Expression of the Steroid Receptor RNA Activator in Human Breast Tumors

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Abstract

The expression of the recently described steroid receptor RNA activator (SRA) was measured by semiquantitative reverse transcription-PCR within 27 independent breast tumors, spanning a wide spectrum of grade and estrogen receptor (ER) and progesterone receptor (PR) levels. Subgroup analysis showed that SRA expression was similar in ER+/PR+ (median = 65.5, n = 8) and in ER−/PR− (median = 94.6, n = 5) tumors. Interestingly, SRA expression in these two subgroups was significantly (Mann-Whitney rank-sum test, P < 0.05) lower than that observed in ER+/PR− (median = 156.4, n = 6) and ER+/PR+ (median = 144.8, n = 8) tumors. A variant form of SRA, presenting a deletion of 203 bp within the SRA core sequence, was also observed in breast tumor tissues. The relative expression of this new SRA isoform correlated with tumor grade (Spearman coefficient r = 0.53, n = 27, P = 0.004). These data suggest that changes in the expression of SRA-related molecules occur during breast tumor progression.

Introduction

Estrogens, through their mitogenic action on breast epithelial cells, regulate the growth and the development of normal as well as neoplastic human mammary tissue (1). The ability of antiestrogens such tamoxifen or raloxifene to antagonize this estrogenic action provides the basic rationale for endocrine therapy and prevention (for review see Ref. 2). Estrogen action is mainly mediated through two ERs (ERα and ERβ (3–5)), which belong to the steroid/thyroid/retinoic acid receptors superfamily (6) and act as ligand-dependent transcription factors. The mechanisms by which steroid receptors modulate the transcription of target genes is under extensive investigation (7). Once bound to the ligand, the receptors undergo conformational changes and dimers of receptors recognize specific regulatory DNA sequences bound to the ligand, the receptors undergo conformational changes

Materials and Methods

Human Breast Tumors. Twenty-seven cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected according to their ER and PR status, as determined by ligand binding assay. Tumors were classified as ER−/PR− (n = 8; ER range, 5–9 fmol/mg protein; PR range, 51–271 fmol/mg protein), ER+/PR− (n = 6; ER range, 59–151 fmol/mg protein; PR range, 5–10 fmol/mg protein), ER+/PR+ (n = 5; ER range, 0–2 fmol/mg protein; PR range, 0–8 fmol/mg protein), and ER+/PR+ (n = 8; ER range, 50–127 fmol/mg protein; PR range, 101–285 fmol/mg protein). These tumors covered a wide spectrum of grade (grades 4–9), determined using the Nottingham grading system (11). Patients were 49–87 years old.

RNA Extraction and RT-PCR. Total RNA was extracted from frozen breast tissue sections using Trizol reagent (Life Technologies, Inc., 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 (12).

Primers and PCR Conditions. The primers used consisted of SRAcoreU primer (sense, 5′-AGGAAAAGCCTTGAGAAGGA-3′, positions 35–53; GenBank accession no. AF092038) and SRA core L primer (antisense, 5′-AGCTC- GGGAGACACCGGAT-3′, positions 696–678; GenBank accession no. AF092038). PCR amplifications were performed and PCR products analyzed as described previously (12), with minor modifications. Briefly, 1 μl of reverse transcription 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 each primer, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). 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. Following electrophoresis, the gels were dried and exposed for 1 h to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA). Amplification of the ubiquitously expressed GAPDH cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as described previously (12). Identity of PCR products was confirmed by subcloning and sequencing, as reported previously (13).

Quantification of SRA Expression. Exposed screens were scanned using a Molecular Imager-FX (Bio-Rad), and the intensity of the SRA corresponding signal 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 (tumor 14) 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 following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software.
independent PCRs were performed. Each GAPDH signal was also expressed as a percentage of the signal observed in the tumor 14. For each sample, the average of SRA signal was then expressed as a percentage of the GAPDH signal (arbitrary units).

Quantification of SRA-Del Relative Expression. It has previously been shown that the coamplification of a wild-type and a deleted variant 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 (13, 14). For each sample, SRA-Del corresponding signal was measured using Quantity One software (Bio-Rad) and expressed as a percentage of the corresponding SRA signal. For each case, three independent assays were performed, and the mean was determined.

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

Results

Detection of SRA and a Variant mRNA Deleted Form (SRA-Del) in Human Breast Tumor Tissues. The existence of three different SRA mRNAs has been reported (9). The sequences of these isoforms differ in their 5' and 3'-terminal regions but are identical within their central region, called the core (Fig. 1). To investigate the expression of all described SRA isoforms in human breast tumor tissues, we designed primers to amplify a 662-bp fragment encompassing almost all of the SRA core region. Total RNA was extracted from 27 human breast tumors and reverse-transcribed, and PCR amplification was performed as described in the “Materials and Methods” using SRA core primers. A 662-bp fragment was obtained in all samples. However, the intensity levels varied from one sample to another (Fig. 2A). This fragment was sequenced and corresponded to the SRA core region. The differences in SRA expression were unlikely to result from different cDNA input, as shown by the similar intensities of GAPDH signal obtained after amplifying GAPDH mRNA in parallel using the same cDNAs (Fig. 2B). An additional fragment, migrating at an apparent size of 459 bp was also observed in most samples. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (corresponding to GenBank accession no. AF092038).

The Expression of SRA Correlates with ER and PR Levels in Subgroups of Human Breast Tumors. For each case, the SRA-corresponding signal was quantified and expressed in arbitrary units, as described in “Materials and Methods.” Results obtained from the 27 cases, grouped according to their ER and PR levels, as determined by ligand binding analysis, are presented in Fig. 3A. When the cohort of cases was considered as a whole (n = 27), no correlation was observed between SRA expression and ER or PR levels. Indeed, similar levels of SRA were found in ER+/PR+ (median = 65.5, n = 8) and ER−/PR− (median = 94.6, n = 5) tumors (Fig. 3A). However, when only ER− tumors were considered (n = 13), a trend toward a positive correlation between SRA expression and PR levels was observed (Spearman coefficient r = 0.527, P = 0.064). SRA expression was higher in ER−/PR+ (n = 8, median = 144.8) than it was in ER−/PR− tumors (Fig. 3A); this difference was statistically significant (two-sided Mann-Whitney rank sum test, P = 0.045). In contrast, within ER+ cases (n = 14), SRA expression negatively correlated with PR levels (Spearman coefficient r = −0.810, P = 0.0004). SRA expression was higher (two-sided Mann-Whitney rank sum test, P = 0.001) in ER+/PR− (n = 6, median = 156.4) than it was in ER+/PR+ cases. In a similar way, SRA expression correlated positively (Spearman coefficient r = 0.735, P = 0.009) and negatively (Spearman coefficient r = −0.532, P = 0.033) with ER levels in PR− (n = 11) and PR+ (n = 16) cases, respectively. SRA levels were higher in ER+/PR− than in ER−/PR− tumors (two-sided Mann-Whitney rank sum test, P = 0.017) and in ER+/PR− than in ER+/PR+ cases (two-sided Mann-Whitney rank sum test, P = 0.047). SRA levels of expression did not correlate with tumor grade scores (Fig. 3B).

The Expression of SRA-Del Correlates with Breast Tumor Grade Scores. For each case, SRA-Del signal was measured and expressed relative to the corresponding SRA signal, as described in the “Materials and Methods.” SRA-Del relative signal did not correlate with ER or PR levels when the cohort of cases was considered as a whole or when ER−, ER+, and PR− subgroups were analyzed. Interestingly, SRA-Del expression positively correlated (Spearman coefficient r = 0.512, P = 0.042) with PR levels in PR+ subgroup (n = 16). However, no statistically significant differences (Fig. 4A) were observed between ER−/PR− (n = 8, median = 2.346), ER+/PR− (n = 6, median = 2.561), ER−/PR− (n = 5, median = 6.571) and ER+/PR+ (n = 8, median = 3.528). By contrast, SRA-Del levels strongly correlated (Spearman coefficient r = 0.530, P = 0.004) with Nottingham grade scores within the whole cohort (n = 27). The level of expression of SRA was significantly higher (two-sided Mann-Whitney rank sum test, P < 0.05) in tumors of high grade (n = 7, median = 6.572) than it was in tumors of low (n = 4, median = 2.192) or intermediate (n = 9, median = 2.588) grade (Fig. 4B).

Discussion

Using primers annealing with the core region of the three previously described SRA isoforms (9), we have investigated SRA expression in 27 independent breast tumors by means of semiquantitative RT-PCR. These SRA isoforms, although different in their 5'- and 3'-terminal regions, are all able to coactivate steroid receptor. Indeed,
SRA core region was found to be necessary and sufficient for the coactivation properties of SRA isoforms (9). Therefore, although PCR performed using primers spanning the SRA core region is likely to recognize different SRA-like molecules, the signal obtained corresponds to molecules that should all have essentially the same function, i.e., coactivation of steroid receptors.

The expression of SRA did not correlate with ER or PR status when the cohort was considered as a whole. This differs from what has been observed for another coactivator, AIB1. Indeed, Anzick et al. (15) first showed that a strong expression of AIB1 that resulted from AIB1 gene amplification was observed in ER+ but not in ER− breast cancer cell lines. More recently, Bautista et al. (16) reported that AIB1 gene amplification correlated with ER and PR positivity. Our results suggest that the pattern of expression of SRA is more complex. Indeed, we found that SRA expression could correlate positively or negatively with ER and PR levels, depending on the subgroup considered. The general trend appeared to be that, in tumors expressing a low level of one receptor (ER or PR), a positive correlation was found between SRA expression and the second receptor (PR or ER). Inversely, in tumors highly expressing one receptor (ER or PR), SRA expression negatively correlated with the level of expression of the second receptor (PR or ER). At this stage of the knowledge of SRA biological function, the interpretation of such an observation is difficult. Indeed, SRA has been shown to be able to coactivate both ER and PR (9). Moreover, progestins are known to decrease the steady state levels of ER-α mRNA and protein, whereas estrogens increase PR expression (17, 18). Therefore, all combinations and cross-talk appear possible. One could speculate that increased levels of SRA in ER−/PR+ cases could partially be responsible, by “boosting” the activity of the weakly expressed ER, of the expression of PR in these tumors. Inversely, in the same ER−/PR+ cases, the strong SRA expression could be responsible for an increased down-regulation of ER by PR. Our results suggest that SRA expression varies from one particular tumor to another. Changes in SRA expression can be associated with known prognostic and predictive factors such as ER and PR in particular tumor subgroups. The question of a direct involvement of SRA in the hormonal status changes occurring during breast tumor progression remains unanswered. Also of interest is the fact that SRA interacts with the activation function 1 of the steroid receptors (9). Activation function 1 is thought to mediate the agonistic effect of antiestrogens such hydroxytamoxifen (19). This agonistic action of antiestrogens is believed to be involved in part in the mechanisms underlying hormone

Fig. 3. Subgroup analysis of SRA expression within 27 human breast tumors. For each case, SRA expression was quantified and expressed in arbitrary units as described in “Materials and Methods.” A, tumors were grouped according to their ER and PR status, as determined by ligand binding assay. □, ER+/PR+ tumors; ▪, ER+/PR− tumors; ■, ER−/PR+ tumors; and ○, ER−/PR− tumors. B, tumors were grouped according to their grade: low (Nottingham grading scores 4–5), intermediate (Nottingham grading scores 6–7), and high (Nottingham grading scores 8–9). The horizontal line represents the median value in each group. P values (two-sided Mann-Whitney rank sum test) are indicated when subgroups were statistically different. ns: no statistically significant differences were found between subgroups.

Fig. 4. Subgroup analysis of SRA-Del relative expression within 27 human breast tumors. For each case, SRA-D3 expression relative to SRA was quantified as described in “Materials and Methods.” A, tumors were grouped according to their ER and PR status, as determined by ligand binding assay. □, ER+/PR+ tumors; ▪, ER+/PR− tumors; ■, ER−/PR+ tumors; and ○, ER−/PR− tumors. B, tumors were grouped according to their grade: low (Nottingham grading scores 4–5), intermediate (Nottingham grading scores 6–7), and high (Nottingham grading scores 8–9). The horizontal line represents the median value in each group. P values (two-sided Mann-Whitney rank sum test) are indicated when subgroups were statistically different. ns: no statistically significant differences were found between subgroups.
resistance in breast cancer. One could speculate that the level of SRA expression might, therefore, modulate and predict the response of a given tumor to hormone therapy. This hypothesis appears to be refuted by the observation of similar levels of SRA in ER+/PR+ and ER−/PR− tumors. But ER+/PR+ tumors, as opposed to ER−/PR− tumors, are likely to respond to endocrine therapy and prevention (see Ref. 2 and references therein). In these cases, the differences in ER levels rather than in SRA expression are more likely involved in the mechanisms underlying endocrine sensitivity. On the other hand, the observation of a higher SRA expression within ER−/PR+ cases, which are more likely to respond to hormone therapy than ER−/PR− tumors (see Ref. 20 and references therein), would be consistent with the hypothesis of a possible involvement of SRA in these mechanisms under some circumstances. One should also note that Berns et al. (21) recently reported that, although no correlation was found between the expression of SRC-1 and ER status, a high expression of this coactivator indicated a favorable response to tamoxifen of patients with recurrent breast cancer. This issue can only be addressed in studies performed on tumors from patients that did and did not respond to endocrine therapy.

We have identified in breast tumor cases a new SRA isoform deleted in sequences from nucleotide 155 to 357 (SRA-DeI). Interestingly, sequence comparison using the BLAST algorithm and the human EST database showed that this deleted SRA isoform has already been found in a pooled cDNA library containing cDNAs from melanocyte, fetal heart, and pregnant uterus (GenBank accession no. AA426601). Because uterus is another steroid target tissue, it could be hypothesized that the source of SRA-DeI in this pooled library was, indeed, uterus. Even though the structure of the SRA gene has not yet been published, SRA-DeI appears to correspond to a perfect exon-3 deleted SRA variant. SRA gene has recently been located on chromosome 5q31.3-32.4 Sequence analysis of the corresponding DNA sequence (chromosome 5, BAC clone 319C17; GenBank accession no. AC005214) revealed that the fragment from nucleotide 155 to 357 corresponds to the third exon. The putative function of SRA-DeI remains to be determined. One should, however, note that a recombinantly developed SRA mutant, deleted of the region 3’ of a Bbox site (position 341) and, therefore, partially deleted of exon 3 sequences, did not coactivate steroid receptors (9). Moreover, exon 3 deletion introduces a shift in the open reading frames, suggested by Lanza et al. (9), and could lead to a premature termination of the putative SRA proteins. One could, therefore, hypothesize that SRA-DeI might interfere with SRA activity. The resulting modifications of the steroid receptor signaling pathways could confer a more aggressive behavior to the tumors expressing higher levels of SRA-DeI. The positive correlation between SRA-DeI levels and tumor grade scores would be consistent with this hypothesis.

Interestingly, modifications of the long arm of the chromosome 5 have been reported in breast tumors. Indeed, Herrmsen et al. (22) found a frequent chromosomal gain in 5q within a subset of 53 lymph node-negative breast carcinomas, whereas Schwendel et al. (23) observed a frequent loss of this region in 39 invasive breast carcinomas. Moreover, among BRCA1 mutation carriers, loss of 5q was observed more frequently than in the control patient (24). One could, therefore, speculate that the loss of SRA is selected for during tumor progression in cells lacking BRCA1 functional gene. Whether changes in SRA expression result from chromosomal abnormalities remains to be determined.

In conclusion, we have shown that SRA is expressed in breast tumors and that its expression correlates with ER and PR levels in particular tumor subgroups. We speculate that changes in SRA expression could be involved in the mechanisms underlying tumor progression and hormone resistance.

References

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