Analysis of the PvuII Restriction Fragment-length Polymorphism and Exon Structure of the Estrogen Receptor Gene in Breast Cancer and Peripheral Blood

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ABSTRACT

The presence of estrogen receptor (ER) is a well-known predictor of clinical outcome in human breast cancer. We examined the ER gene in 26 primary breast cancers (14 ER-positive, 12 ER-negative) to determine if alterations of the gene are associated with the ER-negative status. In tumor biopsies and peripheral blood DNA obtained from the same patients we analyzed the ER exon structure using polymerase chain reaction amplification, restriction endonuclease digestion, and agarose gel electrophoresis. All blood and tumor samples, regardless of ER status, showed a complete set of eight exons of normal sizes, ruling out deletions or rearrangements of the ER gene in excess of ±20 nucleotides. Previous reports indicate that the two-allele ER PvuII polymorphism could be associated with ER expression in breast cancer (Hill et al., Cancer Res., 49: 145—148, 1989) as well as with patient age at time of tumor diagnosis (Parl et al., Breast Cancer Res. Treat., 14: 57—64, 1989). We localized the PvuII polymorphism in intron 1, 0.4 kilobase upstream of exon 2. Sequence analysis showed the polymorphism to result from a point mutation (T→C) at the fifth position of the restriction site (CATCTh). We determined the PvuII restriction fragment-length polymorphism genotype in 257 primary breast cancers and 140 peripheral blood DNA samples obtained from women without breast cancer. The results indicate that the PvuII polymorphism is not associated with ER content or patient age at tumor diagnosis.

INTRODUCTION

The determination of ER3 content by hormone binding assay in cytosol extracts of tumor tissue homogenates has proved useful in selecting patients for hormonal therapy (1). In large series of unselected breast cancer patients about two-thirds of the tumors are ER-positive and one-third are ER-negative (2). When patients with ER-positive primary tumors develop metastases, about 60% will respond to endocrine therapy. In contrast, only 5% of patients with ER-negative cancers respond to hormonal manipulation (3—7). The mechanisms responsible for the resistance to hormonal therapy seen in nearly all ER-negative and about 40% of ER-positive breast cancers are still unknown.

A more comprehensive approach than the measurement of estrogen binding in tumor tissue homogenates will be required to elucidate the molecular mechanisms responsible for resistance to hormonal therapy. Such a study of the molecular mechanisms underlying estrogen resistance has become feasible as a result of recent advances in the characterization of ER. The ER is a member, along with the progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor, androgen receptor, vitamin D3 receptor, and thyroid hormone receptor, of a family of ligand-inducible nuclear receptors, each encoded by unlinked genes (8, 9). The human ER gene is located on chromosome 6q24—q27 (10); it extends over more than 140 kilobases and is split into eight exons (11). The cDNA defines a sequence of 6322 nucleotides encoding a protein of 595 amino acids with a predicted molecular weight of 66,182. The 1785-nucleotide coding region is flanked by untranslated sequences of 232 nucleotides and 4305 nucleotides on its 5' and 3' ends, respectively. Comparative analysis and mutagenesis experiments of this receptor family have led to the identification of three functional domains: (a) a steroid-binding domain in the COOH-terminal region; (b) a DNA-binding domain in the midportion of the molecule; and (c) a transcriptional activation domain in the NH2-terminal region as well as other portions of the molecule (12—17). The interaction of all three domains is necessary for the estrogen-dependent activation of gene transcription, which can be visualized as a series of steps. In the first step estrogen binds to the hormone-binding domain and induces the formation of stable ER homodimers (18—20). In the second step the hormone-activated ER dimer interacts with a regulatory DNA enhancer sequence, called the estrogen response element (21, 22). In the final step, the ER-estrogen response element complex promotes the formation of the transcription initiation complex, conceivably by recruiting transcription factors and/or stabilizing the interaction of transcription factors with the promoter of estrogen-responsive genes (23).

This chain of events leading to a physiological estrogen response has been relatively well defined in target organs such as the uterus and in cultured cell lines, such as MCF-7 breast cancer cells. However, the molecular defect(s) responsible for lack of estrogen response in human breast cancer are still unknown. In other steroid receptors, such as the glucocorticoid, androgen, and vitamin D3 receptors, hormone resistance has clearly been linked to deletions and point mutations of the respective genes (24—27). Since the steroid hormone receptors are closely related in their domain structure and function as ligand-inducible transcriptional regulators, one would expect the type of ER defects associated with estrogen resistance to be analogous to those described for the glucocorticoid receptor, androgen receptor, and vitamin D3 receptor.

In the ER gene, digestion with the restriction endonuclease PvuII identified a single, two-allele polymorphism with fragments of approximately 1.6 and/or 0.7 kilobases. Hill et al. (28) suggested that this RFLP is linked to ER expression in human breast cancer. Analysis of allele distribution and frequency in 188 primary breast cancers showed a correlation between the absence of the 0.7-kilobase allele and the lack of hormone-binding activity. ER-positive tumors were more frequently homozygous for the 0.7-kilobase allele (67%) than for the 1.6-kilobase allele (33%); the converse was true for ER-negative cancers which were homozygous for the 0.7-kilobase
allele in 40% and homozygous for the 1.6-kilobase allele in 60% of cases ($P = 0.014$). Tentatively, Hill et al. (28) located the PvuII RFLP within sequences of the ER gene encoding the DNA- or hormone-binding domains. In a smaller study of 59 breast cancers, we failed to find a correlation between RFLP allele distribution and the results of the hormone-binding assay (29). Instead, we observed a significant correlation between the PvuII RFLP within sequences of the ER gene encoding the estrogen receptor protein and the results of the hormone-binding assay (30). For each tumor, 20 μg of DNA were digested with PvuII, electrophoresed in a 0.8% agarose gel, and transferred to a nylon membrane. Hybridization and autoradiography were performed as previously described (29).

A series of oligonucleotide primers was prepared in the Biosynthesis Laboratory of the Department of Molecular Biology using an Applied Biosystems (Foster City, CA) DNA synthesizer. The primers were designed based on published sequences (11, 33, 34) to study the exon structure of the ER gene by PCR amplification. The primer pairs which bracket coding exons 2 to 7, and part of exons 1 and 8, are shown in Table 1. Genomic DNA (1 μg) was amplified through 30 cycles in a 100-μl volume of 10 mM Tris-HCl, pH 8.3, 40 mM KCl, 4 mM MgCl₂, 0.1% Triton X, 0.01% (w/v) gelatin, and 200 μM each of the four deoxyribonucleotides. Taq polymerase (2 units; Promega Biotech) and one oligonucleotide at 1 μM were used per reaction. Amplification conditions consisted of an initial denaturing step at 95°C for 5 min, followed by annealing at 63°C for 1 min, and polymerization at 72°C for 1 min, followed by 30 cycles of 95°C for 30 s, 63°C for 1 min, and 72°C for 6 min with a 30-s increment/10 cycles in polymerization time. For oligonucleotide pairs 21 and 22, 25 and 26, and 27 and 12 an annealing temperature of 57°C reduced the number of nonspecific fragments. A sample of the PCR mixture was size fractionated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Restriction endonucleases were selected based on the published ER cDNA sequence (33, 34) to further characterize the PCR products of exons 2 to 7. A portion of the PCR product (12 μl) of exons 3, 4, and 5 was directly used for digestion, whereas the entire PCR product (100 μl) of exons 2, 6, and 7 was ethanol-precipitated and then resuspended in 12 μl distilled H₂O for digestion. The samples were completely digested with SstI (exon 2), AluI (exon 3), HindIII (exon 4), XbaI (exon 5), and PstI (exons 6 and 7); separated by electrophoresis in a 4% agarose gel (NuSieve GTG; FMC Bioproducts, Rockland, ME); and visualized by ethidium bromide staining.

Oligonucleotide primers were also prepared for PCR amplification of a region containing the PvuII polymorphic site, which our initial mapping studies had localized to the first intron of the ER gene. The 3′ primer was derived from the published sequence of the second exon (11, 33, 34). To obtain the necessary sequence to design a 5′ primer, the 1.6-kilobase PvuII fragment of the λ clone, λHERC3, containing both exon 2 and a portion of intron 1, was subcloned into Bluescript II KS+ vector (Stratagene, La Jolla, CA). The 5′ region of the intron was then sequenced using the dideoxy sequencing method (35).

These primers (primers 28 and 29; see Table 1) (0.3 μM each) were then used in a PCR amplification reaction consisting of the following steps: denaturation for 30 s at 94°C; annealing at 62°C for 20 s; and polymerase extension at 72°C for 90 s. After amplification, the PCR products were digested with PvuII and electrophoresed in a 1.5% agarose gel to type the samples for the presence of the PvuII restriction site.

To identify the PvuII polymorphic site, the PCR product was cloned into Bluescript II KS+ vector. The PCR product was first incubated with Klenow fragment at 37°C for 30 min in the presence of an additional 22 μM deoxynucleotide triphosphate to convert any overhanging ends to blunt ends. The solution was extracted with phenol/chloroform and ethanol precipitated in the presence of ammonium acetate. The PCR product was ligated with 200 ng of SmaI-cut Bluescript using T₄ DNA ligase in a 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM spermidine, 1 mM ATP, and 1 mM dithiothreitol buffer (36). DH5α competent cells were transformed with the ligation products and plated on LB agar plates containing 40 μg/ml ampicillin. The appropriate clones were identified by blue/white selection, processed for large-scale plasmid purification by CsCl density gradient ultracentrifugation (37), and sequenced as previously described.
Table 1  PCR primers for ER exons and PvuII RFLP genotyping

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Nucleotide position</th>
<th>Sequence</th>
<th>Amplified exon</th>
</tr>
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<tbody>
<tr>
<td>31</td>
<td>174-196</td>
<td>GGTTCTGAGGCTTCTGCGCGT</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
<td>675-684 (in exon 1) plus 10 nt* (in intron 1)</td>
<td>GCCCGGTTACTGTAAGAATG</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>5 nt in intron 1 plus 685-699</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>20 nt at 5' end of intron 2</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>4</td>
</tr>
<tr>
<td>17</td>
<td>20 nt at 3' end of intron 2</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>991 + 992 (exon 3) plus 18 nt in intron 3</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>6</td>
</tr>
<tr>
<td>19</td>
<td>17 nt in intron 3 plus 993-995 in exon 4</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>7</td>
</tr>
<tr>
<td>20</td>
<td>20 nt at 5' end of intron 4</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>8</td>
</tr>
<tr>
<td>21</td>
<td>16 nt in intron 4 plus 1329-1334 in exon 5</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>9</td>
</tr>
<tr>
<td>22</td>
<td>1466 + 1467 in exon 5 plus 18 nt in intron 5</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>16 nt in intron 5 plus 1468-1471 in exon 6</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>11</td>
</tr>
<tr>
<td>24</td>
<td>1600 + 1601 in exon 6 plus 18 nt in intron 6</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
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</tr>
<tr>
<td>25</td>
<td>12 nt in intron 6 plus 1602-1609 in exon 7</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>13</td>
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<tr>
<td>26</td>
<td>20 nt at 5' end of intron 7</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>14</td>
</tr>
<tr>
<td>27</td>
<td>20 nt at 3' end of intron 7</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>15</td>
</tr>
<tr>
<td>28</td>
<td>2022-2041 in 3' tail</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>16</td>
</tr>
<tr>
<td>29</td>
<td>Intron 1 (about 1.3 kilobases from exon 2 boundary)</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>nt 692-727 in exon 2 (8 nt from intron 1)</td>
<td>CACAGCCTTAATTAGTACTAGTAC</td>
<td>18</td>
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* nt, nucleotide(s).

Statistical Analysis. The data were assessed statistically with a full three-way factorial fixed effects analysis of variance (38). In this analysis, the dependent variable was age at breast cancer diagnosis, and the independent variables were RFLP genotype and ER status. The genotype was classified as homozygous 00 (1.6 kilobases), heterozygous 01 (1.6/0.7 kilobase), and homozygous 1 1 (0.7 kilobase). The ER status was dichotomized as negative or positive if the tumors had <3 or >3 fmol/mg cytosol protein. The homoscedasticity of the model was evaluated using Bartlett's test (39). The P value from this test was 0.68, indicating that the data were consistent with the model's assumption of uniform variability of patient ages in subgroups defined by genotype and ER status. The frequency distribution of patients in each subgroup was analyzed using Mantel's trend test (40). Confidence intervals for proportions were calculated using Fleiss's method (41). The 95% confidence interval for each mean age was derived from the SE of the corresponding age estimate using the critical value from the appropriate t distribution.

RESULTS

To analyze the exon structure of the ER gene in normal and malignant cells we extracted genomic DNA from peripheral blood leukocytes and breast cancer biopsies of 26 patients. PCR amplification of leukocyte DNA yielded exon products of the predicted sizes, indicating identical exon structures in blood DNA and MCF-7 cells (11, 33, 34). Of the 26 tumors 14 were ER-positive and 12 were ER-negative by hormone-binding and immunohistochemical assays. Analysis of the genomic DNA from the 14 ER-positive tumors showed ER exon sizes identical to those of the leukocyte DNA samples. PCR amplification of genomic DNA from the ER-negative tumors revealed the presence of all 8 exons in all 12 tumors. The exon sizes were

Fig. 1. Analysis of exon structure of the ER gene from an ER-negative breast cancer and from peripheral blood leukocytes obtained from the same patient. A, ethidium bromide-stained 1.5% agarose gel showing PCR amplification products of genomic ER DNA sequences from ER-negative breast cancer (odd-numbered lanes) and peripheral blood leukocytes (even-numbered lanes). Note that the ER exon sizes are identical for tumor and leukocyte DNA samples. Lanes 1 and 2, exons 1; Lanes 3 and 4, exons 2; Lanes 5 and 6, exons 3; Lanes 7 and 8, exons 4; Lanes 9 and 10, exons 5; Lanes 11 and 12, exons 6; Lanes 13 and 14, exons 7; Lanes 15 and 16, exons 8. B, analysis of PCR products from ER exons 2 to 7 by restriction endonuclease digestion. PCR products shown in A were digested with StyI (exon 2, 107- and 84-base pair fragments; Lanes 1 and 2), AluI (exon 3, 65- and 52-base pair fragments; Lanes 3 and 4); HindIII (exons 4 and 5, 52- and 50-base pair fragments; Lanes 5 and 6), XbaI (exon 5, 101- and 38-base pair fragments; Lanes 7 and 8); PstI (exon 6, 87- and 47-base pair fragments; Lanes 9 and 10); and PstI (exon 7, 124- and 60-base pair fragments; Lanes 11 and 12) and separated on a 2% agarose gel stained with ethidium bromide. Note that the restriction fragments for each of the ER exons are identical for breast cancer (odd-numbered lanes) and leukocyte (even-numbered lanes) DNA samples. Several of the smaller restriction fragments were visible in the original gel but were lost during the reproduction process for the final photograph.
ESTROGEN RECEPTOR GENE IN BREAST CANCER

A. The PvuII site was localized to intron I, 0.4 kilobase upstream of exon 2, using Southern blot analysis. In B, dideoxy sequence analysis indicated that the polymorphism is the result of a T→C point mutation in the fifth position of the PvuII restriction site. In C, genomic DNA from primary breast tumors was PCR amplified using primers 28 and 29. The resulting 1.3-kilobase fragment (0 band) can be cleaved with PvuII into 850-basepair and 450-basepair fragments (1 band) if the restriction site is present on one or both alleles.

identical in ER-negative and ER-positive tumors and in the corresponding peripheral blood leukocytes (Fig. 1A). There was also no evidence of additional variant PCR fragments, even in those ER-negative cancers in which the malignant cells comprised 70 to 90% of the entire cell population. Further analysis of the exon structure by restriction endonuclease digestion yielded fragments of the predicted sizes ranging in length from 38 to 256 base pairs. The respective restriction fragments were identical in size in ER-positive and ER-negative tumors and in the corresponding peripheral blood leukocytes (Fig. 1B).

Eight genomic clones containing the various ER gene exons (11) were digested with PvuII to identify the 1.6-kilobase PvuII fragment containing the ER PvuII polymorphic site. AnGHER3 was found to contain a 1.6-kilobase PvuII fragment which hybridized with the ER cDNA. The 1.6-kilobase fragment was subcloned (pCE133) into the Bluescript plasmid vector (Stratagene, La Jolla, CA). Restriction enzyme mapping of pCE133 revealed that it contained exon 2 and that the polymorphic site was approximately 300–500 base pairs upstream of the 5' end of exon 2 (Fig. 2A). Sequence analysis of pCE133 showed two putative sites 20 base pairs apart, 0.4 kilobase upstream from exon 2, each of which differed by only one base from the PvuII recognition sequence CAGCTG. Sequencing of the 1.6-kilobase allele identified a point mutation (T→C) in the latter recognition site (Fig. 2B). Two independent clones were sequenced from two patients to rule out errors due to Taq polymerase misincorporation. PCR amplification of the genomic DNA fragment containing the PvuII RFLP followed by PvuII digestion and agarose gel electrophoresis showed three genotypic patterns (Fig. 2C). The genotypes were designated 00, 01, and 11, corresponding to the 1.6-kilobase/1.6-kilobase, 1.6-kilobase/0.7-kilobase, and 0.7-kilobase/0.7-kilobase PvuII genotypes of the genomic Southern analysis. A comparison of PCR and Southern analysis of 110 breast cancers showed complete agreement of genotype classification by the two methods.

The PvuII RFLP allele distribution showed no correlation with the results of the ER binding assay or the ERICA analysis (Table 2). However, there was a modest trend suggesting that the prevalence of ER-positive tumors was highest among homozygous 00 women and lowest in homozygous 11 women. The prevalence of ER-positive cancers in heterozygous women was intermediate between those of the two homozygous patient groups.

The PvuII RFLP allele distribution in leukocyte DNA of 140 women without breast cancer was similar to that seen in tumor DNA of breast cancer patients (Table 3). A breakdown of control and patient groups by age showed similar allele distributions in all age groups. A separate analysis of the breast cancer groups from the two hospitals showed that Vanderbilt patients with the homozygous 11 genotype were significantly younger than homozygous 00 or heterozygous 01 patients (P = 0.005). This age difference, however, was not found in Memorial patients, who showed a nonsignificant increase in age in women with the 11 genotype (Table 4). A multiple comparisons test of this association was performed using Bonferroni’s test (42), which reduced the significance of the Vanderbilt age-genotype interaction to P = 0.03. Thus, although unexpected, the observed younger age of homozygous 11 Vanderbilt patients was probably not due to a multiple comparisons artifact. The age at tumor diagnosis was slightly lower for women with ER-

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<table>
<thead>
<tr>
<th>Genotype</th>
<th>00</th>
<th>01</th>
<th>11</th>
<th>Frequency of allele 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>78 (50.3)</td>
<td>35 (22.6)</td>
<td>0.52 [0.47–0.58]</td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>76 (54.9)</td>
<td>27 (26.5)</td>
<td>0.46 [0.39–0.53]</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>82 (52.6)</td>
<td>35 (22.4)</td>
<td>0.51 [0.46–0.57]</td>
<td></td>
</tr>
</tbody>
</table>

* Percentages listed in parentheses; e.g., 27.1% of the 155 tumors that were ER- were in women with the 00 genotype.

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Table 3 Average patient age as a function of PvuII RFLP genotype and hospital

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vanderbilt University Hospital</th>
<th>Nashville Memorial Hospital</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>60.4 [56–65]</td>
<td>59.2 [54–64]</td>
</tr>
<tr>
<td>01</td>
<td>61.0 [58–64]</td>
<td>59.5 [55–64]</td>
</tr>
<tr>
<td>11</td>
<td>52.6 [48–57]</td>
<td>63.1 [58–68]</td>
</tr>
</tbody>
</table>

* Average age in years at time of cancer diagnosis.

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Table 2 Distribution and frequencies of ER gene PvuII alleles as a function of tumor ER/ERICA status

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Fig. 2. Location, sequence, and genotype analysis of the ER PvuII RFLP. In A, the PvuII site was localized to intron I, 0.4 kilobase upstream of exon 2, using Southern blot analysis. In B, dideoxy sequence analysis indicated that the polymorphism is the result of a T→C point mutation in the fifth position of the PvuII restriction site. In C, genomic DNA from primary breast tumors was PCR amplified using primers 28 and 29. The resulting 1.3-kilobase fragment (0 band) can be cleaved with PvuII into 850-basepair and 450-basepair fragments (1 band) if the restriction site is present on one or both alleles.
negative cancers than for women with ER-positive cancers in all subgroups defined by genotype (Table 5). This difference, eight exons and seven introns extending over approximately

**DISCUSSION**

The ER gene on chromosome 6q24–q27 is organized into eight exons and seven introns extending over approximately 140 kilobases (11). One goal of this study was to determine if the genomic ER structure is altered in breast cancer in general and in ER-negative breast cancer in particular. Deletions or rearrangements of the ER gene appear likely when one considers the extensive cytogenetic changes occurring in these neoplasms. DNA flow cytometric studies have shown that between 38 and 100 base pairs, ranging from 117 base pairs for exon 3 to 514 base pairs for the coding portion of exon 1, were identical in ER-positive and ER-negative tumors contained the complete set of ER exons described in MCF-7 cells, ruling out major alterations of the ER gene (44). Detailed karyotype analysis in excess of ±20 nucleotides are responsible for the ER-negative status. Of course, we cannot exclude the possibility of smaller alterations, including point mutations in the ER hormone-binding domain, as the cause of the absent ligand binding. Indeed, in vitro mutagenesis experiments have shown that point mutations can prevent hormone binding (48). We also cannot exclude chromosomal rearrangements which leave the integrity of each of the exons intact.

The ER expression in breast cancer has been linked to a single, two-allele RFLP (28). The restriction endonuclease PvuII cleaves fragments of 0.7 and 1.6 kilobases that identify distinct alleles of the ER gene (alleles 1 and 0, respectively). Hill et al. (28) found that breast cancer patients who were homozygous for allele 0 were more likely to have ER-negative tumors. Before investigating the relationship between ER expression and the PvuII RFLP, we decided to localize the polymorphic site which Hill et al. (28) placed within sequences encoding the DNA- or hormone-binding domains. We identified the PvuII polymorphic site in the first intron, 0.4 kilobase upstream from exon 2, with a point mutation (T—*C) in the recognition sequence CAGCTG responsible for the 0 allele (Fig. 1). The location of the RFLP in the intron makes it unlikely that the polymorphism is correlated with ER expression. However, we cannot rule out the possibility that the polymorphism is in linkage disequilibrium with other ER mutations which do affect ER expression.

The PvuII RFLP allele distribution showed no correlation with the results of the ER hormone binding assay or the immunohistochemical ERICA analysis (Table 2). The prevalence of ER-negative cancers was relatively higher in patients bearing the 1 allele than in those bearing the 0 allele (Table 2). Our findings contradict those reported by Hill et al. (28), who observed a greater percentage of ER-negative tumors associated with the 0 allele. The fact that the two studies disagree and that both showed only a weak correlation between RFLP genotype and ER expression makes it less likely that the RFLP is linked to the ER status. This argument is supported by the observation

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**Table 5 Average age as a function of ER gene PvuII alleles and ER status**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Agea</th>
<th>95% CI</th>
<th>n</th>
<th>Age</th>
<th>95% CI</th>
<th>n</th>
<th>ER—</th>
<th>ER+</th>
<th>(ER+) − (ER+)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>60.4</td>
<td>[58—63]</td>
<td>61</td>
<td>57.8</td>
<td>[56—63]</td>
<td>19</td>
<td>60.7</td>
<td>[57—65]</td>
<td>42</td>
<td>2.56</td>
</tr>
<tr>
<td>01</td>
<td>56.4</td>
<td>[55—62]</td>
<td>134</td>
<td>57.8</td>
<td>[54—62]</td>
<td>56</td>
<td>56.2</td>
<td>[59—66]</td>
<td>78</td>
<td>4.47</td>
</tr>
</tbody>
</table>

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a Average age in years at time at time of cancer diagnosis.

b 95% confidence intervals.

c Number of patients.

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The observed genotypic frequencies for all ages closely fit Hardy-Weinberg expectations. Table 4 shows that the observed genotypic frequencies for all ages closely fit Hardy-Weinberg expectations.
that the 00 genotype occurred with a prevalence (23.4%) in noncancer individuals similar to that in breast cancer patients (23.7%) (Table 4). If the 00 genotype were indeed associated with the failure to express ER, one would have to assume that over 20% of women have nonfunctional ER, which would certainly interfere with normal reproductive function.

We extended our earlier study of the association between the PvuII RFLP genotype and patient age at time of tumor diagnosis (29). As in the previous study, Vanderbilt patients with the 11 genotype were again significantly younger (52.6 years) than women with the 01 or 00 genotypes (61.0 or 60.4 years, respectively) (Table 3). However, patients from Nashville Memorial Hospital failed to show a significant correlation between age at time of tumor diagnosis and RFLP genotype (Table 3).

At present we have no explanation for the different RFLP genotype-patient age association in the patient populations of the two hospitals. Although the existence of this polymorphism may yet prove to have some functional significance, our current data do not show a convincing correlation of the PvuII polymorphism with either ER expression or patient age at time of tumor diagnosis.

ACKNOWLEDGMENTS

We are grateful to Professor Pierre Chambon for the gift of pOR3 and XGHER3. We also appreciate the cooperation of Drs. Vernon Reynolds, William Richards, and John Sawyers of the Department of Surgery at Vanderbilt University Hospital and of Drs. Jere Baxter, Thomas Hanes, and Elsie Ollapally of the Department of Pathology at Nashville Memorial Hospital. William Weller, Nady Roodi, and Lilly Ng provided expert technical assistance.

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