Inhibition of Estrogen Receptor Action by a Naturally Occurring Variant in Human Breast Tumors


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Abstract

It is fairly well accepted that the presence of estrogen receptor (ER) and progesterone receptor (PgR) identifies breast cancer patients with a lower risk of relapse and better overall survival. But patients with discordant receptors, the ER+/PgR− phenotype, are often intermediate in clinical response. We focused upon this group of patients and have identified a truncated ER which is abundant in some ER+/PgR− breast tumors and which inhibits the binding of wild-type ER to its cognate response element. This variant interferes in a dominant negative manner with wild-type ER function and may represent a mechanism for modulation of estrogen responsiveness.

Introduction

ER and PgR belong to a superfamily of ligand-modulated transcription factors that play a role in the progression of breast cancer. Since PgR is induced by estrogen, breast tumors containing estrogen and receptor would be expected to contain PgR (1). However, ER+/PgR− breast tumors are relatively rare. We hypothesized that these tumors could contain an ER unable to function as a transcriptional inducer of PgR expression. Our objective was to determine whether defects in ER DNA binding or transcriptional activity occur in ER+/PgR− tumors. We report here a truncated ER variant lacking exon 7 in these tumors which was unable to induce the transcription of an estrogen-responsive gene construct and which prevented normal, wild-type ER function.

Materials and Methods

Human Tumor Specimens. Frozen human breast tumor specimens, stored at −70°C, were obtained from the San Antonio Breast Tumor Data Network and consist of tissue remaining after receptor assays; no clinical follow-up was available for the tumors examined. All of the ER+/PgR+ and ER−/PgR− tumors were examined by both ligand binding assays and immunohistochemistry using the monoclonal antibody B39 against PgR (3), and a standard streptavidin-biotin-peroxidase detection system (4) to confirm receptor status.

Gel Retardation Assays. Gel retardation assays were performed essentially as described (5) using the consensus vitellogenin A2 ERE oligonucleotide probe labeled with [γ-32P]ATP and T4 polynucleotide kinase. Ten µg of a copolymer of polydeoxyadenylic and polydeoxymethylidylic acids, 0.2 ng probe, in 0.1 M Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 M KCl, and 4% glycerol. After incubation at room temperature for 30 min, 0.1 µg of ER-specific antibody H222, H226, or D75 (2) was added for 2 h on ice, followed by gel electrophoresis in a 4% nondenaturing polyacrylamide gel and autoradiography.

Cloning of a Variant ER Transcript from an ER+/PgR− Tumor. PCR amplification of cDNA was performed as described (6), except that the downstream PCR primer used (5'-TGAAGTAGCGTAAGGAACGT-3') represents the antisense strand of the ER cDNA sequence 1718 to 1739 (7). Two additional nucleotides and an EcoRI site were added to the 5' end of the primers to facilitate cloning. Dideoxy sequence analysis was performed as described (8).

RNase Protection Assay. Total RNA was isolated on a model 340A nucleic acid extractor (Applied Biosystems, Foster City, CA) using 300 mg of frozen, pulverized tumor powder. Residual DNA was removed by DNase treatment. Thirty µg of RNA were hybridized with an antisense RNA probe prepared from the exon 7 ER variant clone, and samples were digested with RNase and electrophoresed as described (9). As an additional screen, immunohistochemical assays were also performed on the ER+/PgR− tumors using the estrogen-regulated pS2 antibody probe (10) in an attempt to select those tumors which may be deficient in ER function. Only those ER+/PgR+ tumors which were negative for PgR and pS2 staining were examined by RNase protection assays.

Yeast Expression Vector System. To construct the exon 7 ER variant (YEPE15) in an inducible yeast expression vector system, a portion of the wild-type ER yeast plasmid YEPE10 under the control of a metallothionein promoter (6) was replaced with a HindIII/BglII PCR-derived fragment from our ER+/PgR− tumor expressing the yeast deletion variant. The sequence of YEPE15 was confirmed by dideoxy sequence analysis. To construct a wild-type ER yeast expression vector (YEPE23), the BamHI/Xhol fragment of YEPE10 was replaced with a similarly digested fragment of pYSK153 (a kind gift from Dr. George Liv, Smith, Kline, and Beecham, Inc.). This construct expresses wild-type ER under the control of a constitutive yeast triosephosphate dehydrogenase promoter (11). To change the selection marker from tryptophan to leucine in this construct, it was next digested with Csp45 and CflI, and the vector fragment was replaced with Klenow, followed by ligation to an end-filled Sall fragment of PM853 (a kind gift of Dr. G. Gorman, Smith, Kline, and Beecham, Inc.), yielding YEPE23. Yeast strains containing the receptor expression plasmids and reporter plasmid were grown in minimal medium in the presence of hormone (17-β-estradiol) for 4 h and copper sulfate overnight at 30°C. Yeast extracts were prepared from these cells and assayed for β-galactosidase activity as described (12). Yeast extracts prepared from cells grown in 25 µM copper were subjected to gel retardation assays as previously described, except that 20 µg of protein extract was used, and pGEM plasmid DNA (Promega) digested with HpaII was substituted for the copolymer of polydeoxyadenylic and polydeoxymethylidylic acids. Western blot analysis using the ER antibody H222 was performed on the yeast extracts as described (12) using yeast transformants grown in 25 µM copper.

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Results and Discussion

Identification of a Truncated ER Variant in ER+/PgR- Tumors. We first examined whether defects in ER DNA binding occur in ER+/PgR- breast tumors. To accomplish this we utilized gel retardation assays with estrogen response element oligonucleotides. The majority of ER+/PgR- tumors contained ER capable of estrogen response element DNA binding; however, 2 of 23 tumors exhibited a binding profile suggestive of a 3' ER truncation (Fig. 1). These two tumors were positive as defined by an upshift with ER-specific antibodies H226 and H222 to the amino terminus and middle of the protein (2) but were negative for upshift using the carboxy terminus antibody D75.

To define this identified alteration, we used primers to the boundaries of the ER ligand-binding region (13) and performed cDNA PCR amplification (14) for cloning and sequence analyses. Full-length wild-type ER was cloned from these two tumors (results not shown); however, we also detected an ER variant which was identical to wild-type sequence for exons 6 and 8 (15) but was missing exon 7 (Fig. 2). The precise deletion of exon 7 is suggestive of alternative RNA splicing; sequence analyses of the exon/intron border of genomic DNA from these tumors is in progress to definitively answer this question. Deletion of exon 7 (nucleotides 1369 to 1548; Ref. 7) predicts a reading frame shift with the inclusion of 10 non-ER residues after codon 457. Thus the ER variant potentially encodes a truncated ER protein with a molecular weight of ~52,000.

Expression of the Exon 7 Deletion Variant in Tumors. We next questioned whether the variant was expressed at levels detectable with conventional RNA techniques, using RNase protection assays to determine the levels of the variant in primary breast tumors (Fig. 3A). If variant RNA is present in tumors, it should protect a fragment of 236 base pairs from RNase digestion. Because the probe is missing exon 7, wild-type ER RNA should be digested into a smaller fragment of 185 base pairs. Both variant and wild-type fragments were detected in tumors. In addition, RNA from normal uterine endometrial tissue hybridized with the variant probe (results not shown). This suggests that the variant is a naturally occurring ER isoform.

Because the ratio of variant to wild type was higher in a ER+/PgR- tumor (Fig. 3A), we next compared ratios in 15 ER+/PgR- and 12 ER+/PgR+ tumors (Fig. 3B). The variant was significantly more abundant in some of the ER+/PgR- tumors with a mean ratio of 0.285, as compared to 0.157 in ER+/PgR+ tumors. These differences in expression led us next to analyze the variant’s functional properties.

Transcription Activity of the Exon 7 ER Variant in Yeast. To test the transcriptional activity of the variant, we expressed the variant receptor in yeast cells under the control of the copper-inducible metallothionein promoter and assayed its ability to induce transcription from a LacZ reporter containing two copies of a vitellogenin A2 ERE (6) (Fig. 4A, YEPE15). The variant was transcriptionally inactive, and this activity was hormone dependent. To study the interaction of the variant with wild-type ER, the plasmid YEPE23 expressing wild-type ER from a constitutive TDH promoter was transformed into yeast cells carrying variant ER and a reporter plasmid. When both wild-type ER and increasing amounts of the variant were coexpressed, wild-type activity was reduced up to 60%. This suggests that the variant exerts a dominant negative effect on wild-type ER activity. Inhibition of wild-type activity was not observed when the ER cDNA was deleted from the variant construct (results not shown).

The dominant negative effect of the variant could be the result of the constitutive DNA binding ability of the variant protein, which interferes with wild-type ER binding to ERE. Alternatively, it may involve mechanisms unrelated to DNA binding such as the formation of inactive heterodimers. To examine these possibilities, we determined the DNA binding activity of the variant protein using a GAL4 transcriptional interference assay.4 In this assay wild-type ER interferes with and reduces basal GAL4 activity up to 85% in the presence of hormone. In cells transformed with the ER variant, GAL4 activity was reduced up to 64% in both the presence and absence of hormone (results not shown). This suggests that the variant may compete for ERE binding and exert its dominant negative effect by saturating ERE binding sites. To determine whether alternate mechanisms were also responsible for this effect, we next performed in vitro DNA binding gel retardation assays using yeast extracts. Wild-type ER bound, but ERE DNA binding of the variant was either deficient or greatly reduced. This was not unexpected, since the variant is truncated within the dimerization domain required for ERE binding (16), but it is in contrast to that predicted from the in vivo GAL4 transcriptional interference assay. When wild-type ER and the variant were coexpressed, wild-type binding capacity was significantly

* McDonnell et al., manuscript in preparation.
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Fig. 2. Cloning of an ER variant missing exon 7 from ER\(^+\)/PgR\(^-\) tumors. RNA was isolated from the two tumors exhibiting the profile shown in Fig. 1, and the ER was cloned and sequenced. Top, wild-type and variant ER sequences; bottom, schematic diagram of the precise deletion of exon 7.

Fig. 3. RNase protection assays detect the exon 7 ER deletion variant in RNA of human breast tumors. In A, RNA from an ER\(^+\)/PgR\(^+\) (517 and 168 fmol ER and PgR/mg cytosol protein, respectively) and an ER\(^+\)/PgR\(^-\) (574 fmol ER/mg cytosol protein) tumor was hybridized with an ER RNA probe prepared from the exon 7 deletion variant cDNA. Lane P, probe alone, corresponding to vector sequences plus ER cDNA from exon 6 (nucleotide 1268) through exon 8 (nucleotide 1739) but lacking exon 7 (16). pBR 322 digested with Msp\(I\) was used as a molecular weight base pair marker (not shown). B, graphic representation of the ratio of the exon 7 deletion variant to wild-type ER obtained by scanning densitometry of signals obtained in RNase protection assays of ER\(^+\)/PgR\(^+\) and ER\(^+\)/PgR\(^-\) tumors.

Reduced. Thus, the variant may not compete for ERE binding in \textit{in vitro} assays but could exert its effect by forming an inactive heterodimer complex with wild-type ER. These results were not the result of excessive variant expression. Western analyses (Fig. 4C) demonstrated that even at maximum copper stimulation, expression of both proteins was equivalent. Thus, the variant appears to be a potent inhibitor of ER action either by competition for ERE binding and/or by heterodimer formation.

This is the first report of a naturally occurring, alternatively spliced ER variant which interferes with normal DNA binding and ER-induced gene transcription. The variant might also interfere with associated transcription factors required for ER activity, but that remains to be tested. Multiple forms of the thyroid hormone receptor generated by alternative mRNA splicing have also been reported to inhibit thyroid hormone action by similar mechanisms (17). Thus the generation of
receptor isoforms by alternative splicing may be a general mechanism for receptor diversity. It appears that alternative ER RNA splicing occurs frequently in human breast tumors. The introduction of a non-ER sequence at the exon 2 and 3/intron boundaries (18), deletion of exon 3 (19), and deletion of exon 5 (6) have all recently been reported. The possibility therefore exists that there may be a deregulation of splicing mechanisms within certain breast tumors.

A dominant negative ER variant in breast tumors could have important effects on hormonal responsiveness. It is well known that steroid receptor status provides a guideline for those tumors which respond to hormonal therapy (20). Approximately 50% of ER+/PgR+ tumors will respond to tamoxifen therapy, but only 27% of ER+/PgR− tumors will similarly respond (21). We suggest that elevated levels of a variant which interferes with normal ER function could render a tumor unresponsive to estrogen and antiestrogens, or functionally receptor negative. Furthermore, this variant may explain certain of the ER+/PgR− tumor phenotypes.

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References

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