue is significantly different from that in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.0414$). C, the relative expression of SRA:AIB1 expression in normal tissue is significantly different from that in the tumor tissues (Wilcoxon matched pairs test, two-tailed; $P = 0.0058$).

sive phenotype associated with poorer-prognosis tumors, which include characteristics such as high grade and large tumor size. A similar relationship of the relative expression of the deleted SRA and grade was also found in a previously described but separate breast tumor cohort (14).

Recently, REA was identified as a specific repressor of ligand-occupied ER ($ER\alpha$ and $ER\beta$, but not other steroid or nuclear receptors) transcriptional activity (8). Furthermore, part of its mechanism appeared to involve competition with coactivators such as SRC-1 (6). It differed from previously identified corepressors such as N-CoR/SMRT (6) because it was selective for ER as opposed to generally effecting members of the nuclear receptor family (8). Because REA was selective for ER, it was relevant to investigate it in breast tissues. Our data suggest that REA expression is not altered in breast tumors compared with normal breast tissues.

Although the assessment of expression by RT-PCR will only allow measurement of global expression of these genes in heterogeneous tissue sections, our *in situ* hybridization data support the conclusion that the major cell type expressing SRA or REA in breast tissue is the epithelial cell, either normal or neoplastic. Previous data have confirmed that AIB1 mRNA is expressed in the epithelial component of both normal and neoplastic breast tissue (10). Therefore, our RT-PCR results likely represent expression differences in the epithelial components of the tissues examined. Furthermore, SRA, AIB1, and REA were shown to be expressed in human breast cancer cell lines in culture (7, 8, 10). Our *in situ* hybridization data are consistent with the RT-PCR data as well. Although further study is needed to confirm the relation between ER and these cofactors within individual cells, the data support the hypothesis that relative changes between coactivators (SRA and AIB1) and a corepressor (REA) can occur in breast tumorigenesis *in vivo*, an important point required to provide *in vivo* relevance for several previously published studies concerning altered coactivators and coregulators using laboratory model systems. Parallel *in situ* studies of AIB1 and REA protein levels, but not SRA (active as an RNA molecule), are required to provide unequivocal evidence of the relative changes between coactivators and corepressors during breast tumorigenesis. Unfortunately, there are presently no commercially available antibodies to REA, and available AIB1 antibodies cannot be used for immunohistochemical analysis. However, the available data based on Western blot analysis of breast and ovarian cancer cell line extracts suggest that there is a quantitative relationship between AIB1 mRNA and protein levels (17, 18).

Recently, a study was published (19) in which both $ER\alpha$ and the coactivator TIF2 were found to be significantly increased in intraductal carcinomas compared with normal mammary gland tissue. This study suggested as well that $ER\alpha$ and a general corepressor N-CoR are reduced in invasive breast cancer compared with DCIS. Although these results are consistent with our data and support the hypothesis that there may be an up-regulation of factors associated with increased ER signaling in breast tumorigenesis, the number of cases screened was small compared with our study, the normal samples and DCIS samples were not matched, *i.e.*, were not from the same patient, to the invasive breast cancer samples, and furthermore not all tumors were $ER+$. These factors introduce biological heterogeneity because the natural history of $ER+$ and $ER-$ breast cancers are distinct, and it is likely that the factors involved in the development of $ER-$ versus $ER+$ breast cancer are different. Also, the lack of matched samples with respect to comparisons among normal, intraductal, and invasive breast cancer introduces significant issues associated with patient-to-patient variability with respect to alterations which may be influenced by age and menopausal and other hormonal status, and may be significantly different between the groups compared and therefore confound the interpretation of the results.

We have used matched normal and breast cancer tissues as surrogates for breast tumorigenesis; however, it is acknowledged that breast tumorigenesis is a complex process, and an investigation of different morphological lesions thought to parallel the evolution of normal breast tissue to invasive breast cancer is necessary before more definite conclusions can be made. However, this study is the first, to our knowledge, that uses multiple matched samples of normal breast tissue and their $ER+$ tumors, and provides evidence that the relative expression of coactivators and corepressors, which are highly relevant with respect to the ER signal transduction pathway, can be significantly altered between normal human breast and breast tumors *in vivo*.

In conclusion, although our study is small, the results presented are consistent with the hypothesis that a significant up-regulation of ER signaling occurs during breast tumorigenesis in $ER+$ tumors. This is reflected not only in the increased expression of $ER\alpha$ shown previously, but now also in an increase in factors that can activate ER activity without a concomitant increase in factors that can repress ER activity. Despite the obvious need to study protein levels where appropriate, when reagents become available, the possibility now exists that an imbalance in the expression of repressors and activators of $ER\alpha$ can occur during human breast tumorigenesis *in vivo* and may

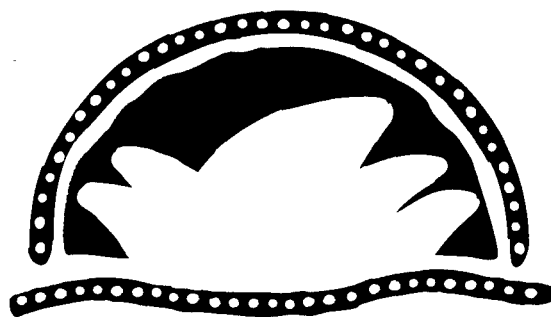
SRA, AIB1, AND REA EXPRESSION DURING BREAST TUMORIGENESIS

contribute to altered estrogen action, which is known to occur during this process.

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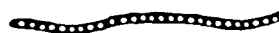


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OVEREXPRESSED PITUITARY TUMOR TRANSFORMING GENE (PTTG) MEDIATES EARLY THYROID TRANSFORMATION AND IS ASSOCIATED WITH FOLLICULAR LESIONS.

Heaney A P*, Nelson V, Horwitz G A, Fernando M, Melmed S. Cedars-Sinai Research Institute-UCLA School of Medicine, Los Angeles, CA.

Pituitary Tumor Transforming Gene (*PTTG*), isolated from GH-secreting adenomas, exhibits *in vitro* and *in vivo* transforming activity and regulates bFGF secretion. Preservation of a C-terminal proline-rich motif (P-X-X-P) is crucial for these functions. As *PTTG* facilitates chromatid separation during mitosis, *PTTG* overexpression may cause aneuploidy, thereby increasing cell susceptibility to oncogenic events. We demonstrated high *PTTG* expression in colorectal tumors, where highest *PTTG* expression was observed in invasive carcinomas. We also observed that *PTTG* is regulated by estrogen, and as thyroid cancer shows a striking female preponderance, and well-characterized phenotypes can be readily identified, we examined *PTTG* expression in 31 thyroid tumors, and matched normal thyroid tissue. In human tumors, increased *PTTG* expression (≥ 2 SD) was observed in 9/13 cases of thyroid hyperplasia (mean $\pm$ SEM *PTTG* fold-increase, 1.9 ± 0.53), 7/9 follicular adenomas (*PTTG* fold-increase, 1.9 ± 0.3), 1/1 follicular carcinoma (*PTTG* fold-increase, 1.4) but only 3/6 papillary carcinomas (*PTTG* fold-increase, 0.84 ± 0.15). In FRTL5 thyroid cells, TSH (10-20 mU/l) induced a 4-fold increase in *pttg* mRNA expression and FRTL5 transfectants overexpressing human *PTTG* formed colonies in soft agar (colony no. mean $\pm$ SEM; vector, 10 ± 3.2 ; wt-*PTTG*, 55 ± 8.6 ; mut-*PTTG*, 9.3 ± 5.4 , $p < 0.001$) and showed increased PCNA immunostaining, compared to cells transfected with vector alone or *PTTG*-constructs bearing mutations in the P-X-X-P motif. Conclusions: *PTTG* is regulated by TSH, transforms thyroid cells *in vitro* and *PTTG* is differentially overexpressed in follicular compared to papillary thyroid cancers. We propose that *PTTG* overexpression in follicular neoplasms leads to increased LOH events, relatively common in thyroid follicular lesions, but infrequent in papillary thyroid cancers. *PTTG* signaling is therefore important as a determinant of the divergence of follicular versus papillary thyroid cancer phenotypes.

OR198

PTOV1, a novel protein overexpressed in prostate cancer, contains a new class of protein modules

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We have isolated and characterized a gene and its encoded protein, not described previously, which we have called PTOV1 (prostate tumor overexpressed).

The cDNA for *PTOV1* was identified in differential display experiments, and shown to be overexpressed in prostate cancer, as determined by semiquantitative RT-PCR. Specific antibodies to PTOV1 were generated, which allowed to study the subcellular localization of this protein, as well as its *in situ* expression in normal and tumor prostate. The subcellular localization of PTOV1 was further confirmed by means of *in vitro* expression of chimeric GFP-PTOV1 constructs. Immunohistochemistry with anti-PTOV1 antibodies confirmed its overexpression in prostate tumors, which affected areas of carcinoma cells as well as areas characterized as prostate intraepithelial neoplasia (PIN), the first morphologically recognizable neoplastic lesion in prostate cancer. Therefore, PTOV1 has been found overexpressed both in early and late stages of prostate cancer.

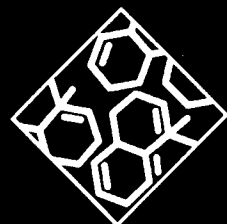
The *PTOV1* gene was isolated, sequenced, and its structure characterized. It consists of 12 exons, and it is localized in chromosome 19q13.3. *PTOV1* mRNA was found in a range of normal human tissues, including brain, heart, skeletal muscle, kidney and liver, with expectedly low levels in normal prostate. Expression of *PTOV1* was found upregulated by androgens *in vitro*. The structure of the PTOV1 protein is remarkable, in that it consists almost entirely of a tandemly repeated domain, separated by a short linker peptide. We have identified a *Drosophila* homolog of PTOV1, which shows also the same repeated domain structure. Furthermore, we have identified a second PTOV domain. We have given the name PTOV2 to this protein. Human and *Drosophila* PTOV2 contain regions extending towards their amino and carboxy termini which show conservation between both organisms, and containing polyglutamine-rich stretches. Extensive database searches have not allowed the identification of any known proteins, domains or motifs with significant similarities to the PTOV domains. Therefore, the PTOV domain is a new class of protein modules present in human, rodent and fly proteins.

OR200

ALTERED EXPRESSION OF ESTROGEN RECEPTOR (ER) COREGULATORS DURING HUMAN BREAST TUMORIGENESIS.

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The hypothesis that altered expression of ER coactivators & corepressors occurs during human breast tumorigenesis *in vivo* is examined in this study. Using quantitative reverse transcription polymerase chain reaction assays, the expression of two coactivators Steroid Receptor RNA Activator (SRA), and Amplified In Breast Cancer 1 (AIB1) and a repressor, Repressor of Estrogen Receptor Activity (REA) were compared between adjacent normal breast tissue and their matched breast tumors. The level of SRA was significantly higher ($n = 19$, Wilcoxon matched test, $P = 0.0004$) in the tumors (median = 63 arbitrary units) compared to their adjacent normal tissue (median = 7 arbitrary units). In the same samples the level of AIB1 mRNA was significantly higher (Wilcoxon, $P = 0.0058$) in tumor samples (median = 67.8 arbitrary units) compared to adjacent normal tissues. In contrast, the level of REA mRNA was not significantly different ($n = 19$, Wilcoxon, $P = 0.0004$) in the tumors (median = 84.6 arbitrary units) from the adjacent normal tissues (median = 69.8 arbitrary units). When the relative expression of SRA and AIB1 mRNA to REA mRNA was compared between breast Tumors and normal tissues, the ratio of SRA/REA was significantly higher (Wilcoxon, $P = 0.0003$) in tumors (median = 37 arbitrary units) compared to normal tissues (median = 12 arbitrary units). Similarly, the ratio of AIB1/REA was significantly higher (Wilcoxon, $P = 0.0414$) in tumors (median = 86.7 arbitrary units) compared to normal tissues (median = 61.3 arbitrary units). Interestingly, the relative expression of SRA/REA was higher (Wilcoxon, $P = 0.0058$) in tumors compared to normal tissues, suggesting the possibility that coactivators with different mechanisms of action were differentially altered during tumorigenesis. Our data support the above hypothesis and suggest that such alterations may have a role in altered estrogen action that occurs during breast tumorigenesis.



hormones and cancer 2000



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	Friday 3rd November
1700-	Registration Radisson Reef Resort
1930- 2130	WELCOME FUNCTION: COCKTAIL PARTY AT RADISSON REEF
	Saturday 4th November
0815	Welcome
0830	<i>Session 1 - Hormones and Cancer Risk</i> Chair: Graham Giles / John Hopper Ron Ross: Epidemiology of breast & prostate cancer James Yager: Oxidative metabolism of estrogens: role in estrogen mediated carcinogenesis Wayne Tilley: Structure and function of the androgen receptor in advanced prostate cancer Gerry Coetzee: Androgen receptor CAG repeat length and breast and prostate cancer risk
1030	COFFEE
1100	<i>Session 2 - Receptor structure, isoforms and function</i> Chair: Dinny Graham/Gerry Coetzee Benita Katzellenbogen: Estrogen receptors: selective ligands, coregulators, and regulated genes in breast cancer Chris Clarke: Expression of progesterone receptors A and B in normal and malignant breast and uterus Geof Green: Lessons learned from the structures of ER α and ER β bound to SERMs
1230	Lunch and Poster Viewing
1400	Plenary 1 Chair: Chris Clarke Kathryn Horwitz: Issues related to Tamoxifen resistance and progesterone gene regulation in breast cancers
1450	<i>Session 3 - Receptor interacting proteins and cancer</i> Chair: Peter Leedman/Tom Ratajczak Michael Stallcup: Cooperative roles for multiple coactivators in steroid receptor signaling and hormonal carcinogenesis Malcolm Parker: Role of p160 coactivators in transcriptional activation by estrogen receptors and cross-coupling to other signalling pathways
1550	COFFEE
1615	<i>Session 4 - Mechanisms of hormone resistance</i> Chair: Richard Santen/Jacky Bentel Rob Nicholson: Endocrine response mechanisms in breast cancer: Role of EGFR signalling Suzanne Fuqua: Estrogen action in premalignant and invasive breast cancer Leigh Murphy: Estrogen receptors (ERs) in human breast tumorigenesis and breast cancer progression
	EVENING FREE

Oestrogen receptors (ERs) in human breast tumorigenesis and breast cancer progression

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During human breast tumorigenesis enhanced and/or altered activity of the ER signaling pathway is thought to occur and be a major driving force in breast tumorigenesis. The assumption derives from the observations that only a minority of normal human breast epithelial cells have detectable ER (7 - 17% ER positive ductal epithelial cells) although greater than 70% of primary breast cancers are ER positive. As well, the majority of proliferating cells in normal human breast tissue is ER negative and estrogen only indirectly effects proliferation in normal mammary tissues. However, estrogen can directly cause proliferation of ER⁺ breast cancer cells and many proliferating cells in breast tumors are ER⁺. Additional alterations in ER signaling are thought to occur during breast cancer progression since many ER⁺ breast tumors are de novo resistant to endocrine therapies and a significant proportion of tumors which originally respond to endocrine therapy, develop resistance to endocrine therapies despite the continued expression of ER. In order to understand the mechanisms underlying these alterations during human breast tumorigenesis and breast cancer progression *in vivo*, we have investigated ER isoforms expressed in human breast tissues and the expression of several coregulators of ER transcriptional activity in human breast tissue. Using semi-quantitative reverse transcription polymerase chain reaction assays (RT-PCR), the expression of ER α , ER β , two coactivators (SRA, AIB1) and one corepressor (REA) of ER activity was compared between ER⁺ breast tumors and their matched adjacent normal human breast tissues. We found that ER α , ER β , and their variant mRNAs are expressed in both normal and neoplastic breast tissues. The relative expression of ER α / ER β is significantly altered between ER⁺ breast tumors and their matched adjacent normal breast tissues. Further, this increase in ER α / ER β ratio is primarily due to a significant increase in ER α mRNA expression in conjunction with a lower ER β mRNA expression in the tumor compared to normal tissue in some but not all ER⁺ cases. Furthermore, the relative expression of both ER α and ER β variant mRNAs changes during breast tumorigenesis. In addition, the levels of the two ER coactivators, SRA and AIB1 mRNA are increased in tumors compared to normal tissues. In contrast, the expression of the ER corepressor, REA mRNA is not significantly different between tumors and normal tissues. These results have been confirmed using *in situ* hybridization analyses. Consistent with these data, the ratios of AIB1/REA and SRA/REA are higher in tumors compared to normal tissues. Furthermore, the ratio of SRA/AIB1 is higher in tumors compared to normal tissues. As well, we have found that the expression of ER β , SRA and REA varies amongst human breast tumors. The expression of several of these modulators of ER α activity was correlated with known treatment response markers, other known prognostic variables and likely tamoxifen sensitivity in human breast cancer. These results suggest that not only are there alterations in ER isoform expression during breast tumorigenesis and breast cancer progression but there are also alterations of the relative expression of coregulators of ER activity during breast tumorigenesis and breast cancer progression *in vivo*. These changes may have a role in altered estrogen action occurring during breast tumorigenesis and breast cancer progression.

2

Multiple Facets of Estrogen Receptor in Human Breast Cancer

*Leigh C. Murphy, PhD, Etienne Leygue, PhD,
Helmut Dotzlaw, PhD, Amanda Coutts, PhD,
Biao Lu, MSC, Aihua Huang, MSC,
and Peter H. Watson, MB*

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INTRODUCTION

Estrogen is a major regulator of mammary gland development and function, and affects the growth and progression of mammary cancers (1,2). In particular, the growth responsiveness of breast cancer (BC) cells to estrogen is the basic rationale for the efficacy of the so-called endocrine therapies, such as antiestrogens. Estrogens mediate their action via the estrogen receptor (ER), which belongs to the steroid/thyroid/retinoid receptor gene superfamily (3). The protein products of this family are intracellular, ligand-activated transcription factors regulating the expression of several gene products, which ultimately elicit a target tissue-specific response (4). Indeed, ER, together with progesterone receptor (PR), expression in human breast tumors, are important prognostic indicators, as well as markers of responsiveness to endocrine therapies (5,6). However, although the majority of human BCs are thought to be initially hormone-responsive, it is well appreciated that alterations in responsiveness to estrogen occurs during breast tumorigenesis. During BC progression, some ER-positive BCs are *de novo* resistant to endocrine therapies, and of those that originally respond to antiestrogens, many develop resistance. This progression from hormonal dependence to independence is a significant clinical problem.

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because it limits the usefulness of the relatively nontoxic endocrine therapies, and is associated with a more aggressive disease phenotype (7). This occurs despite the continued expression of ER, and often PR (8,9). The ER is pivotal in estrogen and antiestrogen action in any target cell, but the nature of the ER is clearly multifaceted.

Until recently, it was thought that only one ER gene existed. However, a novel ER, now referred to as ER β , has recently been cloned and characterized (10,11). Moreover, it has recently been shown that ER β mRNA is expressed in both normal and neoplastic human breast tissue (12-14). This suggests that ER β may have a role in estrogen action in both normal and neoplastic human breast tissue. Furthermore, it has now become apparent that several variant mRNA species of both the classical ER α and ER β can be expressed in human breast tissues, and may therefore have roles in estrogen and antiestrogen signal transduction (13,15-18). The current data suggest that an evaluation of estrogen interaction with human breast tissue needs to include ER α , ER β , and any variant forms of these receptors that may be expressed. The following chapter focuses on the multifaceted nature of the ER in human breast tissues.

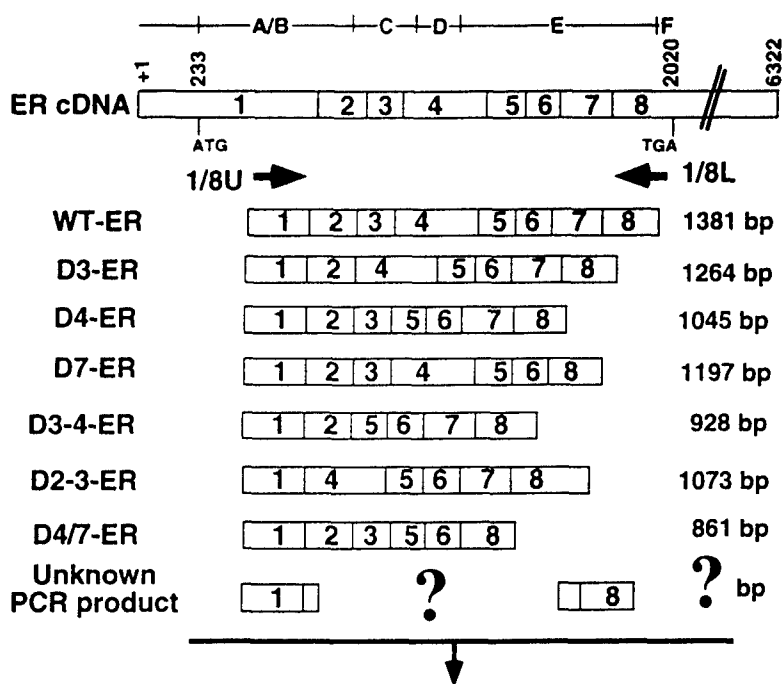
ER α AND ITS VARIANTS

Identification of ER α Variant mRNAs in Human Breast Tissues

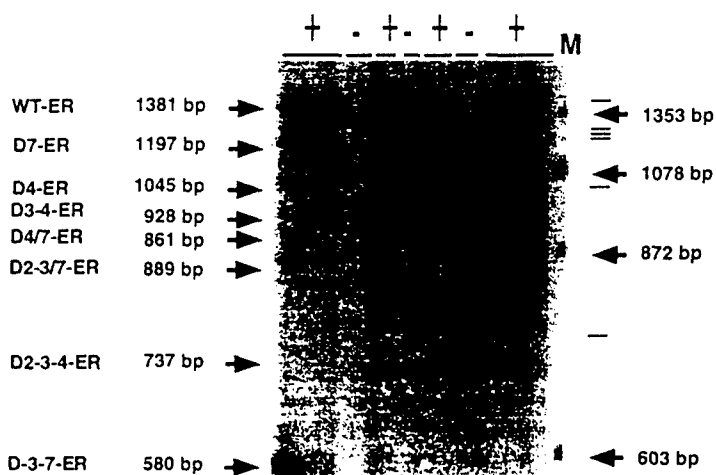
A large body of data has accumulated supporting the existence of ER α variants (19,20). The majority of the data supporting the expression of ER α variants has been at the mRNA level. Two main structural patterns of ER α variant mRNAs have been consistently identified: the truncated ER α mRNAs (21) and the exon-deleted ER α mRNAs (22). The truncated ER α mRNAs were originally identified, by Northern blot analysis, as fairly abundant smaller-sized mRNA species in some human BC biopsy samples (23). The cDNAs of several truncated ER α mRNAs have been cloned and found to contain authentic polyadenylation signals followed by poly(A) tails. The exon-deleted ER α mRNAs have been identified mostly from reverse transcription polymerase chain reaction (RT-PCR) products, using targeted primers.

Multiple ER α variant mRNAs are often detected in individual tumor specimens. In order to determine the relative frequency and pattern of variant expression in a particular sample, an RT-PCR approach was developed that allowed the simultaneous detection of all deleted ER α variant mRNAs containing the primer annealing sites in exons 1 and 8, at levels that represent their initial relative representation in the RNA extract. Since truncated transcripts do not have exon 8 sequences, they will not be measured by this technique. Examples of the results obtained are shown (Fig. 1), and serve to illustrate that

Fig. 1. Top panel. Schematic representation of WT ER α (WT-ER) cDNA and primers allowing co-amplification of most of the described exon-deleted ER α variants. ER α cDNA contains eight different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in transactivating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (AF-2). 1/8U and 1/8L primers allow amplification of 1381-bp fragment corresponding to WT ER α mRNA. Co-amplification of all possible exon-deleted or -inserted variants, which contain exon 1 and 8 sequences, can occur. Amplification of the previously described ER α variant mRNAs deleted in exon 3 (D3-ER), exon 4 (D4-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and exon 7 (D4/7-ER), would generate 1264-, 1045-, 1197-, 928-, 1073-, and 861-bp fragments, respectively. **Bottom panel.** Co-amplification of WT ER α and deleted variant mRNAs in breast tumor samples. Total RNA extracted from ER-positive (+) and ER-negative (-) breast tumors was reverse-transcribed and PCR-amplified, as described (24), using 1/8U



**PCR Co-amplification of WT-ER
and all known and unknown
deleted-ER variant mRNAs**



and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel, and visualized by autoradiography. Bands reproducibly obtained within the set of tumors studied, and which migrated at 1381, 1197, 1045, 928, 889, 861, 737, and 580 bp, were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3, and 4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by dashes (-), barely detectable within the tumor population, i.e., present in less than or equal to three particular tumors, have not yet been identified. M, Molecular weight marker (phi 174, Gibco-BRL, Grand Island, NY). Adapted with permission from ref. 24.

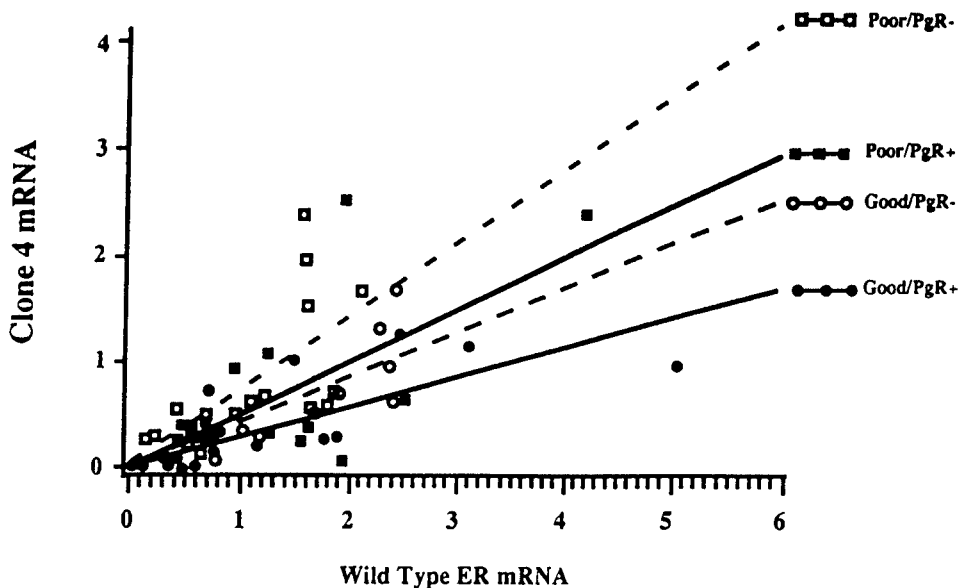


Fig. 2. Linear regression analysis of the relationship between the clone-4-truncated ER α mRNA and the WT ER α mRNA in the various groups. Closed circles represent the good prognosis/ER-positive-PR-positive group; open circles represent the good prognosis/ER-positive-PR-negative group; closed squares represent the poor prognosis/ER-positive-PR-negative group; open squares represent the poor prognosis/ER-positive-PR-negative group. Good vs Poor, $P = 0.0004$; PR-negative vs PR-positive, $P = 0.011$. Reproduced with permission from ref. 25.

a complex pattern of exon-deleted variant ER α transcripts are expressed in any one tumor, that the pattern and relative frequency of detection of ER α variant mRNAs may vary between tumors, and that, in some cases, the relative frequency of detection of individual ER α variant mRNAs may be correlated with known prognostic markers (24).

An example of such a correlation is shown in Fig. 2 (25). The expression of the truncated clone-4 ER α variant mRNA was measured relative to the wild-type (WT) ER α mRNA in a group of breast tumors. The relative expression of the clone-4 variant was significantly increased in those tumors with characteristics of poor prognosis, compared to those tumors with good prognostic characteristics, i.e., clone-4 expression was higher in large tumors with high S-phase fraction, and from patients with nodal involvement, compared to small tumors with low S-phase fraction from patients without nodal involvement. Also, in this group, the relative expression of clone-4 was significantly higher in PR-negative tumors vs PR-positive tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance.

Data support the possibility that ER α variant proteins exist, and that their pattern and frequency are different from different individuals. In some cases, the expression of single ER α variant mRNA species was correlated with known markers of prognosis and endocrine sensitivity. This, in turn, suggested the hypothesis that altered expression of ER α variants may be a mechanism associated with progression to hormone independence.

Putative Biological Significance of ER α Variant mRNAs

EXPRESSION OF ER α VARIANT mRNAs IN NORMAL AND NEOPLASTIC HUMAN BREAST TISSUE

Most studies investigating ER α variant mRNAs have used human BC tissues or cell lines (19). However, it is now known that both truncated and exon-deleted ER α variant

mRNAs can be detected in other tissues, including normal tissues (19). In particular, ER α variant mRNAs have been identified in normal human breast tissue and cells (26–29). Therefore, ER α variant mRNAs are not tumor-specific, are not found in the complete absence of the WT ER α mRNA, and are probably generated by alternative splicing mechanisms.

These observations raised the question of whether the expression of ER α variant mRNAs is altered during breast tumorigenesis and/or progression. When the level of expression of individual variant ER α mRNAs was measured relative to the level of the WT ER transcript, differences between normal and breast tumor tissues were found. The relative expression of clone-4-truncated ER α variant mRNA and the exon-5-deleted ER α variant mRNA, but not the exon-7-deleted ER α variant mRNA, was significantly increased in breast tumors, compared to normal breast tissues obtained from both reduction mammoplasties and normal tissues adjacent to breast tumors (26,27). Preliminary data suggests that this is also true for samples of ER-positive breast tumors and their matched, adjacent normal tissues (29a); there is also evidence suggesting that an exon-3-deleted ER α variant mRNA is decreased in BCs, compared to normal human breast epithelium (29). Because this ER α variant mRNA encodes a protein that can inhibit WT ER α transcriptional activity (30) and causes growth suppression when stably overexpressed in ER-positive MCF-7 human BC cells (29), it was concluded that the exon-3-deleted ER α variant may function to attenuate estrogenic effects in normal mammary epithelium. This function is markedly reduced via decreased exon-3-deleted ER α expression during breast tumorigenesis. In preliminary studies of ER-positive human breast tumor samples and their matched adjacent normal tissues, a statistically significant decreased relative expression of the exon-3-deleted ER α mRNA in the tumor, compared to the normal breast tissues, was noted (29a).

The available data provide evidence for an extensive and complex pattern of alternative splicing associated with the ER α gene, which may be altered during breast tumorigenesis.

SPECIFICITY OF ER α SPLICE VARIANTS IN HUMAN BREAST TUMORS

It is unlikely that the mechanisms generating alternatively spliced forms of ER α result from a generalized deregulation of splicing processes within breast tumors, since similar variants for the glucocorticoid receptor (16,28), the retinoic acid receptors- α and - γ (28), and vitamin D₃ receptor (16) have not been found in breast tumor tissues. However, similar splice variants of PR (*see* subheading Expression of Other Steroid Hormone Receptors, below) were found in both normal and neoplastic breast tissues (31,32).

EXPRESSION OF ER α VARIANT MRNAs DURING BC PROGRESSION

As described above, the relative expression of at least one ER α variant mRNA, i.e., clone-4-truncated ER α mRNA, is significantly higher in primary breast tumors with characteristics of poor prognosis (including the presence of concurrent lymph node metastases), compared to primary tumors with good prognostic markers (including lack of concurrent lymph node metastases) (25). An increased relative expression of exon-5-deleted ER α mRNA has been found in locoregional BC relapse tissue (in the same breast as the original primary tumor, but no lymph node metastases) obtained from patients following a median disease-free interval of 15 mo, compared to both the corresponding primary breast tumor (33) and the primary breast tumor tissue of patients who did not relapse during this period. Although the difference did not reach statistical significance,

these same authors reported a trend toward higher relative expression of exon-5-deleted ER α mRNA in primary tumors of women who relapsed, compared to primary tumors of those that did not relapse. Together, these data suggest that, in addition to altered expression of ER α variant mRNA, which occurs during breast tumorigenesis, further changes in ER α variant expression may occur during BC progression. However, another study (34) has recently found no significant differences in the relative expression of clone-4-truncated, exon-5-deleted, and exon-7-deleted ER α mRNAs, between a series of primary breast tumors and their matched concurrent lymph node metastasis, suggesting that altered expression of ER α variant mRNAs probably occurs prior to the acquisition of the ability to metastasize, and therefore may be a marker of future metastatic potential. This hypothesis remains to be tested.

EXPRESSION OF ER α VARIANT MRNAs AND ENDOCRINE RESISTANCE

The hypothesis that altered forms of ER α may be a mechanism associated with endocrine resistance has been suggested for some time. Moreover, the identification of ER α variant mRNAs in human breast biopsy samples (23,35,36) provided good preliminary data for the hypothesis. In addition, preliminary functional data of the recombinant exon-5-deleted ER α protein suggested that it possessed constitutive, hormone-independent transcriptional activity that was about 15% that of the WT ER (36). The data using a yeast expression system were also consistent with the correlation of relatively high levels of exon-5-deleted ER α mRNA in several human BC biopsy samples classified as ER-negative and PR-positive and/or pS2-positive (36–38). It was also found that the exon-5-deleted ER α mRNA was often co-expressed at relatively high levels with the WT ER α in many human BC that were ER-positive (38). It has been observed that transiently expressed exon-5-deleted ER α has an inhibitory effect on endogenously expressed WT ER α in MCF-7 human BC cells (39), although it does not decrease the WT activity to the same extent as hydroxytamoxifen. In contrast, in human osteosarcoma cells, exon-5-deleted ER α was shown to have little effect alone, but significantly enhanced estrogen-stimulated gene expression by transiently co-expressed WT ER α (40). The limitations of transient expression analysis were addressed by two groups who stably overexpressed the exon-5-deleted ER α in MCF-7 human BC cells (41,42). However, different phenotypes were obtained by the two groups. No effect of the recombinant exon-5-deleted ER α on growth or estrogen/antiestrogen activity in MCF-7 cells was found in one study (41); in the other study (42), the overexpression of recombinant exon-5-deleted ER α in MCF-7 cells was associated with estrogen-independent and antiestrogen-resistant growth. The reasons for the differences between the two studies are unclear, but may be the result of different MCF-7 variants, or changes that could have occurred in the transfectants in addition to transgene expression. The transgene in the Rea and Parker study (41) was episomally maintained; in the study by Fuqua et al. (42), the transgene was presumably integrated into the host chromosomes in a random fashion.

Several laboratories have developed cell culture models of estrogen independence and antiestrogen resistance. Variable results have been obtained when the association of altered ER α variant mRNA expression with estrogen/antiestrogen responsiveness was investigated. An increased relative expression of an exon-3 + 4-deleted ER α variant mRNA was found in an estrogen-independent MCF-7 cell line (T5-PRF) derived by long-term growth in estrogen-depleted medium (43,44). However, this cell line was still sensitive to antiestrogens (43). Although one cell line that was tamoxifen (TAM)-resistant had

differential expression of an exon-2-deleted ER α and an exon-5-deleted ER α mRNA, compared to the parental cell line (45), other independently derived antiestrogen-resistant clones showed no major differences in the expression of ER α variant mRNAs (46,47).

Investigation of ER α splice variants, using clinical tissue samples, has also led to variable conclusions. The relative expression of the clone-4-truncated ER α variant mRNA was significantly increased in primary breast tumors with characteristics of poor prognosis, compared to tumors with good prognostic characteristics (25). Similarly, the relative expression of clone 4 was significantly higher in PR-negative vs PR-positive tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance (25). Furthermore, an increased frequency of detection of ER α variant mRNAs deleted in exons 2–4 and 3–7 was associated with high tumor grade, but an increased detection of an exon-4-deleted ER α variant mRNA was associated with low tumor grade (24). The presence of exon-5-deleted ER α mRNA was found in one study (39) to be associated with increased disease-free survival. However, no difference in the relative expression of an exon-5-deleted ER α variant mRNA was found between all TAM-resistant tumors and primary control breast tumors (37), although, in the subgroup of TAM-resistant tumors that were ER-positive/pS2-positive, the relative expression of the exon-5-deleted ER α was significantly greater than the control TAM-sensitive group.

Although increased expression of any one ER α variant does not correlate with TAM resistance of BCs overall, its association with, and therefore possible involvement in, endocrine resistance in some tumors cannot be excluded. Moreover, the presence of multiple types of ER α variant mRNAs in any one tumor or normal tissue sample has been well documented (24,28), but no data have been published in which total ER α splice variant expression has been analyzed in relationship to endocrine resistance and prognosis. Although mutations have been found in the ER α gene in human breast tumors, they are rare and are not more frequent in TAM-resistant tumors (48).

IDENTIFICATION OF ER α VARIANT PROTEINS

The detection of proteins that correspond to ER α variant mRNAs remains an important issue. It is relevant, therefore, to understand the structure of these proteins. The predicted proteins of some of the most frequently detected ER α variant transcripts are shown schematically in Fig. 3. All of the variant transcripts would encode ER α proteins missing some structural/functional domains of the WT ER α . Although the ER α variant transcripts encode several different types of protein, there are some common themes that emerge. A common feature of these putative proteins is the universal presence of the A/B region, which is known to contain the cell and promoter specific AF-1 function. Exon-4-deleted and exon-3 + 4-deleted ER α mRNAs are in frame and encode proteins that do not bind ligand. However, the majority of the most abundantly expressed variant transcripts, i.e., exon-7-deleted, an exon-4 + 7-deleted, and the clone-4-truncated ER α mRNAs, encode proteins that are C-terminally truncated, and cannot bind ligand. Thus, a common feature of these variants is the inability to bind ligand. The results obtained, in which recombinant techniques were used to measure the function of individual ER α variants *in vitro*, are variable, and often depend on co-expression of the WT receptor. It is difficult to make general conclusions, but many recombinant ER α variant proteins have been observed to modulate the activity of the WT receptor. However, the relevance of the relative levels of expression of WT and variant ER α proteins that are achieved under the experimental conditions used is unclear, because limited data have been published on the

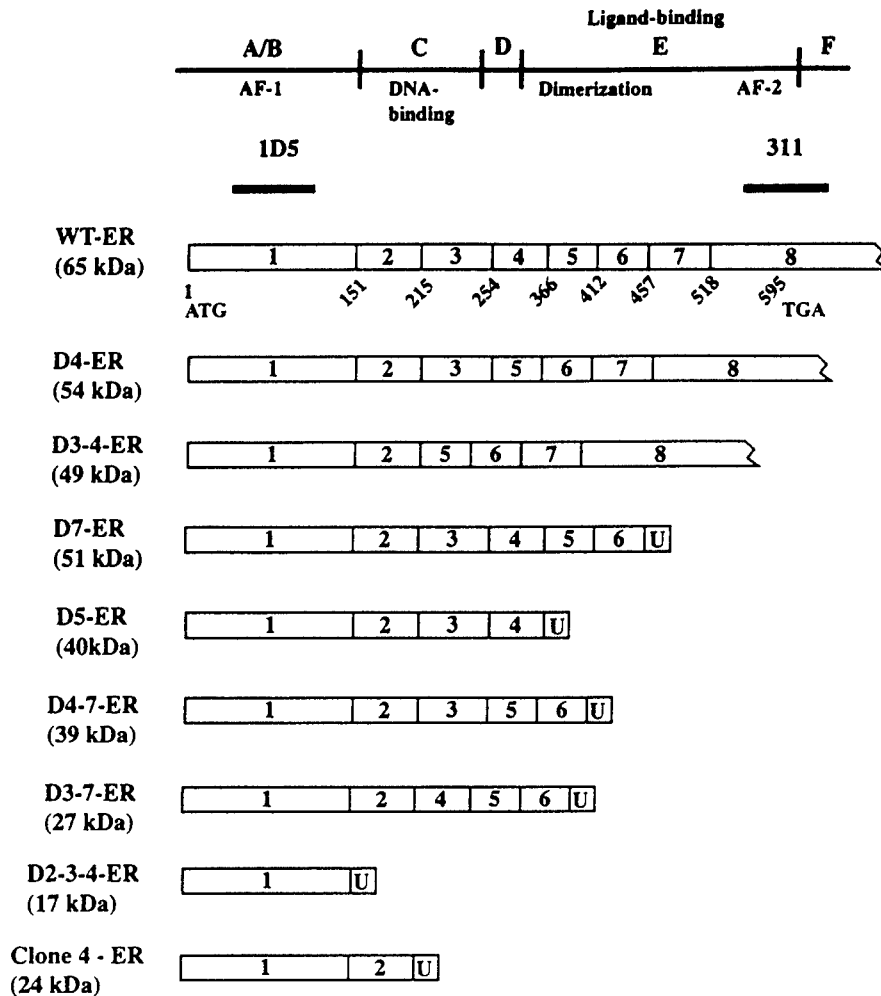


Fig. 3. Schematic representation of the ER α variant proteins predicted to be encoded by ER α variant mRNAs. Identical sequence is depicted by numbered exons. U, amino acid sequence unrelated to WT human ER α amino acid sequence. U sequences are unique to any particular variant. The position of N- and C-terminal epitopes, recognized by 1D5 and AER311 Abs, respectively, are indicated.

detection of ER α variant proteins encoded by known ER α variant mRNAs in tissues or cells in vivo.

From a different perspective, the prediction that the majority of ER α variant proteins are C-terminally truncated has implications for the determination of clinical ER status. Early detection, and changes in clinical practice, have resulted in smaller amounts of breast tumor tissue being available for assay. For this and other reasons, the use of immunohistochemistry (IHC) methods to assess ER status is becoming more common. Therefore, depending on the antibodies (Abs) used, the presence of C-terminally truncated ER α variant proteins could theoretically influence determination of ER status of the tumor sample. The authors have tested this experimentally, by transiently transfecting WT ER α and clone-4-truncated ER α expression vectors into Cos-1 cells, and determining ER status of the cells, using Abs either to the N-terminus of the ER α (Fig. 3, 1D5, Dako) or Abs to the C-terminus (Fig. 3, AER311, Neomarkers). Preliminary data, using

different combinations of WT ER α and variant ER α expression vectors transfected into Cos-1 cells, indicate that the signals (expressed as H-scores, which take into account the intensity of staining and the number of positively staining cells) obtained with the N-terminal and C-terminal Abs, become increasing discrepant (N-terminal > C-terminal signal) with increasing variant expression, presumably because of increased ER α -like proteins containing the N-terminal region, but not the C-terminal region. These preliminary data suggest that increased expression of C-terminally truncated ER α variant proteins could interfere with the IHC determination of ER status.

This possibility was investigated in human breast tumor tissues (49). A series of breast tumors was assayed for ER α , using the set of Abs described above, and the H-scores from each Ab were compared for each tumor. The tumors fell into two distinct groups: one in which the H-scores obtained with each Ab were consistent and not significantly different from each other; and another group, in which the H-scores obtained with each Ab were inconsistent and significantly different from each other. Further, in all but one case, the H-score was higher for the N-terminal Ab, compared to the C-terminal Ab (50). In preliminary experiments using a subset of the original tumor set, the authors found similar results, using another set of N-terminal and C-terminal ER α Abs. Together with the previous experimental data, one interpretation of the tumor data would be that the discrepant tumors had higher levels of C-terminally truncated ER α -like proteins.

To address the hypothesis that the C-terminally truncated ER α -like proteins could correspond to proteins encoded by ER α variant transcripts, the authors compared expression of ER α variant mRNAs in the consistent and inconsistent tumors. The results show a significantly higher relative expression and detection of ER α variant mRNAs that would encode C-terminally truncated proteins in the inconsistent vs the consistent tumors (50). These results suggest that, irrespective of function, the expression of significant amounts of C-terminally truncated ER α variant proteins could interfere with the IHC determination of ER status, which, in turn, might underlie some of the inconsistencies between ER status and clinical response to endocrine therapy. These data are consistent with the hypothesis that ER α variant mRNAs may be stably translated *in vivo*. However, such data are indirect, and other mechanisms, e.g., altered epitope detection, increased proteolytic activity, and so on, may underlie the discrepant ER α H-scores found in some human breast tumors.

More recently, data published from several independent groups support the detection of ER α -like proteins in cell lines and tissues *in vivo*, which could correspond to those predicted to be encoded by previously identified ER α variant mRNAs. The presence of an exon-5-deleted ER α protein was demonstrated immunohistochemically in some human breast tumors, using a monoclonal Ab specific to the predicted unique C-terminal amino acids of the exon-5-deleted ER α protein (39). However, although there was a correlation between IHC detection and presence or absence of exon-5-deleted ER α mRNA determined by RT-PCR, the group was unable to detect any similar protein by Western blotting, suggesting either very low levels, compared to WT ER α , or differential stability of the variant protein relative to the WT ER α during the extraction procedure. In addition, an ER α -like protein, consistent with that predicted to be encoded by the exon-5-deleted ER mRNA, is expressed in some BT 20 human BC cell lines, as determined by Western blot analysis (51). Western blotting of ovarian tissue has identified both a 65-kDa WT ER α protein and a 53-kDa protein recognized by ER α Abs to epitopes in the N-terminus and C-terminus of the WT protein, but not with an Ab recognizing an epitope encoded

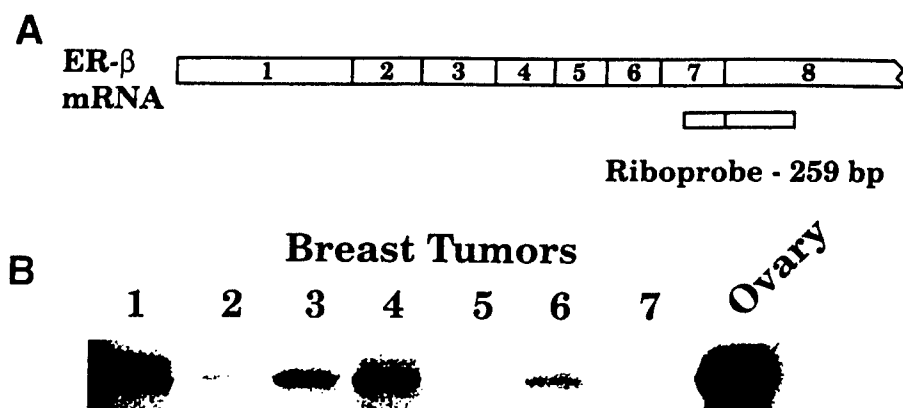


Fig. 4. Detection of ER β mRNA in human breast tumors by RNase protection assay. (A) Schematic representation of hER β mRNA showing various exon sequences, and identifying the riboprobe position and size of the expected protected fragment (259 bp). (B) Total RNA was isolated from seven breast tumor samples, and 25 μ g was used in an RNase protection assay, as previously described (21). Ovarian RNA was used as a positive control.

by exon 4 (52). These results correlated with the presence of both WT and exon-4-deleted ER α mRNAs in these tissues, and suggested that the 53-kDa protein was derived from the exon-4-deleted ER α mRNA.

More recently, a 61-kDa ER α -like protein and a more abundant 65-kDa WT ER α protein were identified in MCF-7 cells (29). The 61-kDa protein is thought to be encoded by an exon-3-deleted ER α mRNA expressed at low levels in these cells, and its co-migration, both before and after dephosphorylation with the recombinant exon-3-deleted ER α protein, when expressed at higher levels after stable transgene expression in another MCF-7 clone, was thought to strongly suggest its identity with the recombinant exon-3-deleted ER α protein.

There is accumulating evidence suggesting that variant ER α proteins, which correspond to those predicted to be encoded by some of the ER α variant mRNAs, can be detected by conventional technologies in clinical specimens.

ER β AND ITS VARIANTS

Identification of ER β mRNA in Human Breast Tissues

With the discovery of ER β , which had properties similar to, yet distinct from, ER α (10, 11, 53, 54), and can interact with the ER α (55, 56), it became important to know whether ER β was expressed in human breast tumors, and, if so, what role it plays in estrogen/antiestrogen action.

The authors have detected the presence of ER β mRNA, both by RT-PCR (12, 14) and by RNase protection assay (Fig. 4; 14), in some human BC biopsy samples and some human BC cell lines. *In situ* hybridization analysis suggested that expression of ER β mRNA could be detected in the BC cells of a human BC biopsy sample (14). Using an RT-PCR approach to analyze both ER β and ER α mRNA expression in a range of breast tumors (12), the following was observed: There was no correlation between ER β expression and ER α expression in breast tumors; in some cases, both ER β and ER α mRNA were expressed in the same tumor; in those tumors in which both ER mRNAs were expressed,

the relative expression appeared to vary widely among tumors. Furthermore, ER β mRNA can be detected in normal human breast tissues by RT-PCR (13) and RNase protection assay (14). Although there are no data reporting the expression of ER β protein(s) in human breast tissues as yet, the available information suggest that ER β may be expressed in both normal and neoplastic human breast tissues, and may have a role in these tissues.

Expression of ER β mRNA During Breast Tumorigenesis

The demonstration of ER β mRNA expression in both human breast tumors and normal human breast tissue suggests that the well-documented role of estrogen in breast tumorigenesis (1,57) may involve both receptors. Using a multiplex RT-PCR approach, it has been shown that the ER α :ER β ratio in a small group of ER-positive human breast tumors was significantly higher than the ratio in their adjacent normal breast tissues (58). The increase in ER α :ER β ratio in breast tumors was primarily the result of a significant upregulation of ER α mRNA in all ER-positive tumors, in conjunction with a lower ER β mRNA expression in the tumor, compared to the normal compartment in some, but not all, ER-positive cases. Preliminary data suggest that the level of ER β mRNA in breast tumors may be correlated with the degree of inflammation (unpublished data). Because *in situ* hybridization data suggest that expression of ER β mRNA could be detected in the cancer cells of a human BC biopsy sample (14), and that human lymphocytes in lymph nodes can also express ER β mRNA (14), it is possible that the cell type contributing to the expression of ER β mRNA may be heterogeneous, depending on the tumor characteristics. If the RNA studies reflect the protein levels of the two ERs, results to date provide evidence to suggest that the role of ER α - and ER β -driven pathways, and/or their interaction, probably changes during breast tumorigenesis.

Identification of ER β Variant mRNAs in Human Breast Tissues

The presence of multiple ER α variant mRNAs in both normal and neoplastic human breast tissues has led to the question of the expression of ER β variant mRNAs. Several ER β variant mRNAs have been detected. The authors have identified an exon-5 + 6-deleted ER β mRNA in human breast tumors (59). This transcript is in-frame, and would be expected to encode an ER β -like protein deleted of 91 amino acids within the hormone binding domain. A human ER β variant mRNA, deleted in exon 5, was identified in MDA-MB231 human BC cells and in some human breast tumor specimens (18). Although that group was unable to detect an exon-5-deleted ER β mRNA in normal human breast tissue, the authors have detected both exon-5-deleted ER β mRNA and an exon-6-deleted ER β mRNA, as well as an exon-5 + 6-deleted ER β mRNA, in normal human breast tissue samples (13), and in some human breast tumors. The exon-5-deleted ER β mRNA and the exon-6-deleted ER β mRNA are out-of-frame and predicted to encode C-terminally truncated ER β -like proteins, which would not bind ligand.

More recently, several exon-8-deleted human ER β mRNAs have been identified (17) from a human testis cDNA library, and by RT-PCR from the human BC cell line MDA-MB435. These variants have been named human ER β 2-5. It should be noted that human ER β 2 is not the equivalent of the ER β variant mRNA with an in-frame insertion of 54 nucleotides between exons 5 and 6 identified in rodent tissues (13,60,61), and also named ER β 2. The authors have been unable to detect an equivalent of the rodent ER β 2 mRNA in any normal or neoplastic human tissue so far studied (13).

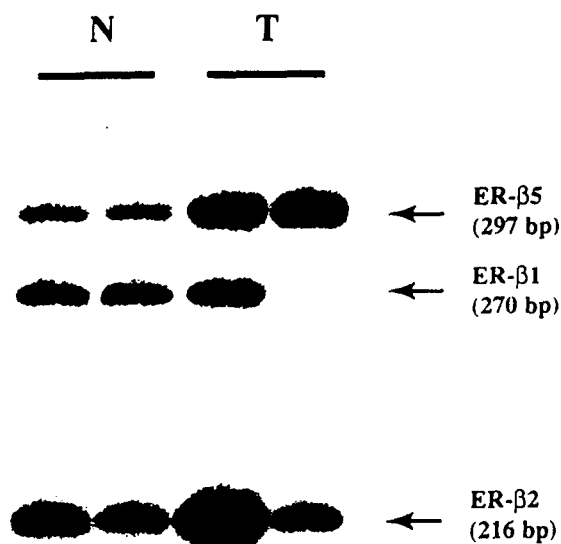


Fig. 5. RT-triple primer PCR analysis (26) of the relative expression of human ER β 1, human ER β 5, and human ER β 2 mRNAs in normal (N) and breast tumor (T) tissue samples.

Several of the human ER β variants deleted in exon 8, specifically hER β 2 and hER β 5, can be detected in normal human mammary gland and in several human BC cell lines (17). The predominant type of hER β exon-8-deleted mRNA present varies among the different cell lines. The authors have confirmed the presence of the hER β 2 and the hER β 5 variant mRNAs in several normal human breast tissue samples from both reduction mammoplasties and normal tissue adjacent to breast tumors (Fig. 5; unpublished data). Moreover, the authors have identified both hER β 2 and the hER β 5 variant mRNAs in several human breast tumor samples (Fig. 5; unpublished data). Using a semiquantitative RT-triple primer PCR approach (26), which simultaneously measures the relative expression of the WT hER β 1 and the two variant hER β 2 and hER β 5 mRNAs, it appears that, in most, but not all, cases, the level of the variant mRNA species exceeds that of the WT hER β 1 (Fig. 5; unpublished data) in both normal and neoplastic human breast tissues. The known sequence of all human ER β -like transcripts is shown schematically in Fig. 6; also shown in this figure are the proteins predicted to be encoded by these variant hER β mRNAs. All the hER β variant mRNAs identified to date are predicted to encode proteins that are altered in the C-terminus in some fashion, and are unlikely to bind ligand (62). However, published data (17) suggest that some of these variant receptors can form homo- or heterodimers among themselves and with WT hER β and hER α , and may preferentially inhibit hER α DNA-binding transcriptional activity (62).

Putative Role of ER β and Its Variants in Breast Cancer

Transient transfection studies have provided data which suggest that ER β 1, i.e., the WT ER β , can only mediate an antagonist response when bound to TAM-like agents, in contrast to the TAM-bound WT ER α , which can mediate either an antagonist or agonist activity on a basal promoter linked to a classical estrogen response element (53,63). This suggests the possibility that altered relative expression of the two ERs may underlie

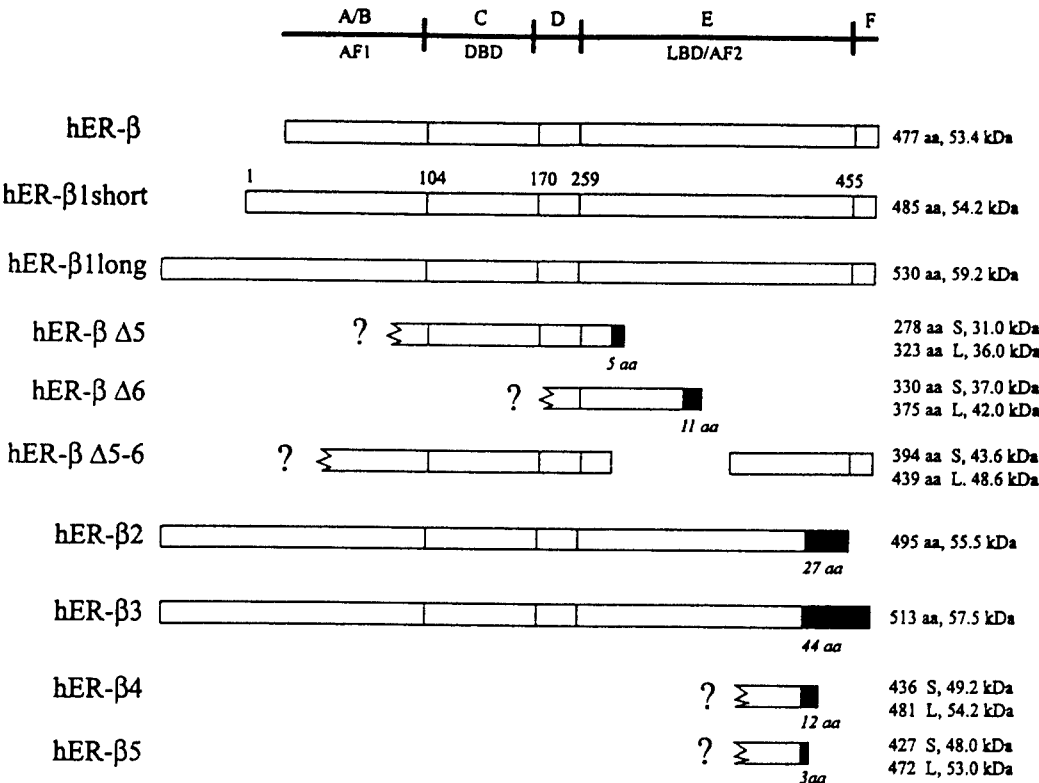


Fig. 6. Human ERβ isoforms. All hERβ isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for the hERβ1 short (14), which contains eight extra N-terminal amino acids, compared to the first hERβ described (10). hERβ1 long (Genbank AF051427) contains 45 additional N-terminal amino acids. hERβ1Δ5 (13, 18), hERβ1Δ6 (13), hERβ2 (Genbank AF051428, AB006589cx), hERβ3 (Genbank AF060555), hERβ4 (Genbank AF061054), and hERβ5 (Genbank AF061055) are truncated, and contain different C-terminal amino acids (black boxes). hERβΔ5-6 (13) (Genbank AF074599) is missing 91 amino acids within the LBD/AF-2 domain. For each receptor, the length (aa) and the calculated molecular mass (kDa), when known or corresponding to the short (S) or the long (L) forms of the putative proteins, are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

altered responses to antiestrogens, and could be a mechanism of altered responsiveness to antiestrogens in human BC. The activity of the estrogen-bound ERβ1 on activating protein 1 (AP-1)-containing promoters is inhibitory, in contrast to that of estrogen-bound ERα, which stimulates transcription (54). Furthermore, antiestrogens of all types demonstrated marked transcriptional activity through ERβ1 on promoters that contained AP-1 sites (54). A nonligand-binding hERβ variant protein, encoded by the variant hERβ2 (also named hERβcx), can heterodimerize with ERβ1, but preferentially heterodimerizes with ERα, and shows a dominant-negative activity only against ERα-mediated transactivation (17,62). It is possible, therefore, that ERβ1 and its variants could have a direct regulatory role on ERα activity. Since the authors have observed an increased ratio of ERα:ERβ mRNA in human breast tumors, compared to their adjacent matched normal tissues, which primarily results from increased expression of ERα mRNA in the breast tumor component (58), it is possible that this may translate into unregulated ERα activity and unregulated growth responses mediated through ERα.

However, there are several issues that must be addressed before anyone can begin to develop rational pathophysiologically relevant hypotheses regarding the role of ER β and/or its variants in human breast tissues. First, it is not yet known whether ER β and ER α are expressed together in the same breast cells, or separately in different normal or neoplastic cell populations. Second, studies so far have only measured mRNA levels. No studies of ER β protein expression in human breast have been published to date. Therefore, the pathophysiological relevance of the relative levels of ER β and ER α expression achieved in transient expression studies, and the resulting functional outcome, are unknown. Third, some in vitro studies have been done using an N-terminally truncated ER β 1 (64), and the functional impact of this is also unknown.

EXPRESSION OF OTHER STEROID HORMONE RECEPTORS AND THEIR VARIANTS IN HUMAN BC

The observation that the PR gene showed a complex pattern of alternative splicing similar to, although not as extensive as, that of ER α , led to the further characterization of PR variants (16,31,32). Two commonly expressed variant transcripts identified in human breast tumors and normal human breast tissue were cloned and sequenced. Variant PR mRNAs with either a precise deletion of exon 6 or exon 4 were identified in most breast tumors examined. PR transcripts deleted in exon 2, exons 3 + 6, or exons 5 + 6, were also found in a few breast tumors (31,32). The exon-6-deleted transcript was the most abundant and frequently expressed PR variant mRNA in the human breast tumors examined, and specific PCR primers were designed to determine the expression of this transcript, relative to the WT PR, using RT-PCR analysis (27). Altered expression of ER α variant mRNAs was observed previously between normal and neoplastic breast tissue; therefore, it was of interest to determine if exon-6-deleted PR mRNA expression was altered during breast tumorigenesis. Using an approach similar to that described previously (27), the relative expression of the exon-6-deleted variant PR mRNA to the WT PR mRNA was examined in 10 normal reduction mammoplasty samples and 17 breast tumors. The relative expression of the exon-6-deleted PR variant to the WT PR mRNA was found to be significantly lower ($P < 0.01$) in normal breast tissues (median = 4.8%) than in breast tumors (median = 13.9%) (unpublished data).

The exon-2-deleted PR mRNA encodes a C-terminally truncated PR-like protein without a DNA or a ligand-binding domain (32). The exon-4-deleted PR mRNA is in-frame, but encodes a protein deleted in exon 4 sequences, missing a nuclear localization signal, and the recombinant protein representing exon-4-deleted PR-A did not bind DNA and had little effect on WT PR-A function (32). Exon-6-deleted PR variant mRNA is out-of-frame and encodes a C-terminally truncated PR-like protein lacking the hormone-binding domain, and the exon-5 + 6-deleted PR variant mRNA is in-frame, but encodes a protein deleted in exon 5 + 6 sequences of the hormone-binding domain (32). Richter et al. (32) have demonstrated that recombinant proteins, representing the exon-6-deleted PR-A and the exon-5 + 6-deleted PR-A are dominant-negative transcriptional inhibitors of both the WT PR-A and PR-B (32). It is possible, therefore, that the presence of PR variant proteins encoded by the identified PR variant mRNAs could modify WT PR activity and influence responses to endocrine therapies. Small, variant PR-like proteins have been identified by Western blotting in some breast tumors (32,65,66), which correspond in size to some of the proteins predicted to be encoded by some of the exon-deleted PR mRNAs. However,

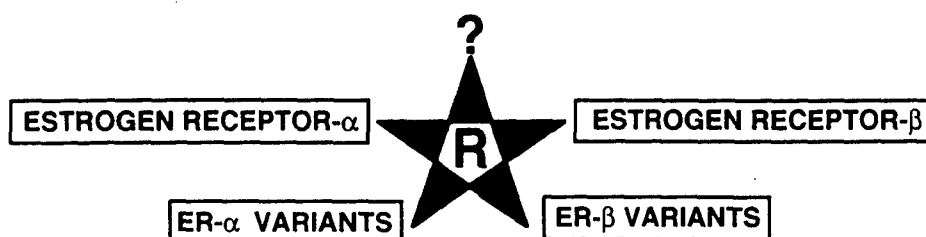


Fig. 7. Schematic representation of the known and unknown (?) multiple facets of the estrogen receptor (R).

some data (66) suggest that the presence and abundance of PR variant mRNAs may not correlate with the detection of these smaller-sized PR immunoreactive species in human breast tumors.

The measurement of PR is an important tool in clinical decision-making with respect to prognosis and treatment of human BC. Furthermore, the level of PR expression provides important clinical information (67). As the use of enzyme-linked immunosorbent assays and IHC assays for PR detection increases, it is likely that variant PR expression will interfere with these assays, whatever their function. PR Ab (AB-52 Ab) used in such assays detect epitopes in the N-terminal region of the WT molecule, which is shared by truncated PR-like molecules. If any or all of the deleted PR variant mRNAs so far identified are translated into stable proteins, they will be co-detected with the WT PR in such assays. Presence of PR variants may also be a factor contributing to discrepancies between biochemical measurement and immunological detection of PR. Indeed, the potential for ER α variant expression to interfere with the IHC assessment of ER status has been documented (49,50,68).

CONCLUSIONS AND CONTROVERSIES

The multifaceted nature of the ER is suggested by the expression of ER α mRNA, ER β mRNA, and their variant mRNAs in both normal and neoplastic human breast tissues (Fig. 7). There is a large body of molecular data that support at least the potential for the multifaceted nature of the ER, and therefore estrogen/antiestrogen signaling in both normal and neoplastic human breast tissues. Alterations in the relative expression of several ER-like mRNAs have been shown to occur during breast tumorigenesis, and the relative frequency of detection and expression of individual ER-like mRNAs can be correlated with different prognostic characteristics in BC. This, in turn, suggests a possible role in breast tumorigenesis and possibly hormonal progression in BC. However, there are still major gaps that need to be filled before there can be a clear idea of the pathophysiological and functional relevance of the experimental results so far in hand. Unequivocal data are required to support the *in vivo* detection of variant ER α , variant ER β , and WT ER β proteins, which correspond to the variant ER α , variant ER β , and WT ER β mRNA species, respectively. There is a need to experimentally determine putative function, using expression levels that reflect pathophysiological levels of expression. There is a need to know if the two WT ERs and/or their variants are co-expressed in the same cells within heterogeneous normal and neoplastic breast tissues. Further, given the detection of multiple forms of variant ER-like species in any one breast tissue sample, the limitations in interpreting data from experimental systems, in which only one variant species is considered in the presence or absence of WT protein, needs to be understood.

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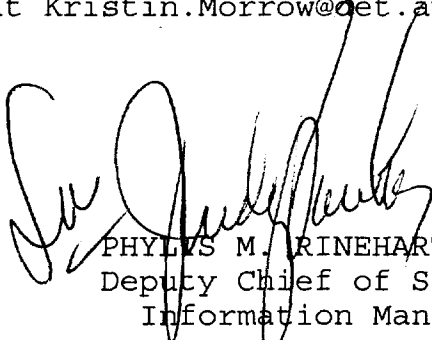
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