Exon 5 Deletion Variant Estrogen Receptor Messenger RNA Expression in Relation to Tamoxifen Resistance and Progesterone Receptor/pS2 Status in Human Breast Cancer

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ABSTRACT

The exon 5 deletion splice variant of estrogen receptor (Δ5 ER), which in vitro is constitutively active in the absence of estrogens, may have a role in conferring both tamoxifen resistance and ER-related phenotype in breast cancer. We have investigated the expression of this variant in vivo (at the level of mRNA) in relation to known tamoxifen resistance and expression of the estrogen-regulated genes progesterone receptor (PgR) and pS2. The amount of Δ5 ER mRNA relative to wild type (WT) ER mRNA (%Δ5/WT) was assessed in 70 tamoxifen-resistant and 50 primary breast carcinomas using reverse transcription/PCR. Both WT and Δ5 ER mRNA were detected in the majority of tumors, although Δ5 ER was detected only in the presence of WT ER. Overall, no significant difference was seen in %Δ5/WT ER between tamoxifen-resistant and primary control tumors (medians, 13 and 15%, respectively). Tumors in both control and resistant groups which expressed PgR/pS2 in the absence of measurable ER protein (ER− PgR+ and ER− pS2+) had significantly higher Δ5 ER mRNA levels compared with other phenotypes (P < 0.002). This association with ER−/pS2+ tumors has not been demonstrated previously. In ER+ tumors which expressed pS2, significantly greater Δ5 ER mRNA expression was observed in tamoxifen-resistant compared with control tumors (P = 0.05). A similar although nonsignificant trend was observed in ER+ PgR+ tumors. While Δ5 ER mRNA is unlikely to be responsible for tamoxifen resistance in most breast cancers, elevated Δ5 ER mRNA levels may be important in some tumors, especially those which continue to express high levels of PgR/pS2.

INTRODUCTION

ER expression has been recognized for many years to be indicative of a good prognosis in breast cancer (1). Endocrine treatment for breast cancer generally involves inhibition of the mitogenic stimulus produced following the binding of estrogens to ER. In the absence of estrogens, ER is present within cells as an inactive complex with a number of heat shock proteins. Upon binding of estrogens ER dissociates from this complex, dimerizes, binds to EREs in the 5' region of ER-responsive genes, and activates transcription of that gene via its two trans-activating factor domains (TAF-1 and TAF-2) (2, 3). The antiestrogen tamoxifen which is widely used in the treatment of breast cancer (4, 5) competes with estrogen for ER, and although it allows ER to dimerize and bind to ER-responsive elements of genes, it causes an altered pattern of gene transcription which results in a decreased mitogenic stimulus to the cell (6, 7). However, of approximately two-thirds of all breast tumors which are classified as ER+, only about 50% respond to tamoxifen treatment. A number of explanations for the nonresponsiveness of these tumors have been suggested, including impaired uptake of drug by the tumor (8), altered metabolism of tamoxifen (9), and variants/mutant forms of ER which are constitutively active in the presence of tamoxifen (10).

PgR and the product of the pS2 gene are two proteins the expression of which is normally associated with an active ER (11, 12). However, in up to 10% of ER− tumors, PgR or pS2 proteins may be expressed in the apparent absence of measurable ER protein (13). As with tamoxifen resistance, variant/mutant forms of ER which would not be detected by conventional methods (EIA and dextran-coated charcoal assay) have been suggested to be present in these tumors and may be responsible for the ER− PgR/pS2+ phenotype (10).

A number of splice variants of ER mRNA have been detected in breast cancer cell lines and tissues (14). Of particular interest as a possible explanation for both tamoxifen resistance in breast cancer and PgR/pS2 expression in ER− tumors is the exon 5 deletion splice variant (Δ5) first identified by Fuqua et al. (10). As a result of a deletion of exon 5 from the mRNA sequence, a missense translation of exon 6 occurs which is prematurely terminated 5 amino acids after the splice site and results in a truncated protein. The major trans-activating function (TAF-2) and the ligand-binding regions of the protein are thereby missing. However, the DNA binding region remains, as does TAF-1, and the translated protein has been shown to be constitutively active in the absence of estrogens in both a yeast system (10) and the breast cancer cell line MCF-7 (15). Preliminary work in MCF-7 cells has also indicated that overexpression of Δ5-translated protein confers resistance to tamoxifen (15). In addition, in a small number of breast cancers, this Δ5 ER mRNA variant was detected in the ER− PgR+, but not ER− PgR−, tumors by RT/PCR (10).

We have investigated the possible role of this exon 5 deletion ER variant both in tamoxifen resistance and in conferring the PgR or pS2 status of breast tumors in vivo. We have previously used RT/PCR followed by Southern blot analysis and sequencing to identify and characterize Δ5 ER mRNA in a number of breast cancer cell lines (16). We now report the use of these techniques to investigate the incidence of Δ5 mRNA compared with WT ER mRNA in breast cancer tissues. WT ER mRNA was detected in the majority of breast tumors irrespective of their ER protein status as measured by EIA. Δ5 ER mRNA was also detected in these tumors, although never in the absence of WT ER mRNA. We have identified a greater Δ5/WT ER ratio in ER− PgR+ and ER− pS2+ tumors compared with other phenotypes and in some ER+ tamoxifen-resistant tumors which continue to express PgR or pS2.

MATERIALS AND METHODS

Tumors. Tumors were obtained from 70 patients with known tamoxifen-resistant breast cancer. These consisted of 47 postmenopausal patients with primary breast cancer who were treated with tamoxifen (20 mg daily) rather than surgery at presentation because of age, tumor size, or advanced local disease. The tumors either were resistant de novo following a median duration of 4 months (n = 14) or responded initially according to International Union Against Cancer criteria but subsequently relapsed with acquired resistance.
following a median duration of 24 months (n = 33). At progression or relapse on tamoxifen, these resistant tumors were treated surgically by either mastectomy or wide local excision. A further 20 patients, all of whom had been treated with adjuvant tamoxifen following surgery for early breast cancer, relapsed following a median of 28 months with either local recurrence in the breast or axilla while still taking tamoxifen and were managed surgically. In addition 3 patients on adjuvant tamoxifen relapsed with metastatic skin nodules, some of which were surgically excised. As a control group 50 tumors were studied which had been excised from postmenopausal women with primary untreated breast cancer.

At surgery in all patients a portion of tumor (approximately 200 mg) was snap-frozen in liquid nitrogen and stored at —80°C for subsequent analysis of WT and ΔS ER. The remainder of the tumor was fixed in formalin and paraffin embedded in wax.

**Cell Lines.** MCF-7 cells were used as a positive control for the RT/PCR. Cells were grown in RPMI 1640 (GIBCO-BRL) containing 10% fetal calf serum (GIBCO-BRL), 2 mM L-glutamine, 5 units/ml penicillin, 5 μg/ml streptomycin, and 12.5 ng/ml amphotericin (Sigma). Upon reaching 80% confluence the cells were collected by EDTA/trypsin treatment, snap-frozen in liquid nitrogen, and stored at —80°C.

**RNA Extraction.** Frozen tumor samples and MCF-7 cells were pulverized in a dismembrator (Braun Biotech UK, Aylesbury, Buckinghamshire, United Kingdom). RNA was purified using RNezol B (Biotecz Laboratory, Houston, TX), as recommended by the manufacturers. Quantification was by spectrophotometry.

**RT/PCR.** Two μg RNA were denatured at 65°C for 3 min and reverse transcribed in the presence of 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 5 mM dithiothreitol (Life Technologies, Ltd., Paisley, Renfrewshire, Scotland), 1 unit/μl RNase inhibitor, 20 μM random primers (Promega, Southampton, United Kingdom), buffer, and 5 units/μl reverse transcriptase (superscript; Life Technologies, Ltd.) for 20 min at 23°C and 1 h at 42°C followed by 10 min at 95°C. PCR was performed using 500 ng concentrations of each primer [CAA GCC GCT TCA TGA TCA (sense; exon 4; 1110—1127 base pairs)]; CAC CAG ACC CTC TAC ACA (antisense; exon 6; 1481—1498 base pairs) and 0.1 unit Taq polymerase (Applied Biosystems Ltd., Woolston Warrington, Cheshire, United Kingdom) in a total of 100 μl. Each PCR consisted of 35 cycles (1 min at 95°C, 1 min at 55°C, 1 min at 72°C) followed by 5 min at 72°C using a Techne PHC-3 thermal cycler. We have previously characterized the WT (388 base pairs) and ΔS (249 base pairs) ER RT/PCR products by Southern blot analysis, differential hybridization with exon 4- and exon 5-specific probes, and DNA sequencing (16).

**Southern Blot Analysis.** PCR product (50 μl) was precipitated using ethanol (100 μl) and glycogen (1 μl of 20-ml/mg solution; Boehringer-Mannheim, Lewes, East Sussex, United Kingdom), for 2 h at —20°C and centrifuged at 14,000 × g for 20 min at 4°C. The pellet was washed with 100 μl 75% ethanol, dried briefly, and resuspended in 10 μl autoclaved water. The DNA samples were loaded onto a 2% agarose gel and electrophoresed for 3 h at 70 V prior to denaturation of the DNA in 0.6 M NaCl, 0.2 M NaOH (45 min) and neutralization in 0.5 M Tris chloride (pH 7.4)-1.5 M NaCl for 30 min. The DNA was transferred onto Hybond N membrane (Amersham International, Amersham, Buckinghamshire, United Kingdom) using 20 × SSC buffer (3 M NaCl-0.3 M sodium citrate, pH 7.0) and was UV cross-linked (Stratagene, Ltd., Cambridge, United Kingdom).

Prehybridization and hybridization (20 h, respectively) were carried out in 7% SDS-10 mM sodium phosphate buffer (pH 7.3) and 1 mM EDTA at 42°C in a Technne hybridization oven. An exon 4-specific probe [CCCT TCC AGT GAA GCT TCG; 1338—1355 base pairs (17)] was end labeled with [γ-32P]dATP (DuPont, Ltd., Stevenage, Hertfordshire, United Kingdom) using T4 polynucleotide kinase (Life Technologies, Ltd.) as recommended by the manufacturers. Stringency washes (5 min at 42°C, 2 washes of 3 min at 42°C) were performed in 5% SDS-10 mM sodium phosphate buffer (pH 7.3) and 1 mM EDTA. The filter was then exposed to X-ray film at —70°C, with intensifying screens to visualize the results. Quantification was by phosphorimaging (Molecular Dynamics), and a ΔS/WT value was calculated.

**Validation of Percentage of ΔS/WT ER.** By expressing the ΔS product relative to WT ER product, account is taken of the efficiency of RT/PCR between different RT/PCR runs. It is widely acknowledged that standardization of RT/PCR is best achieved by amplification with the same set of primers of a control sequence which is as similar as possible to the template of interest, yet which allows PCR products to be distinguished on the basis of size, hybridization, or change of restriction site (18, 19). The coamplification of WT and ΔS can be considered as analogous to this controlled approach. We have previously shown parallel amplification of the WT and ΔS products through both the exponential and plateau phases of the reaction in cell lines and breast tumor tissues (16). In addition each RT/PCR run contained a negative (no RNA) and a positive control (MCF-7 cell RNA). For the positive control, the percentage of ΔS/WT ER values were consistent and reproducible [13.8 ± 0.9% (SE)] between 9 different RT/PCRs.

**ER, PgR, and pS2 Proteins Assays.** For ER and PgR analysis a portion of frozen tumor adjacent to that used for RNA extraction was pulverized in the dismembrator. The powdered tumor was reconstituted 1:8 (w/v) in iced Tris/molybdate buffer [5 mM sodium molybdate, 10 mM monothioglycerol, 1 mM dipotassium chloride EDTA, 3 mM sodium azide, and 10 mM Tris (pH 7.4)]. The ER and PgR status were measured in the extracted tumor cytosol using the respective ELA kits (Abbott Laboratories, Ltd., Maidenhead, Berkshire, United Kingdom). ER values >10 fmol/mg protein and PgR values >15 fmol/mg protein were considered positive.

pS2 was measured immunohistochemically on paraffin-embedded sections of tumor using the BC6 monoclonal antibody (gift from Prof. P. Chambon, Paris, France) in an assay which we have recently validated against the biochemical assay (20). The percentage of invasive carcinoma cells which showed positive cytoplasmic staining for pS2 were counted for each tumor, and tumors were considered positive if >10% cells were positive.

**Statistical Analysis.** Comparative statistical analysis between the groups was conducted using the Mann-Whitney nonparametric test. Comparison between the quantitative ER protein value and mRNA expression was made using linear regression analysis. The frequency of expression of ER, PgR, and pS2 between the resistant and control groups was compared using the χ² test.

**RESULTS**

While the frequency of ER+ tumors as measured by enzyme immunooassay was similar between the control and tamoxifen-resistant groups (54 and 61%, respectively), the ER protein levels were significantly lower in tamoxifen-resistant tumors (Table 1). In contrast, there was no significant difference in either the frequency or quantitative value of PgR and pS2 expression in ER+ tumors from the two groups.

RT/PCR was used to detect WT ER mRNA (388-base pair PCR product) and ΔS ER mRNA (249-base pair PCR product) (Fig. 1). The two bands, generated by the same pair of primers, were characterized by Southern blot hybridization and sequenced, and a measurement of percentage of ΔS/WT ER was used to indicate the relative expression of ΔS ER mRNA, as described previously (16). Using RT/PCR, 76% of ER+ tumors showed positive cytoplasmic staining for pS2 were counted for each tumor, and tumors were considered positive if >10% cells were positive.

**Table 1 Age, ER, PgR, and pS2 expression in primary untreated and tamoxifen-resistant breast cancer**

<table>
<thead>
<tr>
<th>Primary untreated breast cancer</th>
<th>Tamoxifen-resistant breast cancer</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient characteristics</td>
<td>No.</td>
<td>50</td>
</tr>
<tr>
<td>Median age</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>Range</td>
<td>47-81</td>
<td>32-86</td>
</tr>
<tr>
<td>ER+ tumors</td>
<td>No. of ER+</td>
<td>27 (54)*</td>
</tr>
<tr>
<td>ER value (fmol/mg protein)</td>
<td>109.3 ± 24.3d</td>
<td>43 (61)</td>
</tr>
<tr>
<td>PgR value (fmol/mg protein)</td>
<td>185.7 ± 71.3d</td>
<td>50.6 ± 7.9</td>
</tr>
<tr>
<td>pS2 value (percentage of cells)</td>
<td>13/27 (48)</td>
<td>16/43 (37)</td>
</tr>
<tr>
<td>pS2 value (%)</td>
<td>40.8 ± 6.9</td>
<td>51.9 ± 7.1</td>
</tr>
</tbody>
</table>

* Mann-Whitney test.

* NS, not significant.

* Numbers in parentheses, percentage of total.

* Mean ± SEM.
of the 120 breast tumor samples were shown to express WT ER mRNA. All the tumors which were ER+ for protein by enzyme immunobossay expressed WT ER mRNA. In addition 21 of 50 tumors which were ER− by enzyme immunobossay (<10 fmol/mg protein) had WT ER mRNA as detected by RT/PCR, although 12 of these had protein levels >5 fmol/mg protein. Of the tumors which were positive for WT ER mRNA by RT/PCR, 88% also expressed Δ5 ER mRNA by RT/PCR. Δ5 ER mRNA was not detected in the absence of WT ER PCR product.

The median ages of patients in the control and tamoxifen-resistant groups were 64 and 71 years, respectively. Although this was not statistically significantly different, we assessed whether there was a significant relationship between the Δ5/WT ratio and age in either the control or tamoxifen-resistant tumors. No significant relationship was found (r = 0.024 and 0.053, respectively), which indicates that age did not need to be considered as a covariate with receptor expression.

There was no significant difference in the % Δ5/WT ER ratio between the untreated control and tamoxifen-resistant tumors [median percentage of Δ5/WT ER, 15 and 13%, respectively (Fig. 2)]. Similarly no difference was observed between the untreated controls and either the acquired resistant (median, 10%) or the adjuvant relapse (median, 14%) subgroups of tamoxifen-resistant tumors. The percentage of Δ5/WT was higher in the de novo tamoxifen-resistant group (median, 26%), but this did not prove to be significant.

A weak inverse correlation between ER protein concentration and the percentage of Δ5/WT ER was observed for control tumors [r = −0.415, P < 0.01 (Fig. 3A)], but no similar correlation was demonstrated for tamoxifen-resistant tumors [r = −0.073, P not significant (Fig. 3B)]. Six tamoxifen-resistant tumors with ER values >40 fmol/mg protein had percentage of Δ5/WT ER ratios >20%, compared with none of the control tumors.

When the breast tumors were grouped with respect to their PgR or pS2 expression as an indicator of ER function (irrespective of their tamoxifen responsiveness), a variable Δ5/WT ER expression was found (Fig. 4). All possible combinations of ER+ tumors were identified with respect to PgR and pS2 protein expression. However, although there were several ER− tumors which were PgR− pS2−, PgR+ pS2−, or PgR− pS2+, only one ER− tumor was shown to express both PgR and pS2. Significantly higher percentage of Δ5/WT ER levels were demonstrated in both ER− PgR− pS2+ and ER− PgR+ pS2− tumors compared with other phenotypes. Tumors which expressed pS2 alone in the absence of ER protein had a significantly greater percentage of Δ5/WT ER compared with all phenotypes (P < 0.005), except ER− PgR+ pS2−. A significantly higher %Δ5/WT ER was also demonstrated in the tumors which expressed PgR alone in the absence of ER protein compared with the majority of phenotypes (P < 0.01), except ER+ PgR− pS2+ or ER− PgR− pS2−. The median percentage of Δ5/WT ER for ER− tumors expressing either PgR or pS2 or both was 47% compared with 22% for ER− tumors with neither of these proteins expressed (P = 0.0017).

Because the untreated control group would not all be tamoxifen sensitive, we thought that comparison of the two groups in relation to ER phenotype was more appropriate. The percentage of Δ5/WT ER levels in each particular ER/PgR/pS2 phenotype of both the control and resistant breast tumors are shown in Fig. 5. No significant differences between the untreated and resistant tumors was observed in the samples that did not express PgR or pS2 protein (ER+ and ER− tumors) (Mann-Whitney test). As shown above, the ER− tumors which expressed high levels of PgR/pS2 demonstrated elevated percentage of Δ5/WT ER levels, although there was no significant difference between the controls and resistant samples. In contrast, 21% of ER+ PgR+ and 44% of ER+ pS2+ tamoxifen-resistant tumors expressed higher percentage of Δ5/WT ER levels than the upper limit in their respective controls. These differences were significant for pS2 expressing (P = 0.05), but not PgR expressing ER+ tumors. No overall correlation was observed between percentage of Δ5/WT ER levels and the quantitative values for pS2 or PgR protein in either the control or tamoxifen-resistant tumors (analysis not shown).
EXON 5 DELETION VARIANT ER IN BREAST CANCER

A Untreated Control Tumors

![Graph showing relationship between WT ER protein and percentage of Δ5/WT ER ratios in untreated control tumors.](image)

B Tamoxifen-resistant Tumors

![Graph showing relationship between WT ER protein and percentage of Δ5/WT ER ratios in tamoxifen-resistant tumors.](image)

DISCUSSION

Expression of the exon 5 deletion splice variant in breast cancer has been implicated in conferring a PgR+ phenotype to ER− breast tumors and as a possible explanation for tamoxifen resistance in vitro (10, 14). This variant mRNA sequence is thought to code for a truncated protein which lacks both the TAF-2 sequence and the ligand-binding regions but retains its DNA binding and TAF-1 properties. The truncated protein would not be detected by conventional biochemical or immunohistochemical ER assays which depend on an intact ligand-binding domain. If constitutively active, however, the protein may still induce transcription of ER-dependent genes such as PgR or pS2 and thus explain the existence of ER− PgR/pS2+ tumors. In vitro experiments have shown that Δ5 ER confers ligand-independent expression of an ER-dependent reporter gene in yeast (10) and that overexpression of this variant in MCF-7 cells results in an increase in estradiol-independent growth of cells, with resistance to tamoxifen and enhanced expression of PgR (15). However, little work has been performed in human tumors to determine whether tamoxifen resistance is associated with increased expression of Δ5 ER.

We have previously used RT/PCR followed by Southern blot hybridization and DNA sequencing to demonstrate that the Δ5 ER mRNA species is present in a number of breast cancer cell lines (16). In order to quantify the absolute levels of Δ5 ER mRNA in tumor samples using RT/PCR, sampling would have to be performed within the exponential phase and compared to an internal control sequence such as glyceraldehyde phosphate dehydrogenase or actin (21). Alternatively, absolute quantification of message levels by RT/PCR may involve the inclusion of known amounts of exogenous controls which are similar in sequence and bind the same primers as the sequence of interest but are distinguished from it on the basis of size, restriction enzyme digestion, or hybridization. Using this quantification technique it has been demonstrated that valid data can also be obtained during the nonexponential phase of the reaction (18, 19). This current study was not concerned with quantifying absolute levels of Δ5 ER mRNA in the breast tumor samples; rather, we have allowed all our samples to reach the plateau phase, where the reaction is saturated irrespective of the initial amount of mRNA, and have measured the relative expression of the variant message to WT. We have shown previously that the Δ5 and WT ER RT/PCR products were amplified

Fig. 3. Relationship between the amount of WT ER protein and percentage of Δ5/WT ER ratios in untreated control (A) and tamoxifen-resistant (B) tumors. Tumors which expressed measurable amounts of ER (ER+) are those above the dashed line (10 fmol/mg protein). Regression analysis was applied to both populations of tumors.

Fig. 4. Comparison of the percentage of Δ5/WT ER ratios in different ER/PgR/pS2 phenotypes of breast tumors. Positivity for ER, PgR, and pS2 is defined in "Materials and Methods." Statistical analysis was performed using the Mann-Whitney test. Bar, median value. *, P < 0.01 compared to all groups except ER+ PgR− pS2+, ER− PgR− pS2+, ER− PgR+ pS2−, and ER− PgR− pS2+. **, P < 0.005 compared to all groups except ER− PgR+ pS2− and ER− PgR+ pS2+.

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with equal efficiencies in both the exponential and the plateau phases of our RT/PCR reactions, and the ratio of Δ5/WT was not dependent on PCR cycle number (16). Others have also shown coamplification of templates which accumulate in a parallel manner throughout both the exponential and nonexponential phases under certain conditions (18, 19). Within an individual tumor, given the parallel amplification of Δ5 ER and WT ER mRNA, the percentage of Δ5/WT ER ratio may be used as an indication of the relative expression of Δ5 ER. In a tumor where Δ5 ER may be of biological significance, one would expect increased Δ5 ER mRNA expression relative to WT ER mRNA, and therefore the ratio, to be greater in that sample compared with one with low expression.

The majority of tumors (76%) were found to express WT ER mRNA. Detectable WT mRNA was found in all ER protein-positive tumors, while a proportion of ER protein-negative tumors expressed some WT ER mRNA, emphasizing that the difference between the ER− and ER+ breast tumors is quantitative rather than absolute. Although as a result of tamoxifen treatment the ER protein levels (fmol/mg total protein) were significantly reduced in the tamoxifen-resistant compared to the untreated control tumors, PgR and pS2 values did not change significantly. These data are similar to the recent report on ER and PgR in a series of tamoxifen-treated human breast cancers (22). The fact that PgR and pS2 levels were not significantly altered may indicate that some of these tumors remain under hormonal regulation in spite of reduced WT ER. Alternatively a relative increase in expression of the Δ5 form of ER in some tamoxifen-resistant tumors may explain the continued expression of PgR and pS2 under conditions of depleted WT ER.

Our results demonstrate that overall the percentage of Δ5/WT ER ratio was similar between the tamoxifen-resistant and untreated control breast cancer tissues (median values, 13 and 15%, respectively) (Fig. 2). In addition, no significant increase in Δ5/WT ER mRNA was observed between the acquired, de novo, or adjuvant relapse tumors compared with untreated controls. No correlation was observed between the quantitative ER protein levels and the percentage of Δ5/WT ER ratio in resistant tumors while a weak correlation was observed in the control tumors. This loss of correlation could be attributed to the greater number of ER+ tumors expressing higher percentage of Δ5/WT ER levels in the resistant compared to the control tumors and may be suggestive of an association among resistance for tamoxifen, increased Δ5 ER mRNA expression, and the phenotype of the tumors.

PgR and pS2 protein expression can be used to indicate the functional activity of ER, although it must be recognized that factors other than activated ER may influence their expression. Our results indicate that ER− PgR+ pS2− and ER− PgR− pS2+ tumors express significantly greater levels of percentage of Δ5/WT ER compared with other phenotypes. These results confirm and extend the findings of Fuqua et al. (10), who originally identified the Δ5 ER variant in a small number of ER− PgR+ tumor samples. Furthermore, we have identified tumors with an ER− PgR− pS2+ phenotype and demonstrated that these expressed the highest levels of Δ5 ER mRNA (Fig. 4). This phenotype appeared to be more frequent than the ER− PgR+ pS2− tumors, although combined expression of both proteins in ER− tumors occurred in only one case. These results demonstrate that in either ER− PgR+ or ER− pS2+ tumors, Δ5 ER expression may indeed be important in conferring expression of the estrogen-regulated protein supporting the potential functional significance of the variant in breast carcinomas. It is possible that different promoters or transcription factors predominate in addition to Δ5 ER to explain the differential expression of either PgR or pS2 in these tumors. Furthermore, meningioma tissues which express low levels of detectable ER protein but high levels of PgR have been shown to express splice variants of ER mRNA (23, 24), suggesting that this phenomenon may not be confined solely to breast cancer. Although Δ5 ER mRNA has also been demonstrated in normal endometrial tissue (10) its relevance to these findings in breast cancer is unclear due to the differential reported effects of tamoxifen on the breast and endometrium.

In a recent report using the RNase protection assay Zhang et al. (25) found that the majority of breast tumors expressed Δ5 ER mRNA, although they could not demonstrate the presence of this variant in ER− PgR− samples. This is in contrast to our data where several ER− PgR− tumors were found to express Δ5 ER mRNA. They also demonstrated that there was little difference between the relative amounts of Δ5 and WT ER mRNA between the ER/PgR phenotypes by the RNase protection assay and that the majority of tumors expressed equivalent or greater amounts of Δ5 than the WT ER mRNA species. These discrepancies with our results and those previously reported by Fuqua et al. (10) may be a function of the different sensitivities between the RT/PCR and RNase protection assays.

The majority of the ER− untreated control tumors would be expected to be de novo resistant to tamoxifen treatment and, as expected, no significant differences between percentage Δ5/WT ER levels were observed between the ER− control and tamoxifen-resistant samples (Fig. 5, C and D). Most of the ER+ PgR/pS2− control tumors (with presumably nonfunctional receptors) would also be expected to be de novo resistant to tamoxifen, and no significant difference between the controls and resistant tumors was demonstrated with respect to percentage of Δ5/WT ER (Fig. 5B). However, the majority of ER+ PgR/pS2+ control tumors would be expected to respond to tamoxifen


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