Prevalence of Estrogen Receptor Variant Messenger RNAs in Human Breast Cancer

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Abstract

A new approach, based on the competitive amplification of wild-type and exon-deleted estrogen receptor (ER) variant cDNAs, was used to screen 100 human breast tumors for the presence of ER variants. Already described exon 4-deleted ER mRNA was preferentially detected in tumors with lower grades (P < 0.05) or higher progesterone receptor levels (P < 0.01), whereas new ER variants, deleted in exons 2-4 or in regions within exons 3-7 were associated with higher grades (P < 0.025) and higher ERs (P < 0.001). This approach allows investigation of the expression of multiple ER variant mRNAs and may implicate them as new prognostic markers and as possible contributors to tumor progression.

Introduction

Several ER variants have now been detected in both normal and cancerous breast tissues (1-11). Although it is unclear if any or all of these mRNAs are translated in vivo, some of the predicted ER-like proteins, lacking some functional domains (12) of the WT-ER (Fig. 1), exhibit altered functions in vitro. Exon 3- and exon 7-deleted variants may act as dominant negative regulators of WT-ERs (3, 6), whereas exon 5-deleted ER has ligand-independent transcriptional activity (4, 13). Changes in the balance between ER-like molecules could be involved in perturbation of the ER signaling pathway and tumor progression (14-20). Many laboratories have begun to investigate the association between the expression of individual ER variant mRNAs and the loss of hormone-dependent growth (16, 19). However, it is now apparent that several different types of variant ER transcripts and therefore predicted proteins can be expressed together (8, 9), and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript and that would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (17). Thus, signals attributed to the exon 7-deleted ER variant mRNA detected with reverse transcription-PCR using primers in exons 5 and 8 (9) or with RNase protection assays with probes covering the exon 6–8 junction (20) may also include contributions from a variant deleted in both exons 4 and 7 recently identified by Madsen et al. (17). Nevertheless, these molecules may result in quite different proteins which differ in activity and modulate differentially the ER signaling pathway. Moreover, because of the lack of an approach to investigate qualitatively and quantitatively the representation of total ER variant mRNAs within any one given sample, it becomes difficult to evaluate those variants potentially important in vivo either as prognostic markers or as possible contributors to tumor progression. The purpose of this study was to develop a strategy that would allow the investigation of known and unknown exon-deleted or -inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants among themselves and with respect to the WT-ER transcript. The approach used is depicted in Fig. 1. cDNAs corresponding to all exon-deleted ER variants identified to date can be amplified along with the WT-ER mRNA using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences. We assumed that a competitive amplification could therefore occur among all exon-deleted and -inserted ER variant transcripts that would depend on their initial relative representation, the detection of bands corresponding to specific ER variants reflecting the balance between ER variant mRNA species within the sample. Since it is likely that alterations in the coding sequences could be translated into ER-like proteins with altered functions, we have for practical reasons confined our approach to the coding region only. This approach was tested in this pilot study to determine the incidence of ER variants in a set of 100 breast tumors that were selected to represent a wide range of breast cancers with respect to ER and PR levels, size, grade, and axillary nodal status.

Materials and Methods

Human Breast Tissues and Cell Line. All human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank. Tumors (100 cases) were chosen to represent a variety of tumor characteristics represented in the breast tumor population collected in the Manitoba Breast Tumor Bank. Thirty tumors were ER negative (ER < 3 fmol/mg protein), with PR values ranging from 0 to 25 fmol/mg protein, as measured using the ligand-binding assay. Seventy tumors were ER positive (ER ranging from 3.6 to 386 fmol/mg protein), with PR values ranging from 0 to 297 fmol/mg protein. These tumors also spanned a wide range of grades (from 4 to 9), determined using the Nottingham grading system (21), size (ranging from 1 to 6.3 cm), and nodal status (absence or presence of axillary nodes). T-47D-5 cells, which are known to express different ER variant mRNAs (11, 18), were kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). Total RNA was extracted and reverse transcribed in a final volume of 15 μl as described previously (11).

Primers and PCR Conditions. The primers used consisted of 1/8U primer (5’-TGCCTACTACCTGGAGAACG-3’, sense; located in WT-ER exon 1; 615–637) and 1/8L primer (5’-GCCTCCCCCCTGATGTA-3’, antisense; located in WT-ER exon 8; 1995–1978). Nucleotide positions given correspond to published sequences of ER cDNA (22). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, 1 μl of reverse transcriptase mixture was amplified in a final volume of 10 μl in the presence of 10 μM [α-32P]dCTP, 4 ng/μl of each primer, and 1 unit...
of Tag DNA polymerase. Each PCR consisted of 40 cycles (1 min at 60°C, 2 min at 72°C, and 1 min at 94°C). PCR products were then separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. To control for errors in the input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (11). All PCR products were subcloned and sequenced as described previously (11).

RNA Dilution Experiments. Plasmids that contained PCR products subsequently identified as fragments corresponding to exons 3- and 4-deleted ER variant (D3-4-ER) and to variant deleted in exons 2, 3, and 7 (D2-3/7-ER) were linearized with BamHI and gel purified as described previously (11). Corresponding sense RNAs were synthesized using Riboprobe Systems (Promega, Madison, WI) according to the manufacturer’s instructions. One μg of total RNA from T-47D-5 cells was mixed with various amounts of synthetic D2-3/7-ER (ranging from 5 ng to 50 fg) or D3-4-ER RNA (50 fg). These spiked RNA samples were then reverse transcribed and amplified using 1/8U and 1/8L primers as described above.

Statistical Analysis. Each individual tumor sample was analyzed in at least three independent assays. Only bands reproducibly observed in three experiments were considered. The presence of a specific band in a tumor sample was scored only if its signal intensity placed it among the four strongest signals (as assessed by subjective visualization) observed in the corresponding lane. The tumor group in which the band corresponding to the WT-ER mRNA was detected (68 cases) presented the following characteristics: ER level ranging from 0 to 386 fmol/mg protein (average, 111 fmol/mg protein) and PR level ranging from 0 to 297 fmol/mg protein (average, 73 fmol/mg protein). For the purpose of analysis, this group was divided into two subgroups presenting ER, PR, or a grade above or below a point defined as the average of the ER value, PR value, or grade observed within the group. Possible associations between the detection of a particular variant and one particular subgroup were tested using either the χ² test, including Yates’ correction when the estimated frequency was at least equal to 5, or the Fisher exact test (two tailed) in other cases.

Results

Coamplification of WT-ER mRNA and Deleted Variant mRNAs in Breast Tumor Samples. On the basis of the assumption that coamplification of WT-ER mRNA and variant ER mRNAs could effectively occur and therefore allow identification of the frequency and relative expression of variants in breast tumor tissues, 100 breast tumors were selected for analysis that represented a wide range of ER and PR levels, as measured by the ligand-binding assay, grade, nodal status, and size. Total RNA was extracted from each tumor sample and reverse transcribed. PCR was then performed using primers annealing with exon 1 and exon 8 sequences. Fig. 2 shows typical results obtained. Many different PCR products were observed in each of 70 ER-positive tumors but only in 3 of 30 ER-negative tumors. This difference did not result from variable input of cDNA, since similar signals were obtained in all samples after amplification of the housekeeping GAPDH cDNA (data not shown). Two bands that migrated with the apparent sizes of 1381 and 1197 bp were observed in most of the signal-positive tumors. These bands were detectable in 68 and 63 cases, respectively. Following subcloning and sequencing, these bands were shown to correspond to the WT-ER and an exon 7-deleted ER (D7-ER) variant mRNA, respectively. Six other bands that migrated at the apparent sizes of 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were consistently detected within the set of tumors studied, but at an apparently lower frequency. They were observed in 19, 8, 6, 11, 6, and 20 tumors and were found to correspond to ER variant mRNAs deleted in 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 2-4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. Sequences of all of these variants, except D-3-7-ER variant, showed a perfect junction between exons surrounding the deletion area (data not shown). A 801-bp deletion was observed in the D-3-7-ER variant from nucleotides 931 to 1729 (22) located within exon 3 and exon 7, respectively. It should be stressed that some bands, either not consistently observed or specific for less than three tumors, have not yet been assessed further in this study.

Detection of a Particular Variant Depends on Its Initial Representation within the ER mRNAs Population. To determine whether the detection of a variant depended on its initial representation within the ER-like mRNA population, the balance of ER-deleted variants was artificially changed in favor of particular variants. Various amounts of synthetic RNAs corresponding to the D3-4-ER and D2-3/7-ER PCR products were added to total RNA extracted from T-47D-5 breast cancer cells. These RNA preparations were reverse transcribed and subsequently analyzed with PCR using 1/8U and 1/8L primers (Fig. 3). Bands corresponding to WT-ER, D7-ER, D4-ER, and D-3-7-ER were initially detected in T-47D-5. The addition of synthetic D2-3/7-ER RNA, which increased its ability to compete for the binding of 1/8U and 1/8L primers during the PCR reaction, drastically decreased signals corresponding to the initially detectable endogenous variants. The extinction of these signals was directly related to the concentration of the synthetic RNA added. The Addition
of two synthetic RNAs simultaneously resulted in the increased representation of two expected bands.

Detection of Particular Variants May Be Associated with Tumor Characteristics. Detection of ER variants using the approach described here appeared to depend on the initial relative ratio of expression between ER-like mRNAs. It was therefore of interest to search for possible associations between the detection of particular variants and other tumor characteristics. The detection of a specific band in a sample was defined here as its presence as one of the four main signals observed in the corresponding lane. The frequency of detection of each ER variant mRNA within tumors also expressing a detectable WT-ER band is presented in Table 1. Using the mean ER, PR, and grade values as cutoff points for statistical analysis, we found that D-3-7-ER and D2-3-4-ER variants were preferentially detected in the subgroup with higher ER (P < 0.001) and higher grade (P < 0.025), respectively. D4-ER variant was more frequently observed in tumors of lower grade (P < 0.05) or with higher PR levels (P < 0.01).

Discussion

We have used a new approach based on the competitive coamplification of WT-ER and exon-deleted or -inserted ER variant mRNAs to examine the overall expression of these two types of ER variants which encompass the majority of ER variant mRNAs thus far identified (23). Although another distinct group of variants, the truncated ER variants that include the widely expressed ER clone 4 variant (5, 18), cannot be assessed with this analysis, the strategy allows a broad investigation of the ER-like population and the integrity of the entire coding region within this species, without focusing on particular regions. This has enabled us to confirm the existence of four variants already described by others, e.g., exon 7-deleted ER variant (3, 16), exon 4-deleted ER variant (7), exon 3-4-deleted ER variant (9), and a variant deleted in both exons 4 and 7 (17). Beyond these, three new variants were identified. Two of them, deleted in exons 2, 3, and 7 or exons 2–4, correspond to the usual exon-deleted ER variant pattern, i.e., containing a perfect deletion of exon sequences. The third one contained part of exon 3 attached to a sequence beginning inside the seventh exon. It should be noted that very recently, Daffada and Dowsett (24) identified an ER variant presenting a similar pattern of intra-exon deletion between exons 4 and 7. Furthermore, we have been able to detect ER variant mRNA deleted in both exons 4 and 7 for the first time in multiple clinical material, supporting the potential relevance of such a variant in vivo. The function of the putative encoded protein which lacks a nuclear localization signal, all of the hinge domain, and is C-terminal truncated remains to be determined.

Using different RNA preparations, we showed that the detection of
PREVALENCE OF ER VARIANT mRNA IN BREAST CANCER

Table 1  Frequency of detection of ER variant mRNAs within 68 human breast tumors also expressing detectable WT-ER mRNA

<table>
<thead>
<tr>
<th>Tumors expressing</th>
<th>No. of tumors expressing detectable ER variant mRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ER</td>
<td>D7-ER</td>
</tr>
<tr>
<td>ER &lt; 111 (fmol/mg protein)</td>
<td>38</td>
</tr>
<tr>
<td>ER &gt; 111 (fmol/mg protein)</td>
<td>30</td>
</tr>
<tr>
<td>PR &lt; 73 (fmol/mg protein)</td>
<td>41</td>
</tr>
<tr>
<td>PR &gt; 73 (fmol/mg protein)</td>
<td>27</td>
</tr>
<tr>
<td>4 ≤ grade ≤ 6</td>
<td>35</td>
</tr>
<tr>
<td>7 ≤ grade ≥ 9</td>
<td>33</td>
</tr>
</tbody>
</table>

WT and variant ER mRNAs were detected after co-amplification as described in “Materials and Methods.”

* P values calculated using the χ² test with Yates’ correction.

# P value calculated using the Fisher exact test (two tailed).

a variant depended on its initial representation within the ER-like mRNA population. The absence of a prominent signal corresponding to any particular variant could therefore result from its low relative representation. This could explain why variants deleted in either exon 3 or exon 5 were undetectable using our criteria and this approach, although their presence was confirmed by specific PCR amplification in some of the same tumors studied.4 These variants may also correspond to infrequent or poorly represented ER-like mRNAs and therefore PCR products that we have not yet identified. On the other hand, the detection of any particular ER variant mRNA within a tumor sample can result from its overexpression or a change in the balance between all ER variant mRNAs. Using this approach, it is therefore possible to investigate the relative proportion of ER variant mRNAs, and also to compare breast samples regarding the relative expression of their ER-like mRNAs. The set of tumors analyzed in this pilot study was chosen to obtain the widest qualitative representation of important breast tumor characteristics more than to establish statistical associations. The tumor population contained very different tumors spread over a wide range of ER and PR levels, size, grade, and nodal status. It was possible however to establish that detection of particular variants may be correlated with already known prognostic markers. It is interesting to note that the exon 4-deleted variant is associated in this study group with two different markers of good prognosis, i.e., high PR and lower grade. This variant, initially described in breast cancer cell lines (7) and subsequently in vivo in several normal and tumor tissues (9, 10), is expected to encode an ER-like protein lacking most of the hinge domain, which includes an important nuclear localization signal and a part of the hormone binding domain. It might therefore have a cellular distribution and estrogen-binding affinity different from that of the WT-ER. Furthermore, the altered structure of this protein may lead to altered transcriptional activities.

The use of this approach to study a larger set of samples would allow the establishment of a typical pattern of ER variant mRNA expression for each type of tumor. Comparison of such patterns along with the subsequent analysis of the specifically detected transcripts could lead to the discovery of new prognostic factors and the identification of new contributors to tumor progression.

References

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