Advances in Brief

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 (REA) of the estrogen receptor was compared between matched breast tumors and adjacent normal human breast tissue. The levels of SRA and AIB1 mRNA were increased in tumors compared with normal tissues (n = 19; Wilcoxon matched pairs test; P < 0.01). In contrast, the expression of REA mRNA was not different between tumors and normal tissues (n = 19; Wilcoxon; P = 0.11). The ratios of AIB1/REA and SRA/REA were higher (Wilcoxon; P < 0.05) in tumors compared with normal tissues. Furthermore, SRA/AIB1 was higher (Wilcoxon; P = 0.0058) in tumors compared with normal tissues. Although our study is small, these data are consistent with the above hypothesis and suggest that such alterations may have a role in the altered estrogen action occurring during breast tumorigenesis.

Introduction

During human breast tumorigenesis, enhanced activity of the 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 ER+ breast tumors are ERα+ (5).

Factors that enhance and repress receptor activity directly, namely coactivators and corepressors, are now 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, REA (8), at the mRNA level in ER+ human breast tumors and their matched adjacent normal breast tissues. The coregulators studied were chosen because they were identified as either selective for ERs and/or steroid receptors, e.g., SRA (7) and REA (8), or were identified previously to be of relevance in human breast cancer in vivo, e.g., AIB1, which is frequently amplified in breast tumors in vivo (10).

Materials and Methods

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

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

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20-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’-AGGAACCGCGCTGGAACCA-3’); sense; positions 35–53; Gen-6266.
Bank accession no. AF092038) and SRACoreL primer (5'-AGTCTGGGG-GAACCAGGAT-3'; antisense; positions 696–678; GenBank accession no. AF092038); (b) AIB1-U primer (5'-ATA CCT GCT GGA TGG TGG ACT-3'; sense; positions 110–130; GenBank accession no. AF012108) and AIB1-L primer (5'-TCC TTG CTC TCT TAT TTG ACG-3'; antisense; positions 458–438; GenBank accession no. AF012108); and (c) REA-U primer (5'-CGA AAA ATC TCC TCT CCA ACA-3'; sense; positions 385–405; GenBank accession no. AF150962) and REA-L primer (5'-CCT GCT TTG CTT TTA CCA CCA-3'; antisense; positions 781–761; GenBank accession no. AF150962).

Radioactive PCR amplifications for SRA were performed and PCR products were analyzed as described previously (14), with minor modifications. Briefly, 1 μl of RT mixture was amplified in a final volume of 15 μl in the presence of 1.5 μM 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. For AIB1, each PCR consisted of 30 cycles (30 s at 57°C, 30 s at 72°C, and 30 s at 94°C). PCR products then were separated on agarose gels stained with ethidium bromide as reported previously (15).

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

Quantification of 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 measure of the relative expression of the deleted variant (15). For each sample, the signal corresponding to the SRAdel was measured using Quantity One software (Bio-Rad). Three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the SRA signal of one particular tumor measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. In parallel, GAPDH cDNA was amplified, and after analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst (Bio-Rad). Three independent PCRs were performed. Each GAPDH signal was also expressed as a percentage of the signal observed in the same tumor as above. For each sample, the average of the SRA signal was then expressed as a percentage of the GAPDH signal (arbitrary units).

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

Results

Characterization of SRA and REA RNA Expression in Human Breast Tissues by in Situ Hybridization. SRA is functional as an RNA molecule (7), and because no antibodies are available for the immunohistochemical detection of REA, we have therefore used an in situ hybridization approach to determine the cellular localization of expression of SRA and REA RNA in human breast tissues. Fig. 1 shows examples of the results obtained. Antisense RNA probes to SRA showed a strong signal over the epithelial tumor cells of an ER+ human breast tumor section (Fig. 1A), with little, if any, signal obtained when sense SRA probes were used on the adjacent section of the same tumor (Fig. 1C). Low levels of SRA expression were detected mainly over the ductal epithelial cells of normal breast tissue from the same patient. This result paralleled that for AIB1, where it was previously shown using in situ hybridization that AIB1 mRNA expression was significantly increased in breast cancer cells carrying increased copies of the AIB1 gene compared with normal breast epithelial cells, although it was not stated that these samples were from the same patient in this study (10).

In contrast, when the in situ expression of REA mRNA was examined in an ER+ tumor and its matched adjacent normal breast tissue (Fig. 1, D and E, respectively), little difference could be seen between the signal over the epithelial breast tumor cells compared with the normal breast epithelial cells. Furthermore, little if any signal was observed when REA sense probes were used (Fig. 1F). These data suggested that the expression of the steroid receptor-specific coactivator, SRA, in addition to AIB1 (10) was significantly increased in breast tumor cells compared with normal breast epithelial cells, whereas the expression of a specific ER-repressor was not altered in breast tumors compared with normal breast epithelial cells. To investigate this further, we developed a semi-quantitative RT-PCR approach to measure the expression of these coregulators in multiple samples of ER+ breast tumors and their matched adjacent normal breast tissues, as described below.

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

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

Detection of REA mRNA in Normal and Neoplastic Human Breast Tissues. 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 in each tissue sample for all matched pairs is shown in Fig. 3C. REA expression was not significantly different (Wilcoxon matched pairs test; \( P = 0.110 \)) in the tumors (median = 84.6 arbitrary units) compared with the adjacent normal tissues (median = 69.8 arbitrary units). REA expression in the tumors was not correlated with PR status, grade, tumor size, or nodal status.

**Altered Relative Expression of Coactivators and Repressors during Human Breast Tumorigenesis.** The above data suggest that alterations in the relative expression of ER activators and repressor occurred during breast tumorigenesis. To address this question, the relative expression of SRA and AIB1 mRNA to REA mRNA was compared between the breast tumors and the normal tissues. Results are shown in Fig. 4. The ratio of SRA:REA (Fig. 4A) was significantly higher (Wilcoxon matched pairs test; \( P = 0.0003 \)) in tumors (median = 87 arbitrary units) compared with normal tissues (median = 12 arbitrary units). Similarly, the ratio of AIB1:REA (Fig. 4B) was significantly higher (Wilcoxon matched pairs test; \( P = 0.0414 \)) in tumors compared with normal tissues (median = 97 arbitrary units).
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 tumor tissue (T) is shown. Arrows, the expected 662-bp core SRA PCR product (SRA core, confirmed by sequence analysis) and a 459-bp deleted SRA variant PCR product (SRAdel), which was identified by sequence analysis to correspond to the SRA variant deleted in sequences from position 155 to 357 (GenBank accession no. AF092038). B, detection of AIB1 in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA was extracted and assayed for AIB1 expression using RT-PCR as described in "Materials and Methods." After analysis of PCR products on prestained agarose gels, signals were quantified using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of four sets of normal tissue (N) and matched tumor tissue (T) is shown. Arrows, the expected 349-bp AIB1 PCR product (confirmed by sequence analysis). C, detection of REA in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA was extracted and assayed for REA expression using RT-PCR as described in "Materials and Methods." After analysis of PCR products on prestained agarose gels, signals were quantified using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of four sets of normal tissue (N) and matched tumor tissue (T) is shown. Arrows, the expected 397-bp REA PCR product (confirmed by sequence analysis). D, ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. Arrow, the expected 178-bp GAPDH PCR product.

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

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

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

Recently, a study was published (19) in which both ER\(\alpha\) and the coactivator TIF2 were found to be significantly increased in intraductal carcinomas compared with normal mammary gland tissue. This study suggested as well that ER\(\alpha\) and a general corepressor N-CoR are reduced in invasive breast cancer compared with DCIS. Although these results are consistent with our data and support the hypothesis that there may be an up-regulation of factors associated with increased ER signaling in breast tumorigenesis, the number of cases screened was small compared with our study, the normal samples and DCIS samples were not matched, i.e., were not from the same patient, to the invasive breast cancer samples, and furthermore not all tumors were ER\(+\). These factors introduce biological heterogeneity because the natural history of ER\(+\) and ER\(^{-}\) breast cancers 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 ER signal transduction pathway, can be significantly altered between normal human breast and breast tumors in vivo.

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

References
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