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

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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 ERα, ERβ and their variants occurs during breast tumorigenesis. 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 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. Some of these factors e.g. active MAP kinase, are also altered between preinvasive and invasive 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.
4. INTRODUCTION: How the estrogen receptor signal transduction pathway is altered during breast tumorigenesis and if altered ER action increases the risk of developing breast cancer were the overall goals of this project. Estrogens have a major role in both the development and progression of human breast cancer. The estrogen receptor-alpha (ERα), a key component of estrogen signal transduction undergoes an increase in its level of expression during tumorigenesis. We and others have evidence to suggest that the expression of the ER and variant 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 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 then wanted to determine when these changes occur during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and whether they are associated with an increased risk of developing breast cancer. Our specific aim was 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 was to be undertaken in which ER signaling was to be examined 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. ERα 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 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). 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. Blocks within pathology department archives that contain specific breast lesions for study had been previously identified and collected. Details of these cases were shown on attached spread-sheets in
appendix 1 of the 1999 annual report. These tissues were assayed immunohistochemically for ER, PR and Ki-67. Sample analyses of the invasive and DCIS lesions were attached in appendix 1 of the 2000 annual report.

2. Immunohistochemical analyses.

* We have optimized and validated an active MAP kinase antibody for use in our study. Our initial analysis compared adjacent normal breast tissue with matched invasive breast cancer. Using H-score analysis we determined active MAP kinase expression in formalin-fixed-paraffin-embedded tissue sections of 1) human breast tumors and their matched adjacent normal breast tissue as well as 2) primary human breast tumors and their matched lymph node metastases; 3) primary breast tumors from patients who later proved to be sensitive or resistant to tamoxifen treatment. We found that active MAP kinase expression was detected in 48% of primary human breast tumors, and was significantly increased in tumors compared to adjacent normal breast (Wilcoxon test, \( P = 0.027 \)). A significant positive association (\( \chi^2, P = 0.02, n = 55 \)) was obtained between active MAP kinase and presence of lymph node metastases. Moreover, increased active MAP kinase (Wilcoxon test, \( P = 0.0098 \)) was found in concurrent lymph node metastases compared to primary breast tumors. No significant difference in active MAP kinase was found in primary tumors of patients who later responded to tamoxifen or did not respond to tamoxifen. It was concluded that the data suggest that active MAP kinase has a role in breast tumorigenesis, is a marker of breast cancer metastasis and has a role in the metastatic process. However, active MAP kinase is unlikely to be a marker of endocrine sensitivity, or involved in de novo tamoxifen resistance. These data were presented at the Endocrine Society annual meeting this year and have been submitted for publication (see appendix 1).

We have also used the antibody to active MAP kinase to determine expression in the breast lesion cohort collection as described in 1 above. Studies involving DCIS and invasive breast cancer have been completed and the expression of active MAP kinase is significantly higher in invasive breast cancers than ductal carcinoma in situ (\( n = 48 \), unpaired t-test with Welch’s correction, \( P = 0.0233 \)). In a cohort of 27 invasive breast tumors in which there was also a matched DCIS component in the same biopsy sample, active MAP kinase was again significantly increased in the invasive component compared to the DCIS (Wilcoxon test, \( P = 0.0034 \)).

*ERβ : We evaluated a commercially antibody PAI-313 (Affinity Bioreagents) used in breast tissues recently by another group (14), but found that while it is suitable for frozen sections it was unsuitable for formalin fixed paraffin embedded sections. Earlier this year we were given two antibodies: Dr Ho (University of Massachusetts) kindly provided GC17 (14) which detects wild-type ERβ i.e. ERβ1, and Dr Gustafson (Karolinska Institute, Sweden) kindly provided IgY ERB503 (15) which will detect total ERβ isoforms. The GC17 antibody has proven to be a reliable and consistent antibody (see immunohistograms of normal breast tissue, breast ductal hyperplasia and breast tumors in appendix 2 figures 1 and 2). The IgY ERB503 antibody initially only gave non-specific results
initially. However, after contacting other researchers who have successfully used the IgY ERB503 antibody (16), for advice, we now have this antibody working reliably (see appendix 2, figure 2). Our preliminary data suggest that ERβ and/or its isoforms are widely expressed in human breast luminal epithelial cells as well as in the myoepithelial cells surrounding human breast ducts, furthermore expression is still evident but possible decreased in ductal hyperplasia (see appendix 2, figure 2). In invasive breast cancer expression of ERβ is variable, ranging from nondetectable, intermediate expression and high expression (appendix 2, figure 1). These data are consistent with previous data that we have obtained at the RNA level (3,17, appendix 2, figure 3). It was recently published that ERβ expression is progressively decreased from normal breast tissue and in breast lesions that represent breast tumorigenesis (15). The antibody used was IgY ERB503 which detects total ERβ isoforms. We previously published that total ERβ RNA is decreased between adjacent normal breast tissue and matched invasive breast tumors (3), but the relative expression of variants to wild-type is altered during this process (18). Therefore, the results we will obtain with IgY ERB503 (total) antibody will be contrasted and compared with results obtained with the GC17 antibody (wild-type ERβ) to determine a parallel or possibly differential contribution of variant ERβ isoforms during this process.

AIB1: an antibody to undertake this study was developed by Dr J Torchia (University of Western Ontario, London, Ontario). He kindly provided us with an aliquot to test. Previously no antibodies suitable for AIB1 immunohistochemistry were available. However, this antibody has proven to be unreliable and we have not proceeded with this analysis.

cyclin D1: antibodies are available commercially and are being assessed, but this analysis has not been completed due to personnel turnover over during the last year.

We have recently obtained a Ventana instrument with both autostaining and in situ hybridization modules, for our research laboratory only. This was expected to increase our through-put capacity, and decrease assay times. When this equipment is working it gives excellent results, however, the instrument requires a great deal of maintenance and we have not received good service from the company. As a result we have had some significant downtime with respect to our analyses. Further, due to significant staff turnover, and difficulties associated with recruitment as described below in section 3, progress on this project has been slower than anticipated.

3. Previously we had completed preliminary studies to determine if some relevant estrogen receptor coregulators (19), 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 (8,18,20). 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. In situ hybridization analyses were developed for SRA and REA RNA (8) during this preliminary study and were to be used this year to analyze the tissue blocks collected. Due to a significant turnover of personnel in both my laboratory and that of my collaborator Dr Watson, during that time, and the fact that a new individual recruited to do this analysis did not arrive until April/2001, and then was required, due to a seriously ill father, to return to China two months later, and has been on leave of absence ever since, this aspect of the project has not progressed past the point of assay development as described in appendix 2 of the 2000 annual report. Furthermore, this individual was being trained to carry out the RT-PCR analyses for ERα variant and ERβ variant RNAs in the breast samples which had been received from Poland and stored in the NCIC/Manitoba Breast Tissue Bank.
6. KEY RESEARCH ACCOMPLISHMENTS.
* identification of patient cohort for study, retrieval, review and collection of appropriate tissue blocks for analysis, and collection, cataloguing and histopathological analysis of 100 breast tissue specimens received from Poland.
*ERα, PR and Ki67 immunohistochemical assays completed for the initial cohort.
*Optimization and validation of two recently acquired ERβ antibodies. One which detects only wild type ERβ the other which detects total (wild-type and variant isoforms) ERβ.
*validation of an appropriate antibody to measure activated MAP kinase in formalin fixed, paraffin embedded tissues sections completed. Initial IHC study of multiple breast tumors and their adjacent normal breast tissues, and lymph nodes metastasis completed. Data presented in abstract form at an international scientific meeting and manuscript submitted for publication.
*active MAP kinase is significantly increased in invasive versus pre-invasive (DCIS) breast cancer.
* use of the validated active MAP kinase antibody in the immunohistochemical analysis of sections from the previously collected blocks containing specific breast lesions for study.
* establishment of SRA and REA in situ hybridization assays. Data published.
* 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 data published.
* analysis of REA expression in breast tumors correlates with markers of good prognosis i.e. estrogen receptor expression and low grade. Data published.

7. REPORTABLE OUTCOMES/BIBLIOGRAPHY.
*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 3
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 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.

9. REFERENCES.


10. List of Personnel Receiving Payment from the Research Effort.
   Yulian Niu. MD MSc
   Etienne Leygue. PhD.
   Monika Adamczyk. PhD
   Charlene Bergen
   Guang Li. MD
   Christina Walker. BSc
   Antonietta Versace. BSc
   Meghan Webb
Appendix 1
the expression pattern of UGT1A by treating the different cell lines for 12 hours to 3 days at several concentrations of inducers. We showed that UGT1A expressed in MCF-7 cell line were up regulated when cells were treated for 24 hours with 3MC, TCDD and quecrerin. Moreover, the modulation of UGT1A activity in MCF-7 cells was demonstrated by an in vitro glucuronidation assay using 4-methylumbelliferone as a substrate. By contrast, no induction of UGT1A was observed with the same inducers in HEC-1B, Ishikawa and BT-20 cells. These data suggest that UGT1A enzymes are expressed in estrogen target tissues and that they may influence the estrogen concentration by conjugation into polar derivatives.

P2-611

ACTIVATED MITOGEN-ACTIVATED PROTEIN KINASE (ERK1/2) EXPRESSION DURING HUMAN BREAST TUMORIGENESIS AND BREAST CANCER PROGRESSION

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Ligand independent activation of estrogen receptor (ER), due to active mitogen activated protein (MAP) kinase (erK1/2), is thought to be a mechanism underlying development of metastatic resistance. Alterations in estrogen and antiestrogen action occur during breast tumor progression in vivo, using human breast tissue samples and immunohistochemical measurement of active MAP kinase with antibodies specifically detecting dually phosphorylated erk 1/2. Active MAP kinase was detected in approximately 48% of primary breast tumors, and was significantly increased in tumors compared to their adjacent normal breast tissues (Wilcoxon test, P = 0.027). No statistically significant correlations were found with grade, cellular composition, ER or progesterone receptor status. However, a significant correlation (r = 0.38, P = 0.0044, n = 55) was obtained between active MAP kinase and the presence of nodal metastases. Moreover, a statistically significant increase in active MAP kinase expression (Wilcoxon test, P = 0.0098) was found in concurrent nodal metastases compared to matched primary breast tumors. These data support the hypothesis that active MAP kinase is a marker of breast cancer metastasis and has a functional role in the metastasis. We also investigated the possibility that active MAP kinase is a marker of endocrine resistance. No statistically significant difference in active MAP kinase expression was found in ER+, node negative, primary tumors of patients who later were found to respond to tamoxifen treatment (sensitive) or not respond to tamoxifen treatment (resistant). Active MAP kinase is unlikely to be a marker of endocrine sensitivity, or involved in de novo tamoxifen resistance.

P2-612

S-ADENOSYLMETHIONINE DECARBOXYLASE (SAMDC) OVEREXPRESSION REDUCES INVASIVENESS AND TUMORIGENICITY IN NUDE MICE OF HORMONE-RESPONSIVE BREAST CANCER CELLS

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Increased activity of ornithine decarboxylase (ODC), the first and rate limiting enzyme in polyamine (PA) synthesis, has been associated with adverse prognostic features of human breast cancer, including reduced survival. No information, however, is available on the role of SAMDC, another highly regulated enzyme in the PA metabolic pathway mediating the synthesis of the more distal PA spermidine (Spd) and spermine (Spm). To elucidate the role of SAMDC in breast cancer biology, we have generated SAMDC overexpressing MCF-7 breast cancer cells. SAMDC overexpression leads to a selective cellular accumulation of Spm, while (primarily as a result of the compensatory inhibition of ODC) putrescine (Put) is nearly totally suppressed and Spd level is reduced by ∼50%. Control and SAMDC-MCF-7 cells exhibited similar sensitivity in soft agar to Tannoxifen and to the pure antiestrogen ICI-182,780, although SAMDC overexpressing cells were marginally more sensitive to estradiol added in the absence of serum. More importantly, SAMDC-MCF-7 cells exhibited a markedly reduced invasive ability in matrix (p = 0.013). Furthermore, they were less tumorogenic in nude mice. The odds for control clones to form tumors were 3.13 (CI 1.2-8.2, p=0.0184) higher than those for SAMDC clones. The odds ratios were identical in the absence and in the presence of estradiol. In addition, the growth of established tumors was slower for SAMDC than for control clones (p=0.01). Correlation of biologic properties with cellular PA profiles suggests that the less aggressive behavior of SAMDC-MCF-7 cells is mediated primarily by suppression of cellular Put. Our results provide insight into the relative roles of the individual PA in breast cancer biology and indicate that, under these experimental conditions, Put plays a predominant role in influencing breast cancer phenotype.

P2-613

REAL TIME RT PCR ANALYSIS OF RELATIVE PROLACTIN RECEPTOR (PRLR) LEVELS IN HUMAN CANCER CELL LINES

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The prolactin receptor (PRLR) belongs to the cytokine receptor superfamily. The evidence linking PRL to breast cancer development has been drawn in part from findings of high PRLR levels in cancerous tissues. Experimentally, activation of the PRLR induces mammary tumors in mice. In humans, there is a positive correlation between PRLR and estrogen receptor (ER) and progesterone receptor levels, and it is known that sex steroid hormones and PRL interact synergistically to initiate cancerous growth within mammary tissue. There is growing evidence that PRL may also play a role in early transformation events involved in prostate cancer, and that PRLR expression is altered in some neoplasms of the prostate. It is therefore of interest to compare the PRLR status of breast and prostate cancer cell lines, as well as other human cancer cell lines. In this study, we used a one-step real time reverse method to determine relative expression levels of PRLR mRNA in ten human breast cancer cell lines, three prostate cancer and several other cell lines; the housekeeping gene b-actin was used for internal normalization. The results using this method were compared to those published earlier in which Northern blotting and in situ hybridization were used to determine relative levels of PRLR. The human breast cancer cell lines examined, T47D was found to have the highest level of PRLR: 7.89 (+/-0.36 SE) fold higher than that of MCF-7 cells. MB157 cells expressed the lowest levels, 0.18 (+/-0.02 SE) relative to MCF-7 cells. Expression levels in the prostate cell lines were very low but detectable, ranging from approximately 700-fold lower (LMCaP) to 26,000-fold lower (DU145) than MCF-7 levels; HeLa PRLR expression was not detectable. Ultimately, this information will be useful in the selection of model cell lines based on PRLR status.

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P2-614

P53 IS A POTENTIAL MEDIATOR OF HORMONE-INDUCED RESISTANCE TO MAMMARY CARCINOGENESIS

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Reproductive history is a consistent risk factor for human breast cancer. Epidemiological studies have repeatedly shown that early age of first full-term pregnancy is a strong protective factor against breast cancer and provides a physiologically oper-ative model to achieve a practical mode of prevention. In rodents, the effects of full-term pregnancy can be mimicked by a three-week exposure to low doses of estrogen and prog-esterone. The cellular and molecular mechanisms that underlie hormone-induced refracto-teness are largely unknown. Our current studies have demonstrated that an early cellu-lar response that is altered in hormone-exposed mammary cell cultures is the fetal pro liferative burst induced by the chemical carcinogen, methylnitrosourea. The decrease in proliferation observed in the E2P exposed mammary gland is also accompanied by a decrease in the ability of estrogen receptor positive cells to proliferate. RNA expres-sion of several mammary cell-cycle related genes is altered in hormone-treated rats; however, immunohistochemical assays demonstrate that the proliferation and nuclear compartmentalization of the p53 tumor suppressor gene are markedly up regulated as a consequence of hormone treatment. P53 induction is not simply a consequence of mam-mary differentiation since it occurs only in response to E2P treatment and not following differentiation induced by periprostane. Periprostane is a compound that induces alve-olar differentiation but results in no proliferation. These results support the hypothesis that hormone stimulation, at a critical period in mammary development, results in cells with persistent alterations in the intracellular regulatory loops governing proliferation and re- sponse to DNA damage. A corollary to this hypothesis is that the genes affected by E and P are independent of alveolar-differentiation-specific genes. Future experiments are aimed at determining the mechanisms of hormone-induced up regulation of p53 protein expression as a part of the overall goal for identifying and functionally characterizing the genes responsible for the refractory phenotype.

P2-615

PARADOXICAL EFFECT OF ESTRADIOL: IT CAN BLOCK ITS OWN BIOFORMATION IN HUMAN BREAST CANCER CELLS

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Human breast cancer tissue contains all the enzymes: estrogen sulfatase, 17β-hydroxysteroid dehydrogenase, aromatase, involved in the last steps of the estradiol (E2) bioformation in this tissue. A great percentage (95-98%) of human breast cancers are in their early stage hormone-dependent and it is well accepted that E2 plays an important role in the genesis and evolution of this tumor. However, at present there is no information on a direct action of E2 in the transformation from normal to cancerous breast cell. E2 can be synthesized in the breast cancer itself by two main pathways: the "sulfatase" which
Activated Mitogen-activated Protein Kinase Expression during Human Breast Tumorigenesis and Breast Cancer Progression.

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Footnotes:
1. This work was supported by grants from the Canadian Institutes for Health Research/Medical Research Council of Canada and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). P. H. W. is a Medical Research Council of Canada (MRC) Scientist. AA is funded by a Susan Komen Breast Cancer Foundation Fellowship, and more recently by a USAMRMC postdoctoral award. TC is funded by a USAMRMC predoctoral studentship.
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3. Abbreviations used:

Key words: ERK 1/2, active MAP kinase, breast cancer, metastasis, prognostic marker, estrogen receptor, tamoxifen resistance, human breast tissues.
Abstract. **Purpose:** To address the hypothesis that activated MAP kinase (erk1/2) has a role in breast tumorigenesis, breast cancer progression and the development of tamoxifen resistance. **Experimental Design:** H-score analysis and a specific antibody for the immunohistochemical detection of activated MAP kinase in formalin-fixed-paraffin-embedded tissue sections were used to compare expression in 1) human breast tumors and their matched adjacent normal breast tissue; 2) primary human breast tumors and their matched lymph node metastases; 3) primary breast tumors from patients who later proved to be sensitive or resistant to tamoxifen treatment; **Results:** Active MAP kinase expression was detected in 48% of primary human breast tumors, and was significantly increased in tumors compared to adjacent normal breast tissue (Wilcoxon test, P = 0.027). A significant positive association ($\chi^2$, P = 0.02, n = 55) was obtained between active MAP kinase and presence of lymph node metastases. Moreover, increased active MAP kinase (Wilcoxon test, P = 0.0098) was found in concurrent lymph node metastases compared to primary breast tumors. No significant difference in active MAP kinase was found in primary tumors of patients who later responded to tamoxifen or did not respond to tamoxifen. **Conclusions:** These data suggest that active MAP kinase is a marker of breast cancer metastasis and has a role in the metastatic process. However, active MAP kinase is unlikely to be a marker of endocrine sensitivity, or involved in *de novo* tamoxifen resistance.
Introduction.

Ligand independent activation of estrogen receptor (ER) has been extensively documented in experimental models (1). Consequently, it has been speculated that such a mechanism could in part underlie estrogen independent activation of ER and therefore may be associated with altered ER activity that is thought to underlie the altered estrogen action that occurs during human breast tumorigenesis (2) and/or breast cancer progression in particular the development of antiestrogen resistance (1). Previously we had developed an estrogen independent (as defined by loss of growth responsiveness to estradiol) ER+ human breast cancer cell line (T5-PRF) by long-term growth in estrogen depleted media (3). Amongst other changes (3, 4), these estrogen independent cells contained a significant increase in activated MAP kinase (5), as well as an increased apparently ligand independent activity of the endogenous ER (4). Recently, increased activated MAP kinase was found in another cell line model of apparently estrogen independent proliferation (6). MAP kinase has been implicated in the ligand independent activation of ERα since it can directly phosphorylate ERα on serine^{118} leading to ligand-independent ER activation and the loss of tamoxifen inhibition of ER mediated transcriptional activation (7). Furthermore, treatment of cells with EGF or IGF-1 that activate the Ras/Raf/MAPK pathway, also activates the ER in a ligand independent fashion, and this is accompanied by serine^{118} phosphorylation of ER (7). These data suggest the possibility that increased activated MAP kinase in estrogen target tissues in vivo could effect estrogen and antiestrogen responsiveness. Interestingly, an increased expression and activity of MAP kinase in human breast
tumors compared to normal breast tissues was reported (8), although only one breast tumor case was matched to its own adjacent normal breast tissue, all others were independent samples. If confirmed, this observation suggests that increased MAP kinase during human breast tumorigenesis especially in ER+ breast tumorigenesis could also contribute to the altered estrogen action that occurs during this process (9, 10). However, the relationship of activated MAP kinase to steroid receptor status, to other known prognostic variables in breast cancer and to breast cancer progression, in particular antiestrogen sensitivity and resistance, has not been documented. In the following study we have investigated the expression of activated MAP kinase in human breast tissues directly in vivo using immunohistochemistry and assessed the relationship of activated MAP kinase expression with known prognostic variables and progression in human breast cancer.

Materials and Methods.

Human Breast Tissues. All breast samples used for this study were selected from the National Cancer Institute of Canada (NCIC)-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As was previously described (11), tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed-paraffin-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific
paraffin and frozen blocks from cases for this study. For each case interpretation
included an estimate of the cellular composition (including the percentage of invasive
epithelial tumor cells and stroma), tumor type and tumor grade (Nottingham score).
Steroid receptor status was determined for all primary tumor samples by ligand
binding assay performed on an adjacent portion of tumor tissue. Tumors with estrogen
receptor levels above 3 fmol/mg of total protein were considered ER positive, and
tumors with progesterone receptor levels above 10 fmol/mg of total protein were
considered PR positive.

**Cohort 1:** Twenty six primary human breast tumor biopsies were selected. For each
case, matched adjacent normal and tumor 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 previously described (11). The presence of normal ducts
and lobules as well as the absence of any atypical lesion was confirmed in all normal
tissue specimens. In three cases the normal tissue sections did not contain normal
glands and therefore were excluded from the normal versus tumor analysis, leaving 23
cases. The ER levels ranged from 0.8 - 83 fmol/mg protein. Five tumors were ER- and
18 were ER+. The PR levels ranged from 2.2 - 112 fmol/mg protein. Fifteen tumors
were PR+ and 8 tumors were PR- . The tumors spanned a range of grades (grade
scores 5-9) as determined by the Nottingham grading system.

**Cohort 2:** To identify cases that responded divergently to tamoxifen, review of 1000
consecutive cases identified 490 cases that were ER positive and node negative.
Amongst these, 196 cases were identified that had been treated with adjuvant
tamoxifen following surgery +/- local radiation. From these a subset of 15 cases was selected that had shown progression of disease (either died or alive with recurrent disease, referred to as tamoxifen resistant cases). A similar control subset (n = 14) was specifically selected to comprise cases with similar lengths of follow-up (resistant 34 versus sensitive 39 months), ER status, tumor grade and tumor histology, but that had shown no progression of disease (referred to as tamoxifen sensitive cases). The ER levels for the tamoxifen sensitive cases ranged from 4.4 - 146 fmol/mg protein, and the PR levels ranged from 9.5 - 216 fmol/mg protein. One of these tumors were PR - and the rest were PR +. The ER levels for the tamoxifen resistant cases ranged from 4.6 - 107 fmol/mg protein, and the PR levels ranged from 8.8 - 143 fmol/mg protein. One of these tumors was PR- and the rest were PR+. There were no significant differences between the two groups with respect to ER levels or grade scores, however, there was a significantly statistical difference between the groups with respect to PR levels. The tamoxifen sensitive group had a significantly (P = 0.0064, Mann Whitney test, two-tailed) higher median PR level (40.5 fmol/mg protein) than the tamoxifen resistant group (14.8 fmol/mg protein).

**Cohort 3:** Sections from 21 primary human breast tumour samples and their matched lymph node metastases were selected. For the primary tumour samples, the ER levels, determined by ligand binding assays, ranged from 0 fmol/mg protein to 298 fmol/mg protein. Seventeen tumors were ER+ and 4 were ER-. PR levels determined by ligand binding assays ranged from 2.7 fmol/mg protein to 323 fmol/mg protein. Fourteen tumors were PR+ and 7 were PR-.

**Antibodies.** The following antibodies specific for dually-phosphorylated (active)
forms of the MAP kinase isoforms, ERK1 and 2 (p44/42), were used in this study: i) phospho-p44/42 MAP kinase (Thr202/Tyr204) rabbit polyclonal, antibody (#9101S, New England Biolabs, Beverly, MA) ii) phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody (#9106L, New England Biolabs, Beverly, MA); iii) anti-active MAPK rabbit polyclonal antibody (#V8031, Promega, Madison, WI). The antibodies used for immunohistochemistry were validated by the following method. Estrogen depleted MCF-7 breast cancer cells were treated for 3 hours with 50 mM of the MEK1 inhibitor, PD98059 (Calbiochem, La Jolla, CA), 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 (12). Western blot analysis (Fig. 1) showed a significant decrease in the 44/42 kDa ERK 1/2 MAP kinase bands of the PD98059 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 that activates ERK 1 and 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 (polyclonal NEB #9101S and monoclonal NEB #9106L antibodies) to active MAP kinase showed the presence of nuclear and some cytoplasmic staining in some but not all cells. The cell pellet sections were assessed by semiquantitative scoring using an H-score system, as
previously described (13). 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). 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 for further immunohistochemical analysis of activated MAP kinase in formalin fixed paraffin embedded sections of human breast tissues.

**Western Blot Analysis.** Estrogen depleted MCF-7 cells which had been treated or not treated with PD98059, as described above, were extracted using Buffer J (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M DTT) containing 1 tablet per 10 ml of complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany), that was optimized to inhibit proteolysis and phosphatases, as previously described (14). Aliquots of the extracts were analyzed using 10% SDS-PAGE with a 4% stacking gel at 200V for 45 minutes at room temperature according to the Laemmli method (15). Gels were transferred to nitrocellulose using CAPS transfer buffer (10 mM CAPS, pH11, 20% methanol) and transferred for 1 hour at 120V at 4°C. Blots were blocked overnight at 4°C in 0.2% (w/v) l-block (Tropix, Foster City, California) in Tris-buffered saline. Blots were incubated with rabbit anti-active-MAPK antibody (Promega, 1/1000 in 0.2% l-block in TBS containing 0.5% Tween-20) overnight at 4°C, followed by goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-rad, 1/5000) for 1 hour.
at room temperature. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England). The membrane was then stripped (0.2M glycine, 0.1% SDS, 1% Tween-20, pH2.2) for 1 hour at room temperature and blocked for 1 hour at room temperature with 0.2% I-block in TBS. The membrane was then probed with 1/1000 dilution of goat anti-ERK1 (total MAPK, Santa Cruz) in 0.2% I-block overnight at 4°C, followed by incubation with 1/5000 donkey anti-goat antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Detection was with the ECL detection system as described above.

**Immunohistochemistry.** In all cases, tissue samples had been fixed for 18-24h in 10% buffered formalin prior to routine embedding in paraffin wax. 5µm thick sections were cut, mounted on Superfrost/Plus slides (Fisherbrand), dried overnight at 37°C, dewaxed in xylene (4 min) and rehydrated in graded alcohol. DAKO EnVision™ System, Peroxidase (DAKO Diagnostics Canada Inc.) was used for Immunohistochemistry staining. After an initial pilot study using both the monoclonal (NEB #9106L) and polyclonal (NEB #9101S) antibodies, subsequent staining of human breast tissues was done using the polyclonal antibody to MAP kinase. Blocking steps included peroxidase blocking reagent (0.03 % hydrogen peroxide containing sodium azide) for 5 min to block endogenous peroxidase and Universal Blocker (DAKO Diagnostics Canada Inc.) for 15min to prevent non-specific staining with antibody from both mouse and rabbit. Tissue sections were incubated overnight at 4°C with the primary antibody (1:250 dilution in Antibody Diluting Buffer, DAKO Diagnostics Canada Inc.) after an initial incubation, with the same antibody, at 37°C for 30mins. Following the overnight incubation, slides were treated with labeled
polymer (goat anti-rabbit and goat anti-mouse immunoglobulin in Tris-HCl buffer containing carrier protein and anti-microbial agent) for 30 min at RT. Finally, slides were incubated for 10 min with Substrate AEC Chromogen (3-amino-9-ethylcarbazole). Each incubation step was followed by 2 min TBS wash x 2. The slides were counterstained with hematoxylin, immersed in a bath of ammonia water, rinsed in distilled water and a cover-slip applied using an aqueous mounting medium.

Levels of expression were scored semi-quantitatively, under the light microscope, by assessing the average signal intensity (on a scale of 0 to 3) and the proportion of cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an H-score (13).

**Statistical Analysis.** Differences between normal samples and their matched tumors were tested using the Wilcoxon matched pairs test, two-tailed. Correlation between activated MAP kinase expression and tumor characteristics was tested by calculation of the Spearman coefficient r. A Chi-squared test was used to determine statistical significance of the association between active MAP kinase and nodal status. Fisher’s exact test was used to test for differences in frequency of detection of active MAP kinase between groups.

**Results.**

**Activated MAP kinase expression is increased during human breast tumorigenesis.** To confirm and extend previous data suggesting that activated MAP kinase expression is increased in breast tumors compared to normal breast tissue, we
used a polyclonal antibody recognizing only active MAP kinases, ERK1/2, as described and validated above, to compare active MAP kinase expression in 23 breast tumor samples with their matched adjacent normal breast tissues containing normal ductal epithelium. Interestingly, of the 23 cases studied, only 11 (48%) had detectable staining in the tumor epithelium. There did not appear to be any relationship of frequency of detection of activated MAP kinase expression and ER status, although in this cohort only 6 of the tumors were ER-: The tissue sections were subjected to semi-quantitative H-score analysis, using sections obtained from agar embedded cell pellets of PD98059 treated and untreated cell lines as controls (Fig 2B and 2E). An example, of activated MAP kinase staining in a human breast tumor and its matched adjacent normal breast tissue, is shown in Fig 2A and 2D, respectively. The data were analyzed by a Wilcoxon matched pairs statistical test. The expression of active MAP kinase seen in breast tumors (median for all 23 tumors = 0.1; median for the 12 tumors with detectable staining only = 0.6) was significantly increased (P = 0.027, n = 23; P = 0.027, n = 12) compared to their adjacent matched normal breast tissue (median for all 23 normal tissues = 0.0; median for the 12 normal samples whose corresponding tumor had detectable staining = 0.0).

**Activated MAP kinase expression is not altered between breast tumors that are tamoxifen sensitive or tamoxifen resistant.** Ligand independent activation of ER is thought to be, at least in part, a possible mechanism associated with tamoxifen resistance in ER+ breast tumors. To determine if activated MAP kinase expression could be a predictor of tamoxifen sensitivity in primary breast cancers, tumors described under cohort 2 were examined immunohistochemically, as described
above, for activated MAP kinase expression. The tumors were all ER+ and node negative, and were the primary tumors obtained from patients who were later treated with tamoxifen (as described above) and remained disease free (tamoxifen sensitive cases, n = 14) or relapsed (tamoxifen resistance cases, n = 15). Tumor characteristics are detailed in Table 1. It should be noted that there were no statistically significant differences between the ‘sensitive’ and ‘resistant’ groups with respect to ER levels, tumor grade, age or time of follow-up. However, a statistically significant difference was observed between the level of PR in the primary tumors of the ‘sensitive’ and ‘resistant’ cases (Mann-Whitney rank sum test, P = 0.0064). Higher levels of PR were observed in the primary tumors of tamoxifen ‘sensitive’ cases (median 40.5, n = 14) versus tamoxifen ‘resistant’ cases (median 14.8, n = 15).

Ten out of 29 tumors had detectable active MAP kinase staining (7 of 14 ‘sensitive’, and 3 of 15 ‘resistant’), using Fisher’s exact test there was no statistically significant difference (P = 0.128) between the two groups in terms of the frequency of detection of active MAP kinase. When active MAP kinase was quantified using H-score analysis, there was no statistically significant difference in active MAP kinase expression between the two groups (‘sensitive’ median 0.05 versus ‘resistant’ median 0.0, Mann-Whitney rank sum test P = 0.1). It was concluded that activated MAP kinase as measured in primary breast tumors before beginning tamoxifen treatment was unlikely to be a marker of endocrine sensitivity.

**Activated MAP kinase expression is associated with lymph node metastases.** Correlation between activated MAP kinase expression and tumor characteristics was tested by calculation of the Spearman coefficient r, using active
MAP kinase H-scores obtained from all tumors in both cohorts 1 and 2. No statistically significant correlations were found with grade, cellular composition, ER or PR level. However, a significant positive association was obtained between active MAP kinase expression and the presence of lymph node metastases (Chi-square, P = 0.02). This suggested that active MAP kinase may be a marker of metastases and could be associated with progression in human breast cancer.

To investigate this further, an additional cohort of 21 primary human breast tumour samples and their matched lymph node metastases (cohort 3) were provided by the NCIC/Manitoba Breast Tumour Bank (Winnipeg, Manitoba, Canada). Nineteen of 21 primary tumors (90%) had detectable active MAP kinase expression and 19/21 lymph node metastases (90%) had detectable active MAP kinase expression. This increased frequency of detection of active MAP kinase expression as compared to the original cohort 1 (48%) where there was a mixture of node positive and node negative primary breast tumors, is consistent with the statistically significant association of active MAP kinase expression and lymph node involvement identified in cohort 1. This difference in frequency of detection between the two cohorts is significant (P = 0.0034, Fisher’s exact test). Also the pattern of active MAP kinase detection seemed to be conserved between each primary and its matched lymph node metastasis i.e. if the primary tumors (19/21) had detectable active MAP kinase so did its matched lymph node metastases (19/21); if the primary tumor did not have detectable active MAP kinase (2/21) then its matched lymph node metastasis was also negative (2/19).

**Activated MAP kinase expression is increased in lymph node metastases compared to the primary breast tumor.** When the level of active MAP kinase
expression was semi-quantified using H-score analysis as described in the Materials and Methods section, a statistically significant increase (Wilcoxon matched pairs test, \( P = 0.0098 \)) in active MAP kinase expression was found in the lymph node metastases (median score = 1.0, \( n = 21 \)) versus the primary breast tumor (median score = 0.2, \( n = 21 \)). Examples of increased expression of active MAP kinase in lymph node metastases compared to their matched primary breast tumor are shown in Figure 3. These data suggest that not only is active MAP kinase expression a potential marker of metastasis but that it is also increased during breast cancer progression.

**Discussion.**

The data presented here confirm and extend previous data which suggested that increased expression of active MAP kinases, ERK 1 and 2, occurs during human breast tumorigenesis (8). Using multiple samples of human breast tumors and their matched adjacent normal breast tissues together with immunohistochemical detection of dually phosphorylated (active) MAP kinase, our data provide unequivocal evidence that increased active MAP kinase expression occurs in approximately 50% of primary breast tumors compared to their adjacent normal breast tissues. Conclusions reached, from previously published data, were derived from comparisons between breast tumors and independent cases of normal breast tissue and benign breast lesions. In only one case, was the sample matched from the same patient. The combined data suggest that increased expression of active MAP kinase frequently occurs during human breast tumorigenesis, and in part may play a role in this progress. The reasons for increased active MAP kinase expression are unknown, although it may, in part, be
due to increased total expression of MAP kinase (8) in addition to increased activity of growth factor receptor induced cell proliferation pathways (16, 17).

In examining the relationship of active MAP kinase expression in primary human breast tumors with known prognostic variables, increased active MAP kinase expression was unrelated to tumor type, grade or steroid receptor status. However, a positive association of active MAP kinase detection with the presence of lymph node metastases was observed. This suggests that active MAP kinase may be a marker of nodal metastases and a poor prognosis. Furthermore, the level of active MAP kinase in lymph node metastases was significantly increased above that found in their matched primary tumors, suggesting the possibility that increased active MAP kinase may have a mechanistic role in the metastatic progression of breast tumors. Receptor tyrosine kinase activation of signal transduction pathways often includes activation of the ERK 1/2 MAP kinases. Several growth factor receptor pathways are known to be deregulated in breast tumors in particular the c-erbB2 receptor is amplified and associated with a poor prognosis in approximately 30% of human breast cancers (18). Both EGF and IGF I receptor pathways are often increased in human breast tumors, and increased EGF receptor activity is also associated with a poor prognosis (19). In contrast, increased IGF receptor levels are usually associated with a good prognosis (20). Alterations in the extracellular environment (e.g. extracellular matrix components), that occurs during tumorigenesis and metastasis, can also affect MAP kinase activity (16). It is possible, therefore, that the increased active MAP kinase seen in lymph node metastases may be due, in part, to increased and/or deregulated activity of such receptor tyrosine kinase pathways, altered extracellular environment, as well as an
increased level of expression of total MAP kinase (8). Multiple mechanisms are therefore likely to be responsible for the increased active MAP kinase seen during breast tumorigenesis and breast cancer progression. Irrespective of the mechanism(s) by which active MAP kinase is increased, our data suggest that it may be an excellent marker for predicting micrometastases and identifying a subgroup of node negative breast cancers with a poor prognosis. However, this requires further investigation.

The data presented here suggest that active MAP kinase is increased in breast tumors compared to their matched, adjacent normal breast tissues, suggesting that active MAP kinase is increased during breast tumorigenesis and may have a role in breast tumorigenesis. A more detailed study of pre-neoplastic breast lesions would be necessary to support this hypothesis and identify the stage at which this increase occurs. However, in ER+ breast tumorigenesis such an increase in activated MAP kinase may have a role in the deregulated and altered action of estrogen thought to occur during this process (2). Although our data suggest that increased active MAP kinase expression was unrelated to steroid hormone receptor status in primary breast tumors, we have found a proportion (%) of ER+ tumors that can contain increased active MAP kinase expression. Since active MAP kinase is able to directly phosphorylate ERα (7) and apparently induce ligand independent activation of the receptor, the hypothesis has been suggested that this may underlie, in part, the development of tamoxifen resistance. To address this issue we compared the expression of active MAP kinase in primary tumors of patients, that later were classified as tamoxifen sensitive or resistant. These breast tumors were all ER+ and node negative. We found no significant differences in either the frequency of detection or the
level of active MAP kinase expression between the primary tumors from patients that subsequently were shown to be tamoxifen sensitive and the primary tumors from patients that subsequently were shown to be tamoxifen resistant. These data suggest that active MAP kinase expression in ER+ primary human breast tumors is not a marker of endocrine responsiveness, and is unlikely to be involved in de novo tamoxifen resistance. We cannot, however, exclude the possibility that altered active MAP kinase expression may be involved in acquired tamoxifen resistance. In this study we have only measured active MAP kinase in the primary tumors of ER+ patients as yet unexposed to any form of adjuvant treatment. Previous data and our current data show that not all ER+ breast cancers will respond to tamoxifen treatment despite never having seen tamoxifen before. This type of resistance is referred to as de novo resistance. To study acquired tamoxifen resistance it would be necessary in future studies to acquire biopsy material from breast cancer metastases that develop in patients whose original breast cancer responded to tamoxifen and then disease recurrence occurred during tamoxifen treatment.

In summary, we have investigated the expression of the active MAP kinase, ERK 1 and 2, during human breast tumorigenesis and breast cancer progression. Significantly increased active MAP kinase was found in primary breast tumors compared to their adjacent matched normal breast tissues, was correlated with nodal metastasis when detected in primary breast tumors, and was significantly increased in lymph node metastases compared to their matched primary breast tumors. These data suggest that not only is active MAP kinase a marker of progression in human breast
cancer, but may also have a role in both breast cancer progression as well as breast tumorigenesis.
References.


Legends to Figures.

Figure 1. Western blot analysis of T-5 human breast cancer cells with and without PD98059 treatment. T-5 human breast cancer cells were exposed to 50 mM PD 98059 for 3 hours and proteins extracted (14). Proteins were resolved by 10% PAGE/SDS as described in the materials and methods section. A) Expression of active MAP kinase visualized using anti-active MAPK rabbit polyclonal antibody (#V8031, Promega) and chemiluminescence. B) Blots from A were stripped and reprobed with goat anti-ERK1 (total MAP kinase, Santa Cruz) and visualized via chemiluminescence.

Figure 2. Active MAP kinase expression in primary invasive breast tumors and matched adjacent normal breast tissues determined immunohistochemically. A) active MAP kinase expression in an invasive breast tumour detected using rabbit polyclonal, antibody (#9101S, New England Biolabs); B) active MAP kinase expression in vehicle alone treated T-5 cells using rabbit polyclonal, antibody (#9101S, New England Biolabs); C) active MAP kinase expression in vehicle alone treated T-5 cells using E10 monoclonal antibody (#9106L, New England Biolabs); D) active MAP kinase expression in the matched adjacent normal breast tissue using rabbit polyclonal, antibody (#9101S, New England Biolabs); E) active MAP kinase expression in PD98059 treated T-5 cells using rabbit polyclonal, antibody (#9101S, New England Biolabs); F) active MAP kinase expression in PD98059 treated T-5 cells using E10 monoclonal antibody (#9106L, New England Biolabs). Magnification at microscopy x 200.
Figure 3. Active MAP kinase Expression in Primary Breast Tumors and Matched Concurrent Lymph Node Metastases. Immunohistochemistry with rabbit polyclonal antibody (#9101S, New England Biolabs); on primary tumors (A, B, and C) and their matched lymph node metastases (D, E, and F, respectively), showing no staining (A and D), moderate staining (B and E) and intense staining (C and F) for MAP kinase. ** = Lymphocytes. Magnification X 200.
Figure 1

A. VEHICLE  PD 98059

- ERK 1 (p44)
- ERK 2 (p42)

B. VEHICLE  PD 98059

- ERK 1 (p44)
- ERK 2 (p42)
Appendix 2
**Figure 1**

- ERβ (wild-type) in normal human breast tissue
- ERβ (wild-type) negative human breast tumor
- ERβ (wild-type) intermediate expressing human breast tumor
- ERβ (wild-type) high expressing human breast tumor
Normal Human Breast Tissue
IgYERβ503 (total)

Human Breast Ductal Hyperplasia
GC-17 rabbit antibody to ERβ
(wild-type or ERβ1)
Fig. 3. Normal human mammary epithelial cells obtained from reduction mammoplasties. 1-6 are cells isolated from 6 different patients. The cells were sorted by flow cytometry (reference Stingl et al., Differentiation 63: 210-13, 1998) on the basis of differential expression of markers α-6 integrin (top line), CALLA/CD10 (bottom line) as well as Epithelial-specific antigen (ESA), shown to identify different epithelial populations as determined by phenotypic and functional characterization. L = luminal, M = myoepithelial enriched. RNA was extracted and analyzed for ER expression by RT-PCR using primers specific for each ER. In C, ERβ isoforms were measured by RT and TP-PCR as in Leygue et al Cancer Res 59: 1175-79, 1999.
Appendix 3
Expression of a Repressor of Estrogen Receptor Activity in Human Breast Tumors: Relationship to Some Known Prognostic Markers

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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 $r = 0.3233$; $P = 0.042$) and that the median level of REA mRNA was significantly (Mann-Whitney two-tailed test, $P = 0.042$) 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 ER$\alpha$ (2). Recently, a highly specific repressor of the transcriptional activity of ligand-occupied ERs (ER-$\alpha$ and ER-$\beta$) 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 involved appearance of 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, 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. 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 as >10 fmol/mg of protein). These tumors spanned a wide range of grade (grades 4–5), 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 $1 \times 10^6$ 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 passing stock cells twice in phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum, 100 nm glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin (5% charcoal-stripped fetal bovine serum) as described previously (5). Cells were then plated at above in 5% charcoal-stripped fetal bovine serum and 2 days later treated with 10 nm estradiol-17β and harvested for analysis at various times (1–48 h). The steroids were added directly from 1000X stock solutions in ethanol to obtain the required concentrations. The cells were harvested by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at $-70^\circ$C until RNA was isolated.

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2 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.
RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20-μm 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 μg of total RNA was reverse transcribed in a final volume of 25 μl as described previously (6).

Primers and PCR Conditions. The primers used were primer REAU (5′-CGA AAA ATC TCC TCC CCT ACA-3′; sense; positions, GenBank Accession No. AF150962) and primer REAL (5′-CCT GTG CTT TTT CTA CCA-3′; antisense; position, GenBank Accession No. AF150963). PCR amplifications were performed and PCR products analyzed as described previously (7) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl in the presence of 4 ng/μl 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 the 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 prestained 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 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
Epithelium, or percentage of stromal or fat cell content within the tumor sections analyzed.

Discussion

Our data show that the level of REA 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 REA mRNA expression and known prognostic and treatment response markers in human breast cancer biopsies. The positive correlation of REA and ER expression (a good prognostic variable and a marker of response to endocrine therapies) together with inverse correlation of REA expression and grade suggests that REA 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 REA 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 REA. This latter suggestion together with the lack of correlation of absolute ER levels and REA mRNA in ER+ tumors would be consistent with our observation that REA expression, at least at the RNA level, was found not to be regulated by estrogen.

REA has been identified as a protein that interacts in a yeast-two hybrid system with a dominant negative mutant ERs (3). It was shown to be a selective repressor of ER (both ERα and ERβ) transcriptional activity as determined in transient transfection assays using several estrogen-responsive element-containing promoters regulating a chloramphenicol acetyltransferase reporter gene. Cotransfection of a REA expression vector enhanced the potency of antiestrogens such as 4-hydroxytamoxifen andICI 182,780. Furthermore, REA competitively reverses coactivator, i.e., SRC-1, transcriptional enhancement of ER activity. Together these data suggest that REA 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.

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 REA, 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 REA 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.

References
Altered Expression of Estrogen Receptor Coregulators during Human Breast Tumorigenesis

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Abstract

The hypothesis that altered expression of specific coactivators/corepressors 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 (REAI) 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 REAI mRNA was not different between tumors and normal tissues (n = 19; Wilcoxon; P = 0.110). The ratios of AIB1:REAI and SRA:REAI were higher (Wilcoxon; P < 0.05) in tumors compared with normal tissues. Furthermore, SRA:AIB1 was higher (Wilcoxon; P = 0.0008) 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 ERα 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 ERα (7-17% ERα+ ductal epithelial cells; Ref. 1), whereas >70% of primary breast cancers are ERα+ (2). Furthermore, the majority of proliferating cells in normal human breast tissue is ERα+, 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 ERα+ breast tumors are ERα+ (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 ERα levels often increased during breast tumorigenesis (9), but it is likely that other factors which modulate ERα activity might also be altered during breast tumorigenesis with an outcome of enhancement or deregulation of ERα signaling that may underlie alterations of estrogen responsiveness from indirect in normal breast epithelium to direct in ERα+ breast tumor cells. We have addressed this hypothesis by investigating the expression of two known coactivators of ERα, SRA (7) and AIB1 (10), and a repressor of ERα activity, REAI (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 ERα and/or steroid receptors, e.g., SRA (7) and REAI (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 micro-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-μm breast tumor and normal 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-REAI, consisting of pGEM-T-easy plasmid containing a 399-bp insert of the human REAI cdNA (from nucleotide 385 to 783, numbered according to GenBank accession no. AF150562), 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 hematoxylin.

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20-μm 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 μg of total RNA was reverse-transcribed in a final volume of 25 μl as described previously (13).

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

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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, ductal carcinoma in situ.
Bank accession no. AF092038) and SRAcoreL primer (5'-AGTCTGGG-GAACCCGAGGAT-3'; antisense; positions 696–678; GenBank accession no. AF092038) and AIB1-U primer (5'-CTG GTA GG ACG-3'; sense; positions 110–130; GenBank accession no. AF092108) and AIB1-L primer (5'-TCC TTG CTT TTT TAT TCG ACG-3'; antisense; positions 458–438; GenBank accession no. AF092108); and (c) REA-U primer (5'-CGA AAA ATC TCC TTC CCT ACA-3'; sense; positions 385–405; GenBank accession no. AF130662) and REA-L primer (5'-CTC CCT TTG CTT TTT CTA CCA-3'; antisense; positions 781–761; GenBank accession no. AF130662).

Radioactive PCR amplifications for SRA were performed and PCR products were analyzed as described previously (14), with minor modifications. Briefly, 1 μl of RT mixture was amplified in a final volume of 15 μl in the presence of 1.5 μCi of [α-32P]dCTP (3000 Ci/mmol), 4 ng/μl 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 μl of RT mixture was amplified in a final volume of 20 μl, in the presence of 4 ng/μl of each primer and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.).

For AIB1, one 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 were then 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 the intensities of PCR products on pre-stained 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 deleted 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 pre-stained 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., log[AIB1: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
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 coactivator, 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 repressors 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; \( P = 0.0141 \)) in
**SRA, AIB1, and REA Expression During Breast Tumorigenesis**

**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. 2. A, detection of SRA and SRAdel 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 tumors (T) is shown. Arrows, the expressed 662-bp core SRA PCR product (SRA core, confirmed by sequence analysis); and a 459-bp deleted SRAdel variant PCR product (SRAdel), which was identified by sequence analysis to correspond to an SRA variant deleted in sequences from position 155 to 327 (GenBank accession no. AF590238). 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 precast 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 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 precast 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 379-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. Arrows, the expected 178-bp GAPDH PCR product.

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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 □ and the tumor samples by ○. 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).
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 □ and the tumor samples by ○. 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 tissue (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 tissue (Wilcoxon matched pairs test, two-tailed; P = 0.0014). C, the relative expression of SRA:AIB1 expression in normal tissue is significantly different from that in the tumor tissue (Wilcoxon matched pairs test, two-tailed; P = 0.0058).

Recently, a study was published (19) in which both ERα 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 ERα 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 ERα 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 is 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 ERα 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 ERα occurs during breast tumorigenesis in ER+ tumors. This is reflected not only in the increased expression of ERα 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 ERα can occur during human breast tumorigenesis in vivo and may
contribute to altered estrogen action, which is known to occur during this process.

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

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Pituitary Tumor Transforming Gene (PTTG), isolated from GH-secreting adenomas, exhibits in vitro and in vivo transforming activity and regulates hMG-β secretion. Preservation of a C-terminal proline-rich motif (P-X-X-P) is crucial for these functions. As PTTG facilitates chromatin 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 expression 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 (at ≥ 2 SD) was observed in 9/13 cases of thyroid hyperplasia mean ± SEM PTTG fold-increase, 1.9 ± 0.33), 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) induced a 4-fold increase in pttg mRNA expression and FRTL5 transfectants overexpressing human PTTG formed colonies in soft agar colony number mean ± SEM; vector, 10 ± 3.2; wt-PTTG, 55 ± 8.6; mut-PTTG, 9 ± 3.4, 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.

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ALTERNED 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 a quantitative reverse transcription polymerase chain reaction assay, the expression of coactivators (ER corepressor RNA Activator (SRA), and Amplified In Breast Cancer 1 (AIB1)) in a prostate cancer (PC-3), and a breast cancer cell line (MCF-7) was assessed. The level of SRA mRNA was significantly higher in PC-3 compared to MCF-7. The relative expression of SRA and AIB1 mRNA was compared between breast tumors and normal breast tissue (n = 19, Wilcoxon matched pair test, P = 0.0004) in tumors (median = 62 arbitrary units) compared to their adjacent normal tissue (median = 7 arbitrary units). In the same samples the AIB1 mRNA was significantly higher (Wilcoxon, P = 0.0058) in tumor samples (median = 67.8 arbitrary units) compared to adjacent normal tissue. In normal tissue the SRA mRNA was significantly different (n = 19, Wilcoxon, P = 0.001) in tumors (median = 84.6 arbitrary units) compared to adjacent normal tissue. The ratio of SRA/REAl mRNA was significantly higher (Wilcoxon, P = 0.0003) in tumors (median = 67 arbitrary units) compared to normal tissues (median = 12 arbitrary units). Similarly, the ratio of AIB1/REAI mRNA was significantly higher (Wilcoxon, P = 0.0041) in tumors (median = 87 arbitrary units) compared to normal tissues (median = 61.3 arbitrary units). Interestingly, the relative expression of AIB1/REAl was higher (Wilcoxon, P = 0.0058) in tumors compared to normal tissues, suggesting that coactivators and corepressors act in different mechanisms of action.
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<td>Registration Radisson Reef Resort</td>
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<td>WELCOME FUNCTION: COCKTAIL PARTY AT RADISSON REEF</td>
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## Saturday 4th November

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<td><strong>Ron Ross:</strong> Epidemiology of breast &amp; prostate cancer</td>
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<td><strong>James Yager:</strong> Oxidative metabolism of estrogens: role in estrogen mediated carcinogenesis</td>
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<td><strong>Wayne Tilley:</strong> Structure and function of the androgen receptor in advanced prostate cancer</td>
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<td><strong>Benita Katzellenbogen:</strong> Estrogen receptors: selective ligands, coregulators, and regulated genes in breast cancer</td>
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<td><strong>Chris Clarke:</strong> Expression of progesterone receptors A and B in normal and malignant breast and uterus</td>
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<td><strong>Rob Nicholson:</strong> Endocrine response mechanisms in breast cancer: Role of EGFR signalling</td>
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<td><strong>Suzanne Fuqua:</strong> Estrogen action in premalignant and invasive breast cancer</td>
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<td><strong>Leigh Murphy:</strong> Estrogen receptors (ERs) in human breast tumorigenesis and breast cancer progression</td>
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Oestrogen receptors (ERs) in human breast tumorigenesis and breast cancer progression

Murphy J.C.
Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba, Canada. R3E 0W3

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% of 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 once 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 ERα, ERβ, 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 ERα, ERβ, and their variant mRNAs are expressed in both normal and neoplastic breast tissues. The relative expression of ERα / ERβ is significantly altered between ER+ breast tumors and their matched adjacent normal breast tissues. Further, this increase in ERα / ERβ ratio is primarily due to a significant increase in ERα mRNA expression in conjunction with a lower ERβ mRNA expression in the tumor compared to normal tissue in some but not all ER+ cases. Furthermore, the relative expression of both ERα and ERβ 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 ERβ, SRA and REA varies amongst human breast tumors. The expression of several of these modulators of ERα 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.
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/retninoid 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.
because it limits the useful 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 ERβ, has recently been cloned and characterized (10,11). Moreover, it has recently been shown that ERβ mRNA is expressed in both normal and neoplastic human breast tissue (12–14). This suggests that ERβ 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 ERα and ERβ can be expressed in human breast tissues, and may therefore have roles in estrogen and antiestrogen signal transduction (13,15–19). The current data suggest that an evaluation of estrogen interaction with human breast tissue needs to include ERα, ERβ, 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.

**ERα AND ITS VARIANTS**

**Identification of ERα Variant mRNAs in Human Breast Tissues**

A large body of data has accumulated supporting the existence of ERα variants (19,20). The majority of the data supporting the expression of ERα variants has been at the mRNA level. Two main structural patterns of ERα variant mRNAs have been consistently identified: the truncated ERα mRNAs (21) and the exon-deleted ERα mRNAs (22). The truncated ERα 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 ERα mRNAs have been cloned and found to contain authentic polyadenylation signals followed by poly(A) tails. The exon-deleted ERα mRNAs have been identified mostly from reverse transcription polymerase chain reaction (RT-PCR) products, using targeted primers.

Multiple ERα 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 ERα 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

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**Fig. 1. Top panel.** Schematic representation of WT ERα (WT-ERα) cDNA and primers allowing co-amplification of most of the described exon-deleted ERα variants. ERα 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). 18/8 and 18/18 primers allow amplification of 1381-bp fragment corresponding to WT ERα 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 ERα variant mRNAs deleted in exon 3 (D3-ERα), exon 4(D4-ERα), exon 7(D7-ERα), both exons 3 and 4 (D3-4-ERα), exons 2 and 3 (D2-3-ERα), exons 4 and exon 7 (D47-ERα), would generate 1264-, 1045-, 1197-, 928-, 1073-, and 861-bp fragments, respectively. **Bottom panel.** Co-amplification of WT ERα 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 18/8U
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, 898, 861, 872, 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 (D3-3-7-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 (phi174, Gibco-BRL, Grand Island, NY). Adapted with permission from ref 24.
Fig. 2. Linear regression analysis of the relationship between the clone-4-truncated ERα mRNA and the WT ERα 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 ERα transcripts are expressed in any one tumor, that the pattern and relative frequency of detection of ERα variant mRNAs may vary between tumors, and that, in some cases, the relative frequency of detection of individual ERα 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 ERα variant mRNA was measured relative to the wild-type (WT) ERα 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 ERα variant proteins exist, and that their pattern and frequency are different from different individuals. In some cases, the expression of single ERα variant mRNA species was correlated with known markers of prognosis and endocrine sensitivity. This, in turn, suggested the hypothesis that altered expression of ERα variants may be a mechanism associated with progression to hormone independence.

**Putative Biological Significance of ERα Variant mRNAs**

**Expression of ERα Variant mRNAs in Normal and Neoplastic Human Breast Tissue**

Most studies investigating ERα variant mRNAs have used human BC tissues or cell lines (19). However, it is now known that both truncated and exon-deleted ERα variant
mRNAs can be detected in other tissues, including normal tissues (19). In particular, ERα variant mRNAs have been identified in normal human breast tissue and cells (26–29). Therefore, ERα variant mRNAs are not tumor-specific, are not found in the complete absence of the WT ERα mRNA, and are probably generated by alternative splicing mechanisms.

These observations raised the question of whether the expression of ERα variant mRNAs is altered during breast tumorigenesis and/or progression. When the level of expression of individual variant ERα 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 ERα variant mRNA and the exon-5-deleted ERα variant mRNA, but not the exon-7-deleted ERα 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 ERα variant mRNA is decreased in BCs, compared to normal human breast epithelium (29). Because this ERα variant mRNA encodes a protein that can inhibit WT ERα 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 ERα variant may function to attenuate estrogenic effects in normal mammmary epithelium. This function is markedly reduced via decreased exon-3-deleted ERα 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 ERα 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 ERα gene, which may be altered during breast tumorigenesis.

**Specificity of ERα Splice Variants in Human Breast Tumors**

It is unlikely that the mechanisms generating alternatively spliced forms of ERα 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 ERα Variant mRNAs During BC Progression**

As described above, the relative expression of at least one ERα variant mRNA, i.e., clone-4-truncated ERα mRNA, is significantly higher in primary breast tumors with characteristics of poor prognosis (including the presence of concurrent lymph node metatstases), compared to primary tumors with good prognostic markers (including lack of concurrent lymph node metastases) (25). An increased relative expression of exon-5-deleted ERα 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 ERα 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 ERα variant mRNA, which occurs during breast tumorigenesis, further changes in ERα 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 ERα mRNAs, between a series of primary breast tumors and their matched concurrent lymph node metastasis, suggesting that altered expression of ERα 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 ERα Variant mRNAs and Endocrine Resistance**

The hypothesis that altered forms of ERα may be a mechanism associated with endocrine resistance has been suggested for some time. Moreover, the identification of ERα 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 ERα 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 ERα 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 ERα mRNA was often co-expressed at relatively high levels with the WT ERα in many human BC that were ER-positive (38). It has been observed that transiently expressed exon-5-deleted ERα has an inhibitory effect on endogenously expressed WT ERα 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 ERα was shown to have little effect alone, but significantly enhanced estrogen-stimulated gene expression by transiently co-expressed WT ERα (40). The limitations of transient expression analysis were addressed by two groups who stably overexpressed the exon-5-deleted ERα 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 ERα 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 ERα 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 transfecants in addition to transgene expression. The transgene in the Rie and Parker study (41) was episomal; 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 ERα variant mRNA expression with estrogen/antiestrogen responsiveness was investigated. An increased relative expression of an exon-3 + 4-deleted ERα 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 ERα and an exon-5-deleted ERα mRNA. Compared to the parental cell line (45), other independently derived antiestrogen-resistant clones showed no major differences in the expression of ERα variant mRNAs (46,47).

Investigation of ERα splice variants, using clinical tissue samples, has also led to variable conclusions. The relative expression of the clone-4-truncated ERα 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 ERα 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 ERα variant mRNA was associated with low tumor grade (24). The presence of exon-5-deleted ERα 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 ERα 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 ERα was significantly greater than the control TAM-sensitive group.

Although increased expression of any one ERα 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 ERα 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 ERα splice variant expression has been analyzed in relationship to endocrine resistance and prognosis. Although mutations have been found in the ERα gene in human breast tumors, they are rare and are not more frequent in TAM-resistant tumors (48).

**Identification of ERα Variant Proteins**

The detection of proteins that correspond to ERα 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 ERα variant transcripts are shown schematically in Fig. 3. All of the variant transcripts would encode ERα proteins missing some structural/functional domains of the WT ERα. Although the ERα 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 ERα 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 ERα 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 ERα 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 ERα 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 ERα 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 ERα variant proteins predicted to be encoded by ERα variant mRNAs. Identical sequence is depicted by numbered exons. U, amino acid sequence unrelated to WT human ERα 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 ERα variant proteins encoded by known ERα variant mRNAs in tissues or cells in vivo.

From a different perspective, the prediction that the majority of ERα 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 ERα variant proteins could theoretically influence determination of ER status of the tumor sample. The authors have tested this experimentally, by transiently transfecting WT ERα and clone-4-truncated ERα expression vectors into Cos-1 cells, and determining ER status of the cells, using Abs either to the N-terminus of the ERα (Fig. 3, 1D5, Dako) or Abs to the C-terminus (Fig. 3, AER311, Neomarkers). Preliminary data, using
different combinations of WT ERα and variant ERα 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 ERα-like proteins containing the N-terminal region, but not the C-terminal region. These preliminary data suggest that increased expression of C-terminally truncated ERα 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 ERα, 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 ERα 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 ERα-like proteins.

To address the hypothesis that the C-terminally truncated ERα-like proteins could correspond to proteins encoded by ERα variant transcripts, the authors compared expression of ERα variant mRNAs in the consistent and inconsistent tumors. The results show a significantly higher relative expression and detection of ERα 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 ERα 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 ERα 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 ERα H-scores found in some human breast tumors.

More recently, data published from several independent groups support the detection of ERα-like proteins in cell lines and tissues in vivo, which could correspond to those predicted to be encoded by previously identified ERα variant mRNAs. The presence of an exon-5-deleted ERα 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 ERα protein (39). However, although there was a correlation between IHC detection and presence or absence of exon-5-deleted ERα 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 ERα, or differential stability of the variant protein relative to the WT ERα during the extraction procedure. In addition, an ERα-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 ERα protein and a 53-kDa protein recognized by ERα Abs to epitopes in the N-terminus and C-terminus of the WT protein, but not with an Ab recognizing an epitope encoded
by exon 4 (52). These results correlated with the presence of both WT and exon-4-deleted ERα mRNAs in these tissues, and suggested that the 53-kDa protein was derived from the exon-4-deleted ERα mRNA.

More recently, a 61-kDa ERα-like protein and a more abundant 65-kDa WT ERα protein were identified in MCF-7 cells (29). The 61-kDa protein is thought to be encoded by an exon-3-deleted ERα mRNA expressed at low levels in these cells, and its co-migration, both before and after dephosphorylation with the recombinant exon-3-deleted ERα 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 ERα protein.

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

**ERβ AND ITS VARIANTS**

**Identification of ERβ mRNA in Human Breast Tissues**

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

The authors have detected the presence of ERβ 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 ERβ mRNA could be detected in the BC cells of a human BC biopsy sample (14). Using an RT-PCR approach to analyze both ERβ and ERα mRNA expression in a range of breast tumors (12), the following was observed: There was no correlation between ERβ expression and ERα expression in breast tumors; in some cases, both ERβ and ERα 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, ERβ 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 ERβ protein(s) in human breast tissues as yet, the available information suggest that ERβ may be expressed in both normal and neoplastic human breast tissues, and may have a role in these tissues.

Expression of ERβ mRNA During Breast Tumorigenesis

The demonstration of ERβ mRNA expression in both human breast tumors and normal human breast tissues 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 ERα:ERβ 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 ERα:ERβ ratio in breast tumors was primarily the result of a significant upregulation of ERα mRNA in all ER-positive tumors, in conjunction with a lower ERβ 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 ERβ mRNA in breast tumors may be correlated with the degree of inflammation (unpublished data). Because in situ hybridization data suggest that expression of ERβ 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 ERβ mRNA (14), it is possible that the cell type contributing to the expression of ERβ 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 ERα- and ERβ-driven pathways, and/or their interaction, probably changes during breast tumorigenesis.

Identification of ERβ Variant mRNAs in Human Breast Tissues

The presence of multiple ERα variant mRNAs in both normal and neoplastic human breast tissues has led to the question of the expression of ERβ variant mRNAs. Several ERβ variant mRNAs have been detected. The authors have identified an exon-5 + 6-deleted ERβ mRNA in human breast tumors (59). This transcript is in-frame, and would be expected to encode an ERβ-like protein deleted of 91 amino acids within the hormone binding domain. A human ERβ variant mRNA, deleted in exon 5, was identified in MDA-MB-231 human BC cells and in some human breast tumor specimens (18). Although that group was unable to detect an exon-5-deleted ERβ mRNA in normal human breast tissue, the authors have detected both exon-5-deleted ERβ mRNA and an exon-6-deleted ERβ mRNA, as well as an an exon-5 + 6-deleted ERβ mRNA, in normal human breast tissue samples (13), and in some human breast tumors. The exon-5-deleted ERβ mRNA and the exon-6-deleted ERβ mRNA are out-of-frame and predicted to encode C-terminally truncated ERβ-like proteins, which would not bind ligand.

More recently, several exon-8-deleted human ERβ mRNAs have been identified (17) from a human testis cDNA library, and by RT-PCR from the human BC cell line MDA-MB-435. These variants have been named human ERβ2-5. It should be noted that human ERβ2 is not the equivalent of the ERβ 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 ERβ2. The authors have been unable to detect an equivalent of the rodent ERβ2 mRNA in any normal or neoplastic human tissue so far studied (13).
Several of the human ERβ variants deleted in exon 8, specifically hERβ2 and hERβ5, can be detected in normal human mammary gland and in several human BC cell lines (17). The predominant type of hERβ exon-8-deleted mRNA present varies among the different cell lines. The authors have confirmed the presence of the hERβ2 and the hERβ5 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 hERβ2 and the hERβ5 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 hERβ1 and the two variant hERβ2 and hERβ5 mRNAs, it appears that, in most, but not all, cases, the level of the variant mRNA species exceeds that of the WT hERβ1 (Fig. 5: unpublished data) in both normal and neoplastic human breast tissues. The known sequence of all human ERβ-like transcripts is shown schematically in Fig. 6: also shown in this figure are the proteins predicted to be encoded by these variant hERβ mRNAs. All the hERβ 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 hERβ and hERα, and may preferentially inhibit hERα DNA-binding transcriptional activity (62).

**Putative Role of ERβ and Its Variants in Breast Cancer**

Transient transfection studies have provided data which suggest that ERβ1, i.e., the WT ERβ, can only mediate an antagonist response when bound to TAM-like agents, in contrast to the TAM-bound WT ERα, 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 hERβ 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, AB006589x), hERβ3 (Genbank AF060555), hERβ+ (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 ERβ and/or its variants in human breast tissues. First, it is not yet known whether ERβ and ERα 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 ERβ protein expression in human breast have been published to date. Therefore, the pathophysiologically relevant levels of ERβ and ERα 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 ERβ1 (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 ERα, 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 ERα 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,
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 ERα 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 ERα mRNA, ERβ 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 ERα, variant ERβ, and WT ERβ proteins, which correspond to the variant ERα, variant ERβ, and WT ERβ 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.
ACKNOWLEDGMENTS

This work was supported by grants from the Canadian BC Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). LCM is a Medical Research Council of Canada (MRC) Scientist, PHW is a MRC Clinician-Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship, AC is a recipient of a Manitoba Health Research Council (MHRC) Studentship.

REFERENCES


**FINAL PROGRAM**

*Simultaneous translation (English / French)*  
available in sessions marked with *

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**Thursday, May 3, 2001**

3:00 - 6:00 pm  
Reception

5:00 - 6:00 pm  
Opening Reception - Sponsored by AVON  
Foyer, Convention Floor

Carol Storoz, CBCRl Board Chair - CBCRl's unique partnership

6:00 pm  
Opening of Conference  
Grand Ballroom

Welcome and moment of silence

Why reasons for hope?  
Kathleen Prichard, MD - Scientist perspective

Carol Ann Cole - A survivor's perspective

6:30 - 9:00 pm  
Evening event - Speaker themes: hormone

Chairs:  
Jacques Simard PhD and Joy Yorath

Speakers:

Malcolm Pike PhD - Overview of prevention/epidemiology

Susan Davis MBBS, PhD, FRACP - Hormone replacement therapy and breast cancer: an Australian experience

Gabriel André MD - Hormone replacement therapy and breast cancer: the European and French experience

Evan R. Simpson PhD - Molecular insights into the roles of estrogen in the aging woman

Fernand Labrie MD, PhD - Selective estrogen receptor modulators and women's health

9:00 - 9:30 pm  
* Panel discussion
Saturday, May 5, 2001

7:00 - 8:00 am  Continental breakfast  
                Foyer, Convention Floor

8:00 am  * Welcome and moment of silence  
          Grand Ballroom

8:00 am  * Keynote address: Alan Bernstein PhD, FRSC  
          President, Canadian Institutes of Health Research

8:30 - 10:15 am  * Plenary session: New and novel targets for treatment of breast cancer  
                  Grand Ballroom
          Chairs: Tak Mak O.C., PhD, DSc, MD, FRSC, FRS and Romy Litwin
          Speaker 1: Gabriel Leckey Ph.D.* - The role of tumour initiating lymphocyte-induced
          homing and dose chemotherapy as a novel treatment strategy
          Speaker 2: Paul Gray* - Oncolytic viruses as cancer therapeutics
          Speaker 3: Marie-France Lee PhD* - Reovirus and breast cancer

10:15 - 10:45 am  Panel discussion

10:45 - 11:15 am  Nutrition break  
                  Foyer, Convention Floor

11:15 am - 12:45 pm  Concurrent sessions:  
                      Presentations by CBCRI grantees and others
                      1. Theme: Oncogenes & tumour suppressor genes
                         Chairs: Adam Müller PhD and Barbara Gauthier
                         Speaker 1: Trevor Shepherd BSc - Role of the pea3 subfamily ets genes in
                                   mammary oncogenesis
                         Speaker 2: Don White MSc - Induction of mammary tumours in transgenic mice
                                   expressing the integrin-linked kinase (ILK) in the mammary epithelium
                         Speaker 3: Eldad Zacksenhaus PhD - Constitutively active Rb transgenes induce
                                   breast cancer
2. Theme: *Hormonal factors*

*Suzor-Côté Room*

**Chairs:** Vincent Giguere PhD and Sandra Fusco

**Speakers:**
- Trevor Archer PhD - Transcription regulation in breast cancer cells
- Norman Boyd MD, DSc, FRCP(C) - Risk factors for breast cancer as determinants of blood level growth hormone, IGF-1 and prolactin
- Harriet Kinyamu PhD - Selective activation of the glucocorticoid receptor by steroid antagonists in cancer cells
- Jean Latreille MDCM, FRCP(C) - Testosterone, a hormonal marker for breast cancer in postmenopausal women: preliminary results of a case-control study in Montreal
- Leigh Murphy PhD - Estrogen receptors (ERs) and beyond (cogs, wheels and kinases) in human breast tumorigenesis

3. Theme: *Communications, information dissemination & education*

**Chairs:**

**Speakers:**
- Jacqueline Frasson - Media exposure to treatment and therapy intervention
- Soffia Fransessen MSc - Breast disease awareness: influence of medical knowledge: a randomized trial to assess the impact of a home study module on medical-students' performance
- Joanne Galajda BA - Innovative training for the physician: communication with breast cancer patients
- Andrew Horgan - A randomized controlled trial
- Dawn Stacey RN, MScN - The development and validation of a preventive decision aid for women at high risk for breast cancer
- Patrice Watras MD - Community study of a physical activity aide (booklet & audiotape) for women with a family history of breast cancer
- Tim Whelan MD, MSc - Development and evaluation of computer-based version of the research support for cancer

4. Theme: *Clinical Trials*

**Chairs:**

**Speakers:**
- Robert Martz SRM, MSc - Double-blind randomized trial of tamoxifen versus placebo in patients with node positive or high risk node negative breast cancer who have completed CMF, CEF or AC adjuvant chemotherapy.
- Michael Pollak MD - A randomized trial of antiestrogen therapy versus combined antiestrogen and octreotide LAR therapy in the adjuvant treatment of breast cancer in post-menopausal women
- Kathleen Pritchard MD, FRCP(C), FACP - Disease-free & overall survival after cyclophosphamide, Adriamycin, 5-FU, & tamoxifen (CAFT) compared to T alone in postmenopausal, receptor (+), node (+) breast cancer: new findings from phase III southwest oncology group intergroup trial S8814 (INT-0100), (NCIC CTG MA.9)
ESTROGEN RECEPTORS (ERs) AND BEYOND (COGS, WHEELS AND KINASES) IN HUMAN BREAST TUMORIGENESIS.

LC Murphy PhD¹, P Watson MD², E Leygue PhD¹, H Dotzlaw¹, S Simon BSc¹, A Parkes BSc¹, B Lu MD¹, T Cherlet MSc¹, A Adeyinka MD², Y Nui MSc¹, L Snell², S Troup BSc².

Department of Biochemistry and Medical Genetics¹ and Department of Pathology², University of Manitoba, Winnipeg, Manitoba, Canada. R3E 0W3.

Altered estrogen action is thought to occur and be a major driving force in breast tumorigenesis. The assumption derives from observations that 1) only a minority of normal breast epithelial cells have detectable ER (7 - 17%) but > 70% of primary breast cancers are ER+; 2) estrogen is an indirect mitogen in normal breast, but is a direct mitogen in ER+ breast tumors. To determine mechanisms underlying altered estrogen action during tumorigenesis in vivo, we investigated ER isoform expression in human breast tissues, expression of coregulators of ER transcriptional activity in human breast tissues, and expression of activated MAP kinases (erk1 and 2), that are known to activate ER by phosphorylation.

Using semi-quantitative reverse transcription polymerase chain reaction assays (RT-PCR), the expression of ERα, ERβ, 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 the relative expression of ERα and ERβ is significantly altered between ER+ breast tumors and their matched adjacent normal breast tissues. This difference is due to a significant (P<0.05) increase in ERα mRNA expression in conjunction with a lower ERβ mRNA expression in the tumor compared to normal tissue in some but not all ER+ cases. In addition, the levels of the two ER coactivators, SRA and AIB1 mRNA are increased in ER+ tumors compared to normal tissues (P < 0.01). In contrast, the expression of the ER corepressor, REA mRNA is not significantly different between tumors and normal tissues. These results were confirmed using in situ hybridization analyses. Consistent with these data, the ratios of AIB1/REA and SRA/REA are higher (P < 0.05) in tumors compared to normal tissues. Furthermore, the ratio of SRA/AIB1 is higher (P = 0.0058) in tumors compared to normal tissues. Using immunohistochemistry we examined the expression of activated (phosphorylated) MAP kinases (erk1/2) between breast tumors and their adjacent normal breast tissues. Activated MAP kinase expression was significantly increased in breast tumors compared to their normal adjacent breast tissue (P = 0.027).

These results suggest that not only are there alterations in ER isoform expression during breast tumorigenesis but there is also increased expression of several factors capable of activating ERα with little, if any, increase in factors thought to negatively regulate ERα. These changes may have a role in altered estrogen action occurring during breast tumorigenesis.
Appendix 4
Dr Leigh Murphy
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Dear Dr. Murphy,

Our editorial policy is to publish in each issue of "Biomedicine & Pharmacotherapy" articles that we consider to be of particular interest to our readers.

Elsevier Publishers will publish a special issue on "Steroids and nuclear receptors in cancer" scheduled for February 2002.

We would appreciate if you could provide us with a review article of 6 to 8 pages on "Steroid receptors in human breast tumorigenesis and breast cancer progression".

If positive, you will receive by regular mail a copy of "Instructions to authors" in order to provide us with a manuscript by December 15 2001.

Looking forward to your participation,
Yours sincerely,

Jacques-Michel Renoir
Haim Tapiero

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Printed for "Leigh C. Murphy" <lcmurph@cc.umanitoba.ca>
Dear Dr Tapiero: I will be happy to accept your invitation to write this review article for Biomedicine & Pharmacotherapy: "Dossier Steroid Hormones".

Yours sincerely, Leigh Murphy
Dear Dr Murphy,
Thank you for your positive answer. Please find downloaded the "Instruction to authors"

Best Regards

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