Estrogen Receptor Mutations in Tamoxifen-resistant Breast Cancer

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Abstract

Clinical resistance to antiestrogens like tamoxifen is a major problem in the treatment of hormone-dependent breast cancers. Since the estrogen receptor plays a central role in mediating the effects of estrogens and antiestrogens, we hypothesized that mutations in the estrogen receptor could be one mechanism by which breast tumors evolve from a hormone-dependent to a hormone-independent phenotype. The eight exons of the estrogen receptor complementary DNA from 20 tamoxifen-resistant and 20 tamoxifen-sensitive tumors were screened by Single Strand Conformation Polymorphism (SSCP), and the variant conformers were sequenced to identify the nucleotide changes. A 42-base pair replacement was found in exon 6 of a tamoxifen-resistant tumor. A single base pair deletion in exon 6 of a tamoxifen-resistant metastatic tumor but not in the primary tumor was detected in another case. If translated, both these mutations could generate truncated receptors with an intact DNA-binding domain and a defective hormone-binding domain that could constitutively activate transcription of previously estrogen-responsive genes. The remaining 18 of 20 tamoxifen-resistant tumors did not contain mutations in any of the 8 exons of the estrogen receptor complementary DNA. These results suggest that mutations in the estrogen receptor occur at a low frequency and do not account for most estrogen-independent, tamoxifen-resistant breast tumors.

Introduction

Breast cancer is a major source of morbidity and mortality among women in North America and Europe. The recognition that many breast cancers are dependent on estrogen for growth and progression has been the basis for the continuing interest in the role of ER in the prognosis of this devastating disease (1). A characteristic feature of breast cancer is that the disease evolves into an estrogen-independent growth phenotype, a change that often marks the beginning of a more aggressive growth phase that is nonresponsive to antiestrogen therapy (2). Resistance to antiestrogens like tamoxifen is therefore a major problem in the treatment of hormone-dependent breast cancers. About 30–40% of ER-positive tumors fail, from the outset, to respond to antiestrogen therapy and are considered to be estrogen-independent and tamoxifen-resistant (2). Moreover, the majority of ER + tumors that initially respond to antiestrogen therapy will eventually develop resistance to this treatment without necessarily altering their ER profile (2).

The ER is a member of the steroid and thyroid hormone receptor superfamily that function as ligand-dependent transcription factors. These receptors contain an NH2-terminal domain that affects transcription efficiency, a central DNA-binding domain that binds to the target gene hormone response element and thereby determines target gene specificity, and a COOH-terminal HBD (3). Studies on the role of ER in breast cancer have focused on measurements of ER by ligand binding assays or immunohistochemistry (4). It is clear from in vitro mutagenesis that ligand binding and immunoreactivity are not adequate indicators of a functional ER since deletion of all or part of the DNA binding domain renders the ER transcriptionally inactive, despite its ability to bind estrogen with high affinity. It is remarkable that the mutant ERs that lack the HBD can constitutively activate transcription even in the absence of hormone (3). Indeed, androgens (5, 6), vitamin D3 (7), and glucocorticoid (8) resistance syndromes are all associated with the inheritance or acquisition of mutant forms of cognate receptors. In contrast, hereditary estrogen resistance syndromes are rare and could prove to be lethal to the organism (9). However, acquired estrogen resistance as seen in human breast tumors could arise due to mutant ERs. Several ER variants with different functional capabilities (9–11) and putative RNA splicing variants of ER (9, 10, 12) are described in breast cancer cell lines and breast tumors. These mutant ERs might confer either dominant positive or dominant negative effects on cancer cells, thereby generating the hormone-independent phenotype of the breast tumors. Intrigued by the possibility that tamoxifen-resistant human breast cancer might contain ER mutations that alter ER function or inactivate it and thereby affect the cancer cell phenotype and its response to antiestrogens, we initiated a study to screen for mutations in the ER cDNA of tamoxifen-resistant and -sensitive breast cancer tissue specimens. The results show that although mutations in ER do occur in tamoxifen-resistant tumors, they are quite rare and do not account for the majority of tamoxifen-resistant breast cancers.

Materials and Methods

Human Breast Tumor Tissue and Clinical Data

Breast tumor tissue was obtained from the tissue procurement core, Department of Pathology, Cleveland Clinic Research Foundation and characterized by clinical and histopathological features. All the tumors were radioimmuno logically determined to be estrogen receptor-positive and progesterone receptor positive/negative and were from postmenopausal women with Stages II, III, and IV breast cancer. The classification of tumors based on their response to tamoxifen was as follows:

Group 1: Paired Primary and Metastatic Tumors from Patients Receiving Adjuvant Tamoxifen. These patients received adjuvant tamoxifen therapy after initial surgery and relapsed with metastatic disease in their ipsilateral axillary lymph nodes or in the supraclavicular lymph nodes while still receiving tamoxifen. We have analyzed 5 tumor pairs belonging to this group (10 tumors).

Group 2: Tumor Tissue from Patients with Metastatic Disease (Stage III or IV) Receiving Tamoxifen. The patient’s clinical response status is described below.

Tamoxifen Resistant. Patients with Progressive Disease while Receiving Tamoxifen. These tumors were biopsied just prior to initiation or during therapy. We have analyzed 15 tumors belonging to this category.

Tamoxifen Sensitive. Tumors biopsied before initiation of tamoxifen therapy from patients who had an objective response or prolonged disease stability (>4 months) with tamoxifen. We have analyzed 15 tumors belonging to this category.

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\[\text{3 The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; HBD, hormone binding domain; cDNA, complementary DNA; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.}\]

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**Reverse Transcription-PCR Amplification**

Total cellular RNA was extracted from cryostat sections of breast tumors (snap-frozen in liquid N₂ and stored frozen at ~80°C) with RNAzol (Biotex Laboratories, Houston, TX) according to the recommendations of the supplier. RNA (500 ng) was reverse transcribed at 42°C for 1 h following the instructions of the Gene Amp RNA PCR system (Perkin Elmer Cetus, Norwalk, CT). First strand cDNA was directly used for 30 cycles of PCR amplification (13) of the 8 ER exons using Gene Amp PCR reagent kit (Perkin Elmer Cetus). A pair of primers was synthesized (Fig. 1) for each ER exon based on the cDNA sequence (14). To ensure cDNA and not genomic DNA amplification, the primer pairs for each exon were designed on adjacent exons. PCR was carried out in a Thermocycler (Eriomp, Inc., San Diego, CA) in a final volume of 100 µl containing 250 µM per liter each of dATP, dGTP, and TTP; 20 µM per liter of dCTP; 10 µCi [³²P]dCTP; 50 pmol each of PCR primer; 2.5 units of Taq polymerase; and 10 µl of Taq polymerase buffer. PCR cycles included denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and polymerization for 1.5 min at 72°C, followed by one cycle of extension reaction at 72°C for 15 min. We ruled out the possibility of misincorporation errors due to Taq polymerase by performing a minimum of three independent PCR reactions for each exon. Single Strand Conformation Polymorphism (SSCP) (15, 16) of the PCR products was performed to detect possible deletions, insertions, or point mutations.

### SSCP

Following PCR, a 5-µl aliquot of the amplified product was diluted with 45 µl of 0.1% sodium dodecyl sulfate-10 mM EDTA; 2 µl of this mixture was added to 2 µl of Sequenase stop mix (95% formamide-20 mM EDTA-0.05% bromophenol blue-0.05% xylene cyanol; United States Biochemical Corporation, Cleveland, OH). The cDNA samples were denatured at 95°C for 3 min and immediately placed on ice before being run on 6% polyacrylamide non-denaturing gels with or without 10% glycerol. Electrophoresis was performed in 0.09 M Tris base-0.09 M boric acid-2.5 mM EDTA running buffer either at 40 W at room temperature, or at 40 W at 4°C for 4–6 h, or at 5 W at room temperature for 16 h. The gels were dried and exposed to XAR-5 film (Kodak) for 24–48 h at room temperature. The plasmid HEGO (cloned ER cDNA; gift from Dr. Geoffrey Greene) served as control on SSCP gels.

### DNA Sequencing

The variant SSCP bands were cut out, extracted with 100 µl distilled water for 15 min at room temperature, and reamplified as described above. The PCR products were purified on Magic DNA purification columns and sequenced using the fmol DNA sequencing system from Promega (Madison, WI), according to the manufacturer's protocol. Both the sense and antisense strands were sequenced.

### Results

#### A 42-bp Pair Replacement in ER Exon 6 of a Metastatic Breast Tumor

All eight exons of the ER cDNA could be amplified from the total RNA of the 40 breast tumors (described in “Materials and Methods”) with the primers described in Fig. 1. Deletions of entire exons were not observed in any of the 40 tumors examined in this study. PCR amplification of exon 6 from human breast cancer tissue specimens using primers ER 16 (5'-CTGTTTGCTCCTAACTTGCT-3') and ER 17 (5'-GGTGCTGACAGAAATGTG-3') resulted in the expected fragment length of 191 base pairs in all of the tumors (data not shown). However, PCR-SSCP analysis (Fig. 24) demonstrated variant conformers (indicated by arrows) in the tamoxifen-resistant tumor 17 compared to the wild-type pattern (cloned ER cDNA). The presence of more than one allele in the same specimen suggested that the tumor contained a heterozygous somatic mutation. Sequence analysis of the variant conformer (Fig. 2B) indicated that 47 nucleotides (1271–1318) of exon 6 were substituted or replaced by 42 nucleotides (1148–1190) of exon 5 (Fig. 2B). Since the 42-nucleotide exon 5 fragment was flanked on either side by exon 6 sequences and the PCR fragment was flanked by the appropriate primers, we ruled out the possibility of this being a PCR artifact. Several independent RNA-PCR and SSCP analyses confirmed the presence of this mutation in tumor 17. As shown in Fig. 2C this 42-base pair substitution generates a 5-base pair deletion, resulting in a frame shift that generates an ER with a translation termination at codon 454 in exon 6. Since all of the other ER exons in this tumor are wild type, on translation this mutation could give rise to a truncated protein with an intact DNA-binding domain and a defective HBD that would be unable to bind hormone. Such mutant proteins could constitutively activate ER-responsive genes and induce ER-independent growth of breast tumors. Tumor 17 (ER+PR−) was indeed from a patient who rapidly progressed on tamoxifen and was resistant to the drug from the outset inspite of high ER values (ER value of 500 as determined by radioimmunoassay).

**Single Nucleotide Deletion in the Metastatic but not in the Primary Tumor from the Same Patient.** SSCP analysis also showed a variant conformer in addition to the wild-type conformer (Fig. 3A) in exon 6 of the metastatic tumor 10. Interestingly, the primary tumor 9 from the same patient contained only the wild-type conformer under two different SSCP conditions (Fig. 3, A1 and A2). Sequence analysis of the variant band (Fig. 3B) revealed a single nucleotide (T) deletion at codon Ser 432 of exon 6 (Fig. 3B). This generates a frame shift and a translation termination codon at 437. Sequencing of the opposite DNA strand confirmed these results. The sequence of remaining exons was identical to the wild-type sequence, indicating that exon 6 of tumor 10 differed from the wild-type sequence by only a single base deletion. Sequence analysis of exon 6 in the primary tumor showed the wild-type sequence with Ser 432. The observation that this patient relapsed while on adjuvant tamoxifen therapy suggests that either the cancer cells acquired the mutation during tumor progression or that tamoxifen therapy selects for breast cancer cells that express mutant forms of estrogen receptors.
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Not All ER Mutations Are Associated with the Resistant Phenotype and Not All Tamoxifen-resistant Tumors Contain ER Mutations. Using the same techniques, we have also observed a substitution in ER exon 4 of a tamoxifen-sensitive tumor (tumor 2, from a patient with Stage II disease). This substitution alters the amino acid Glu 352→Val, a drastic change from acidic to basic amino acid (data not shown). However, this patient responded to adjuvant tamoxifen therapy and remains disease free. This suggests that not all ER mutations are associated with a tamoxifen-resistant phenotype. Another such example is the mutation Gly 400→Val in the ER cDNA isolated from the cell line MCF-7. This mutation only reduces the affinity of the receptor for estrogen at 37°C but not at 4°C (17). We have also detected silent mutations in the tamoxifen-resistant tumor 6 (exon 2, Gly 276), in the tamoxifen-resistant tumor 28 (exon 6, Ala 505), in the tamoxifen-sensitive tumor 3 (exon 5, Lys 472) and in the tamoxifen-sensitive tumor 35 (exon 7, His 577; Table 1).

Surprisingly however, the remaining 18 of 20 ER+, tamoxifen-resistant tumors that we analyzed did not contain mutations in any of the eight exons of the ER cDNA but contain wild-type estrogen receptors at levels comparable to tamoxifen-sensitive tumors. Our results show that mutations in ER do occur in tamoxifen-resistant tumors; however, they are quite rare and thus do not account for the majority of tamoxifen-resistant breast tumors. Other mechanisms must therefore be responsible for the tamoxifen-resistant and hormone-independent phenotype seen in these tumors.

Discussion

Estrogen receptor mutations have been reported in several breast cancer cell lines (9) and breast tumors (10–12 and references therein), although their correlation with clinical history has not been determined. Our study of 20 tamoxifen-sensitive and 20 tamoxifen-resist-
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Fig. 3. A. PCR-SSCP analysis of ER exon 6 in tumor 9 (primary) and tumor 10 (tamoxifen-resistant, metastatic tumor) from the same patient. Tumor 11 and wild-type (cloned ER cDNA) served as negative controls. The SSCP gels were run at 5 W at room temperature for 16 h and 40 W at 4°C for 4 h. Tumor 10 shows variant SSCP conformers that are indicated by arrows, whereas the primary tumors 9 and 11 show a wild-type SSCP mobility pattern. SS and DS, single-stranded and double-stranded DNA, respectively. B. Sequence analysis of ER exon 6 coding part of HBD in tumors 9 and 10. Sequence analysis was performed as described in "Materials and Methods." A single base pair deletion of T in the codon Ser 432 generates a frame shift and a translation termination codon at 437 in the metastatic tumor 10, whereas the primary tumor 9 from the same patient shows a wild-type sequence.

Table 1  ER mutations in breast tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>ER/PR status</th>
<th>Response to tamoxifen</th>
<th>Description of tumor</th>
<th>Mutations in the ER cDNA</th>
<th>Nucleotide change</th>
<th>Codon change</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>ER+/PR+</td>
<td>Sensitive</td>
<td>Substitution of GAG→GTG</td>
<td>Glu 352→Val</td>
<td>Misssense</td>
<td>Silent</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ER+/PR+</td>
<td>Sensitive</td>
<td>Substitution of AAG→AAA</td>
<td>Lys 472 (None)</td>
<td>Silent</td>
<td>Silent</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ER+/PR+</td>
<td>Resistant</td>
<td>Substitution of GCC→GGT</td>
<td>Gly 276 (None)</td>
<td>Silent</td>
<td>Silent</td>
<td></td>
</tr>
<tr>
<td>9b</td>
<td>ER+/PR+</td>
<td>Resistant</td>
<td>Deletion of T at Ser 432</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>10b</td>
<td>ER+/PR+</td>
<td>Sensitive</td>
<td>Nucleotides 1271–1318 of exon 6 replaced with exon 5 nucleotides 1148–1190</td>
<td>Termination codon at 437</td>
<td>Truncated ER with defective HBD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ER+/PR-</td>
<td>Resistant (Inherent)</td>
<td>Substitution of GCC→GCG</td>
<td>Ala 505 (None)</td>
<td>Misssense</td>
<td>Silent</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>ER+/PR-</td>
<td>Resistant</td>
<td>Substitution of CAT→CAC</td>
<td>His 577 (None)</td>
<td>Silent</td>
<td>Silent</td>
<td></td>
</tr>
<tr>
<td>35c</td>
<td>ER+/PR-</td>
<td>Sensitive</td>
<td>All other tumors were ER+/PR-, were either tamoxifen-resistant or sensitive, and did not contain any nucleotide changes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Substitution.
b Primary and metastatic tumors from the same patient.
c All other tumors were ER+/PR-, were either tamoxifen-resistant or sensitive, and did not contain any nucleotide changes.
tumor progression, the mutation frequency could be higher in late stage disease (19). However, in our study this possibility is unlikely since we analyzed 20 tumors with metastatic disease (progressive or recurrent disease) and did not find a significant increase in frequency of mutations in ER. It is noteworthy that Mullick and Chambon (20) reported that the ER in the tamoxifen-resistant cell line LY2 is structurally and functionally indistinguishable from that of the parental tamoxifen-sensitive MCF-7 cells. They demonstrated that the antiestrogen resistance of the LY2 cells corresponds in fact to the estrogen-independent growth of these cells (20). Possible reasons for the failure to detect mutations in the remaining tamoxifen-resistant tumors that we analyzed are: (a) the mutation is present in a very small number of cells and cannot be detected by SSCP, or (b) ER mutations are rare and occur at a very low frequency. Our ability to detect mutations depends not only on the use of optimal conditions for SSCP but also on the proportion of sample that contains mutant cDNA and on the sensitivity of detecting that cDNA. We rule out the possibility that we failed to detect mutations due to insensitivity of our techniques because three different SSCP conditions were used to screen each exon. SSCP has been widely applied for detecting mutations in various systems and has been reported to detect 80–90% of all mutations (16). The other issue concerns the proportion of cells in the tumor that contain the mutation. Breast tumors contain nonmalignant supporting stroma and blood vessels (18). Therefore, the detection of a somatic mutation depends on the proportion of tumor and non tumor cells in the specimen and on the proportion of tumor cells with the mutation. Detection of variant and wild-type conformers on an SSCP gel is diagnostic of a somatic mutation but does not indicate whether the wild-type allele is present in malignant cells that lack the mutation, is present in nonmalignant cells, or is present in both. In tumors where we detected mutations, both the wild-type and mutant bands were evident on SSCP gels, indicating that the mutations are somatic. The presence of a major wild-type band suggests that many cells contain wild-type ER. While some of these cells are nonmalignant, others could be malignant cells without the mutation.

Inherited germ-line mutations have been extensively described in the androgen, glucocorticoid, and thyroid resistance syndromes, but germ-line mutations of estrogen receptor are not known and could be lethal to the organism (9). Several ER gene mutations (reviewed in Ref. 10) and even posttranslational modifications of ER have been reported (11). Graham et al. (9) have described a frame-shift/termination mutant in the ER-positive tamoxifen-resistant cell line T47DCO. This mutant was generated by a point deletion in the HBD just upstream of the end of exon 5. This could give rise to an ER protein truncated in the middle of the HBD amino acid 417. Fuqua et al. (12) have reported truncated ER variants lacking exon 5 (10) and exon 7 (12) that could have been generated by transcriptional splicing errors. Although several other examples of variant ERs exist in the literature (10), this report is the first extensive study that has attempted to correlate the clinical history and therapeutic outcome of breast tumors with ER mutations. Our results suggest that ER mutations occur at a low frequency that is probably comparable to the mutation frequency of any other mammalian gene. While mutant estrogen receptors could represent a definitive mechanism for ER-independent and tamoxifen-resistant growth of tumors 10 and 17 that we have described, our study clearly shows that only 2 of 20 tamoxifen-resistant tumors contain ER mutations. The remaining 18 tamoxifen-resistant tumors contain wild-type estrogen receptors. Other mechanisms must therefore be responsible for the estrogen independence and the clinical resistance to tamoxifen observed in these tumors.

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References

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