

A Hypersensitive Estrogen Receptor- α Mutation in Premalignant Breast Lesions¹

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Abstract

The best current model of breast cancer evolution suggests that most cancers arise from certain premalignant lesions. We have identified a common (34%) somatic mutation in the estrogen receptor (ER)- α gene in a series of 59 typical hyperplasias, a type of early premalignant breast lesion. The mutation, which affects the border of the hinge and hormone binding domains of ER- α , showed increased sensitivity to estrogen as compared with wild-type ER- α in stably transfected breast cancer cells, including markedly increased proliferation at subphysiological levels of estrogen. The mutated ER- α exhibits enhanced binding to the TIF-2 coactivator at low levels of hormone, which may partially explain its increased estrogen responsiveness. These data suggest that this mutation may promote or accelerate the development of cancer from premalignant breast lesions.

Introduction

Studies in colon carcinoma have demonstrated a series of genetic alterations that are closely associated with morphological tumor progression (1). There is also epidemiological evidence for a similar model of breast cancer evolution. In this model (2), breast cancer is hypothesized to evolve from normal ductal epithelium to typical hyperplasia to atypical hyperplasia to carcinoma *in situ* to invasive carcinoma and, finally, to metastatic carcinoma. Recent data also suggest that the majority of hyperplasias share molecular alterations with invasive disease in the same breast (3), providing genetic evidence that they are related. Unlike colon cancer, very little is known about the specific molecular changes associated with the earliest stages of breast cancer evolution. However, there is a great deal of evidence to support the hypothesis that estrogens are important because they are potent mitogens for normal breast epithelial cells, and it is believed that the duration of breast epithelium exposure to estrogen is a significant risk factor for breast cancer development. It is also generally agreed that expression of the ER³ is relatively low in normal breast epithelium but higher in certain premalignant lesions (e.g., typical hyperplasias; Ref. 4), leading us to hypothesize that inappropriate overexpression of WT ER and the appearance of an altered ER might be early events in breast cancer evolution. In this report, we provide evidence of a somatic ER mutation occurring at high frequency in breast hyperplasias that has altered responsiveness to hormone.

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³ The abbreviations used are: ER, estrogen receptor; WT, wild-type; GST, glutathione S-transferase; RT-PCR, reverse transcription-PCR; Var, variant, TIF-2, transcription intermediary factor.

Materials and Methods

Sample Preparation and Nucleotide Sequence Analysis. Histological slides from archival, clinical specimens were screened microscopically for evidence of hyperplasia. Microdissection of specimens was performed on 55 samples using serial sections from formalin-fixed, paraffin-embedded tissue blocks as described previously (5). Briefly, alternative 3- and 10- μ m-thick sections were cut from the blocks and float-mounted on glass slides. The 3- μ m-thick slides were stained with H&E and examined under the light microscope to locate regions of normal and hyperplastic tissues, and these areas were outlined with a felt-tipped pen. The marked slide was then used as a template to guide manual microdissection from the corresponding regions of the unstained 10- μ m-thick sections. It was possible to obtain distant normal tissue from four of the patients with hyperplasia. DNA was liberated from the microdissected specimens using a modification of the procedure of O'Connell *et al.* (5). Genomic sequencing was then performed using PCR amplification of isolated DNA using ER primer 1 (nucleotides 1093–1112, 5' primer) and ER primer 2 (nucleotides 1231–1250, 3' primer) of the ER gene (6). An aliquot of this amplification was then used to perform single-stranded PCR amplification using ER primer 3 (nucleotides 1220–1239, 3' primer) of the ER gene. After precipitation of the single-stranded PCR amplification product, dideoxysequence analysis was performed using ER primer 4 (nucleotides 1101–1130, 5' primer). Genomic DNA was isolated from normal blood samples of 80 healthy women and used for genomic sequence analysis as described above. RNA was also isolated from four additional frozen hyperplastic lesions and used for RT-PCR amplification, cloning, and sequence analyses as described previously (7).

Stable Transfection and Cell Growth Analyses. The WT ER expression construct was prepared in the pcDNA1 vector as described previously (8). Site-directed mutagenesis of this construct was then used to generate the A908G transition, and the entire coding sequence of ER was verified by dideoxysequence analysis in this clone. The generation of stable transfectants was performed as described by Oesterreich *et al.* (9) using cotransfection with the G418-selectable expression vector pSVneo at a ratio of 25:1 with the ER plasmids into MCF-7 breast cancer cells. To analyze for expression of WT or Var sequences, Western blot analyses were performed using the 6F11 antibody (DAKO). Twofold to threefold elevated levels of total ER protein were detected in the two WT ER clones and the three Var clones (data not shown). In addition, RT-PCR amplification of cDNA from the transfectants (7), followed by dideoxysequence analysis, confirmed that exogenous WT and Var RNA were expressed in the stable transfectants. Furthermore, the relative levels of WT or Var sequences were determined by genomic sequence analysis as described above; the ER Var transfectants contained both the WT nucleotide (A) and the Var nucleotide (G) sequence in approximately equal ratios on the sequencing gels. For cell growth studies, cells were plated at a density of 2×10^4 in media containing 10% charcoal-stripped, estrogen-free FCS and either left untreated or treated with the indicated increasing estradiol concentrations of 1×10^{-12} , 1×10^{-11} , or 1×10^{-9} M. The medium was replaced every 48 h, and the cells were harvested and counted on days 2, 4, 6, and 8, respectively.

Statistical Methods. After taking logarithms to stabilize within-group variances, as determined to be appropriate by Box-Cox analysis (10), one-way ANOVA was used to detect estrogen dose-related differences in growth on day 8 (i.e. 0 versus 10^{-12} versus 10^{-11} versus 10^{-9} M) and to detect differences

among estrogen doses (10^{-12} versus 10^{-11} versus 10^{-9} M). The Student-Newman-Keuls multiple range test ($\alpha = 0.02$) was used to determine which doses were different from each other. Analyses were performed using SAS Software (V6.12; SAS Institute, Cary, NC).

GST Pull-down Assays. Bacterial expression vectors for GST-WT ER and GST-mutant ER were constructed by PCR amplification of the hinge and hormone-binding domains of WT ER- α and the A308G ER- α using a sense primer (nucleotides 756–775) and an antisense primer [nucleotides 1788–1769; Ref. 6], and then cloning these products into the *Bam*H1-*Eco*RI sites of pGEX-2kt GST gene fusion vector (Pharmacia). The GST pull-down assays were performed as described previously (11) using recombinant TIF-2 (pSG5-human TIF-2; a kind gift from Dr. Ming-Jer Tsai) translated *in vitro* using the TNT-coupled Reticulocyte Lysate System (Promega, Madison, WI). The reactions were allowed to bind the glutathione-Sepharose 4B beads (Pharmacia) for 1.5 h in the presence of increasing amounts of estradiol at 4°C. Samples were subsequently analyzed by SDS-PAGE.

Results and Discussion

To test the hypothesis that there was an altered ER in early breast disease, we first prepared cDNA by reverse transcription of RNA from four typical hyperplasias of the breast, followed by PCR amplification using primers specific for the entire coding domain of ER- α . The PCR products were then cloned and sequenced. We found the WT ER sequence in two of these premalignant lesions (Fig. 1). However, in the other two lesions, we also found an ER- α variant with an A-to-G bp transition at nucleotide 908 (Fig. 1, top). This transition introduces a Lys-to-Arg substitution at residue 303 within exon 4, at the border between hinge domain D and the beginning of hormone-binding domain E of ER- α (Fig. 1, bottom). Although this substitution represents a conservative amino acid change, we were encouraged to enlarge the size of our study because there are data suggesting that the NH₂-terminal region of the ER- α hormone-binding domain is important in the generation of a complete transcriptional response in cells (12). We therefore microdissected archival histological sections of 55 additional typical hyperplasias, isolated DNA, and performed direct genomic sequencing using primers bordering ER- α nucleotide 908. We found the same ER- α alteration in 18 of 55 of these additional premalignant lesions. Thus, the A908G ER- α alteration was present in a total of 20 of 59 (34%) of the hyperplasias examined.

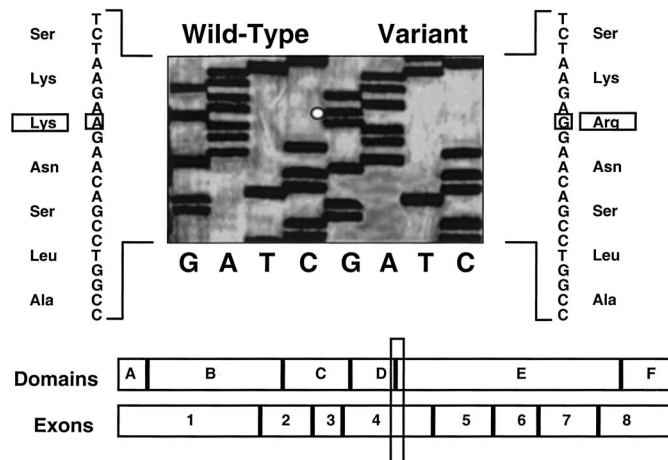


Fig. 1. Sequence analysis of ER Var and WT cDNAs isolated from frozen breast hyperplastic tissue. RNA isolation, RT-PCR amplification of the ER across nucleotides 118–1234, and cloning and sequencing of ER were performed as described previously (7), except that restriction sites were incorporated into the primers to facilitate cloning into pGEM7zf(+) (Promega). A portion of the sequencing products is shown for WT and Var clones, demarcating the location of the G transition and Arg substitution. ER domains A–E and the exons across these domains are shown on the bottom, with the location of the Lys-to-Arg change demarcated with a box across exon 4 at the end of domain D.

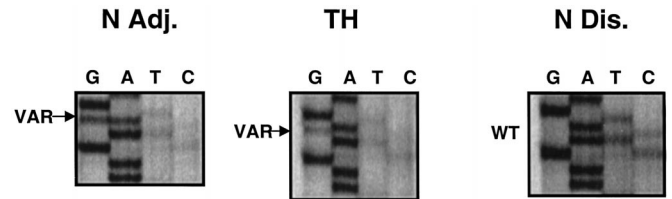


Fig. 2. Detection of the ER Var sequence in archival breast specimens. Identification of WT and Var ER sequences in one patient with typical hyperplasia (TH). Manual microdissection on a light box under a dissecting microscope was performed to microdissect archival, formalin-fixed, paraffin-embedded tissue blocks and was precise enough to ensure at least 50% cellularity. DNA was liberated from the microdissected specimens, and direct genomic sequence analysis was performed. Normal adjacent breast epithelium (N Adj.), typical hyperplasia, and distant normal epithelium (N Dis.) were all available for analysis from this patient. The position of the A908G sequence is indicated by arrows.

We also prepared DNA from normal breast epithelium adjacent to the hyperplastic lesion of those samples that contained the A908G ER- α alteration. The ER- α Var sequence was detected in the normal adjacent epithelium of some of the samples tested (data not shown). Thus, it appears that the A908G ER- α transition is frequently present in premalignant lesions of the breast and may also occur in the adjacent normal-appearing breast epithelium.

To address whether the ER alteration might represent a somatic change in the breast rather than a germ-line alteration or a naturally occurring polymorphism within ER- α , we microdissected distant normal epithelium from 4 of the 20 patients with the A908G ER alteration in their hyperplastic lesion. Only four of the patients had sufficient normal distant tissue for analysis. Genomic sequencing of one patient's samples is shown in Fig. 2. Variant A908G ER- α sequence was detected along with WT sequence in the normal adjacent DNA (N Adj.) and the typical hyperplasia (TH) DNA from this patient, but the normal distant tissue (N Dis.) displayed only WT ER- α sequence. All four of the patients with the Var ER- α sequence in their hyperplastic lesion exhibited the WT sequence in their distant normal tissue. To further strengthen this observation, we also examined normal DNA by direct genomic sequencing of 80 blood samples collected from patients without breast disease. We did not detect the ER- α Var sequence in any of these normal samples (data not shown). We therefore conclude that the A908G ER- α alteration is a somatic mutation appearing frequently in association with breast hyperplasia. Thus, just as LOH can occur in morphologically normal ductal epithelium adjacent to breast cancers (13) and may therefore demarcate a localized region predisposed to the development of breast cancer, we suggest that a somatic mutation in ER- α within a localized region of normal breast epithelium might define a region of increased risk if the mutation confers a selective advantage to these cells.

To test the hypothesis that this ER mutation might confer such an advantage, we examined the proliferative response to hormones in breast cancer cell transfectants containing the mutation. We first prepared a cytomegalovirus-driven mammalian expression vector for WT ER- α and used site-directed mutagenesis (Promega) to generate the K303R substitution. We then stably introduced the mutant expression vector in the ER-positive MCF-7 breast cancer cell line that normally expresses WT ER- α . We chose this cell line because we found that WT ER- α was expressed along with the mutant in the original two of four typical hyperplastic lesions we examined. As a control, we also stably transfected the expression vector alone into MCF-7 cells. Transfected clones were then cultivated in estrogen-depleted medium ($-E_2$) or medium supplemented with increasing amounts of estradiol (10^{-12} to 10^{-9} M). Both nontransfected MCF-7 cells (Fig. 3A) and vector-alone-transfected cells (Fig. 3B) exhibited typical estrogen dose-response growth curves. Minimal cell growth stimulation was seen with 10^{-12} M estradiol in these cells. Because it

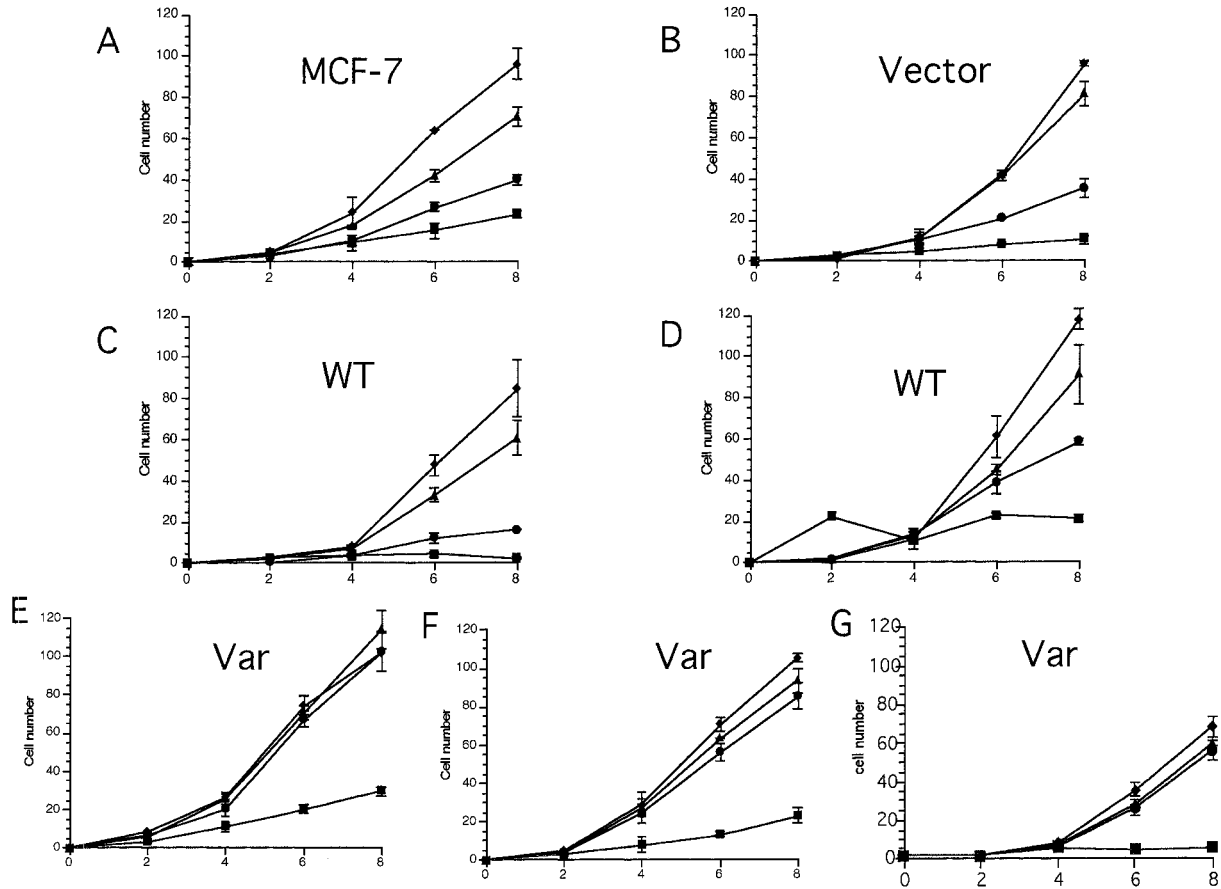


Fig. 3. Growth curves of stable MCF-7 transfectants in response to increasing concentrations of estradiol in the media. Cells were plated at a density of 2×10^4 in media containing 10% charcoal-stripped, estrogen-free FCS and either left untreated (■) or treated with the indicated estradiol concentrations [1×10^{-12} (●), 1×10^{-11} (▲), 1×10^{-9} (◆) M]. The medium was replaced every 48 h, and the cells were harvested and counted on days 2, 4, 6, and 8, respectively. Cell number $\times 10^4$ is shown. A, untransfected, parental MCF-7 cells. B, vector-alone stably transfected cells. C and D, cells stably transfected with WT ER. D–F cells stably transfected with the mutant ER.

was possible that overexpression of the receptor alone might stimulate the growth of these cells, we also transfected MCF-7 cells with the expression vector for WT ER- α , but their estrogen dose-response curves (Fig. 3, C and D) were not different from those of the controls (9). In contrast, three independent clones expressing the ER- α mutation responded to extremely low levels of hormone (10^{-12} M, Fig. 3, E–G) with nearly the same highly proliferative response seen at the highest concentration of estradiol used (10^{-9} M).

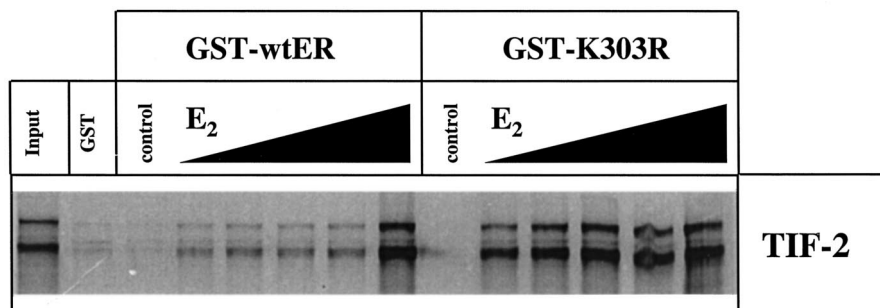
Using ANOVA (10), we determined that these were highly significant estrogen dose-response in the MCF-7, vector-alone transfected, and WT ER- α -transfected cells ($P = 0.001$) but that there was little or no difference in response to differing concentrations of estradiol in each of the three mutant ER- α -transfected clones ($P = 0.41, 0.015, \text{ and } 0.09$, respectively, for clones E, F, and G). The growth-stimulatory effects of low levels of hormone in cells expressing the ER- α mutation were even more evident when doubling times were calculated from the growth curves. For example, the doubling time for MCF-7 cells in 10^{-12} or 10^{-9} M estradiol is 2.2 versus 1.3 days, respectively. The doubling times for cells expressing the ER- α mutant is the same (1.3 days) at either 10^{-12} or 10^{-9} M hormone. We conclude from this data that expression of the ER- α mutation confers a hypersensitivity to estrogen with an ability to be maximally stimulated in response to physiological levels (10^{-12} to 10^{-11} M) of hormone. Thus, the A908G ER- α mutation is a gain-of-function mutation that could have a significant biological role in early breast disease.

One mechanism by which the ER- α mutation might confer hypersensitivity to low levels of hormone would be an increased binding

affinity for estradiol. However, no differences in estradiol affinity were detected between the WT ER- α and the A908G ER- α mutation using saturation binding Scatchard analyses, nor were there differences in affinity for the antiestrogen tamoxifen (data not shown).

Another mechanism might be altered affinity for ER coregulators. It is now understood that many of the cell type- and tissue-specific effects of ER- α are dependent on the cellular pool of coregulatory factors that bind to and influence its transcriptional activity (reviewed in Ref. 14), many of which act as signaling intermediates between the ER and the general transcriptional machinery or directly have enzymatic activities such as histone acetyltransferase activity. The A908G ER- α mutation occurs in a region implicated in binding to certain of these coregulatory proteins, such as L7/SPA (15) and the SRC-1 family of coactivators (16). For example, efficient interaction of SRC-1 with the progesterone receptor hormone-binding domain requires the presence of hinge sequences (16). Thus, we compared the ability of WT and mutant ER- α to interact with TIF-2 (17), a member of the SRC-1 family, using *in vitro* GST pull-down assays (11). GST-WT ER- α and GST-ER- α mutant fusion constructs containing the hinge and hormone-binding domains were prepared. Full-length TIF-2 was synthesized *in vitro* in the presence of [35 S]methionine and then tested for specific hormone-dependent binding to the immobilized GST-ER fusion proteins (Fig. 4). Both receptors bound TIF-2 in the presence (10^{-6} M) but in not the absence of estradiol. However, the mutant required much less hormone for efficient binding. Even at the lowest estradiol concentration tested, 4×10^{-8} M, the mutant ER efficiently bound TIF-2, whereas WT ER- α exhibited negligible binding at this concentration. Similar data were obtained with the SRC-3 coactivator

Fig. 4. Interaction of the WT and mutant ERs with TIF-2 *in vitro*. Full-length TIF-2 was synthesized *in vitro* in the presence of [35 S]-methionine and then incubated with Sepharose beads containing immobilized GST, GST-WT ER, and GST-A908G mutant ER with or without estradiol. Bound TIF-2 was eluted and observed by SDS-PAGE and autoradiography. Input TIF-2 is shown (10%), as is nonspecific GST binding in the absence of estradiol. Increasing levels of estradiol used were 4×10^{-8} , 5×10^{-8} , 6×10^{-8} , 7×10^{-8} , and 1×10^{-6} M.



(data not shown). These data suggest that the K303R substitution enhances TIF-2 binding by lowering the concentration of hormone required to facilitate the formation of the coactivator:ER hydrophobic groove binding surface (18) within the ER hinge/ligand-binding domain. An additional mechanism is that this residue in the ER may be a potential site for acetylation. An Arg substitution at this site could render it incapable of being acetylated, and/or the substitution could reduce the net negative charge if the surrounding Lys residues in the ER are indeed acetylated. These biochemical possibilities will be pursued in future studies. Altered coactivator binding has also been reported for a Y537N ER- α mutation (19) that we identified in a metastatic bone lesion from a breast cancer patient (20). Thus, it may be clinically relevant that both of these *in vivo* ER- α mutations drastically affect the ability of the receptor to bind to coregulatory proteins. A detailed study of the biological and clinical significance of both of these ER- α mutations in breast cancer patients is currently under way.

In summary, we have shown that a large proportion of premalignant breast hyperplasias express an altered ER- α that is hypersensitive to the effects of estrogen. Furthermore, the alteration results from a somatic mutation in the breast, with this mutation affecting the ability of the receptor to bind to the TIF-2 coactivator. There is an increasing body of evidence, both epidemiological (2) and molecular (3), suggesting that these premalignant lesions are both risk factors and direct precursors of invasive breast cancer. However, hyperplasias are relatively common in the breast, and only a small fraction of them will progress to cancer. Currently, we are unable to differentiate which of these lesions are genetically stable or the biological differences driving some of them to progress. An ER- α mutation that confers a proliferative advantage, such as hypersensitivity to hormone, could provide a favorable cellular environment to accelerate the accumulation of additional genetic events important for tumor progression.

Premalignant breast lesions are microscopic masses with a positive growth imbalance, and the hypersensitive ER- α mutation could be an important factor contributing to this imbalance. Hormone levels normally fluctuate during the menstrual cycle in premenopausal women, and levels are considerably lower in postmenopausal women. An ER mutation hypersensitive to estradiol could provide a continuous mitogenic stimulus to the breast epithelium even during phases of low circulating hormone, especially in postmenopausal women, thus elevating their risk for breast cancer. This hypothesis will have to be tested in different clinical data sets of breast cancer patients. A large clinical follow-up study will be needed to definitively address this important question. If there is indeed a correlation between risk and expression of this ER- α mutation, genetic analysis for the mutation in premalignant lesions might help to identify patients who would benefit from preventive measures.

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