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Estrogen Receptor Variants in Epithelial Compartment of Normal Human Breast

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Estrogen receptor (ER) splice variants have previously been identified in normal human breast. Normal breast, however, comprises many cell types including fat cells, fibroblasts, endothelial cells, and a variety of inflammatory cells besides breast epithelial cells. The objective of our study was to demonstrate the existence of several variants in the epithelial compartment of the normal human breast. To this end, highly enriched breast epithelial cells from reduction mammoplasty specimens were isolated using Percoll gradient centrifugation. We analyzed the presence of both wild-type (WT) and variant receptors in human breast epithelial cells using the seminested polymerase chain reaction and direct automated sequencing of the amplified products. We demonstrated that a number of spliced variants of the ER coexist with the WT receptor. Variants lacking exons 2, 4, 5, and 7 are detected in the breast epithelial compartment of the normal human breast.

Key Words: Estrogen receptor; estrogen receptor variant; normal human breast; normal breast epithelial cells; estrogen receptor gene polymorphism.

Introduction

Estrogen is involved in both normal breast development and breast cancer pathogenesis. This effect is mediated via the estrogen receptor (ER), which is a transcriptional activator and exists in multiple isoforms. Human breast cancers have been shown to express several ER variants resulting from different splicing events *(1–3)*. Although a role of ER variants in human breast pathogenesis has been suggested, these variants have also been identified in normal human breast tissues *(4–6)*. In fact, both the wild-type (WT) and variants exist in normal and cancerous human breast tissues. As part of our ongoing project to analyze the regulation of ER in normal human breast epithelial cells, both in our primary cell culture *(7,8)* and nude mice systems *(9,10)*, we analyzed the presence of ER splice variants in dissociated human breast epithelial cells from reduction mammoplasty specimens. This approach differs from previous approaches that have utilized total RNA extracted from the entire breast tissues *(4,5)*. Normal human breast tissues comprise various cell types in varying proportions. Breast epithelial cells are embedded in a collagenous matrix that also contains other cell types including fibroblasts, endothelial cells, and inflammatory cells. Because there is limited information available on ER variants in the breast epithelial compartment of the normal human breast, we analyzed the presence of these variants in highly enriched human breast epithelial cells using the seminested polymerase chain reaction (PCR) and direct automated sequencing of the amplified products. The present study broadens our knowledge of the distribution of ER variants in the epithelial compartment of the normal human breast.

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Results

Cell dissociation of human breast tissues followed by separation using Percoll gradient centrifugation removes nonepithelial cell types including fibroblasts, endothelial cells, and blood cells, resulting in highly enriched clumps of human breast epithelial cells. When examined under phase contrast microscopy, ducts and lobules are evident in this preparation (Fig. 1). Total RNA was extracted from this preparation in order to analyze the existence of ER in the epithelial compartment of the human breast.

The structure of ER mRNA from normal human breast epithelial cells was analyzed using the primer sets that encompass the entire coding sequence. The PCR products obtained from these normal cells were compared with those obtained from the MCF-7 cell line. The PCR products from all five sets of primers were of the expected size, and they aligned exactly with the PCR product from MCF-7 (Fig. 2). With primer set 5 (U5/D5), an additional smaller band was seen in all specimens. Direct sequencing of this product demonstrated that the PCR product lacked exon 7, as discussed subsequently.

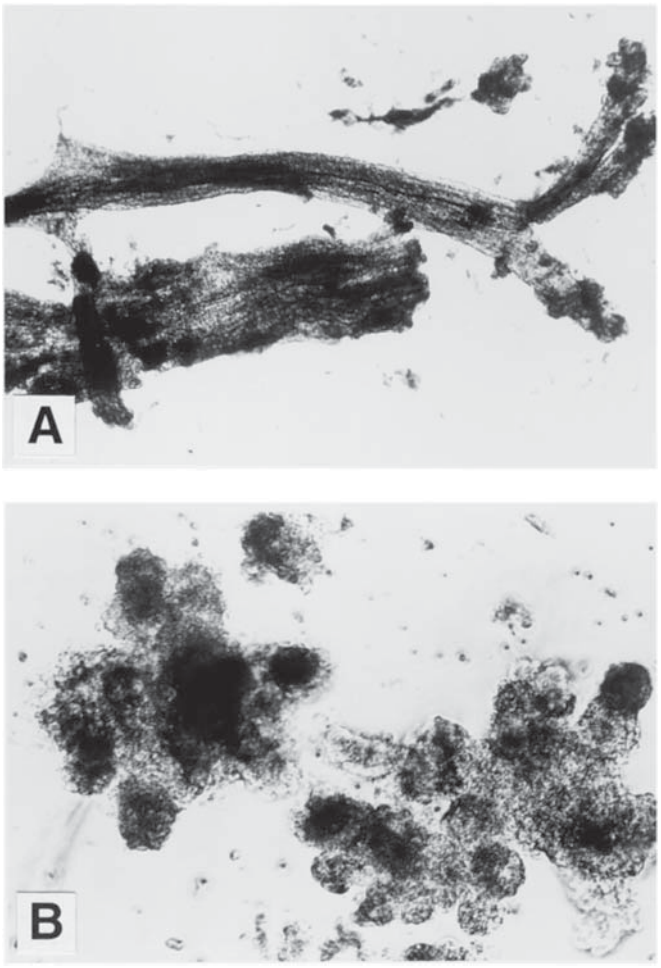


Fig. 1. **(A)** Ducts and **(B)** lobules seen in the final preparation after dissociation of human breast tissue from reduction mammoplasty specimen and enrichment of epithelial clumps by Percoll gradient centrifugation. (A):×10; (B):×40.

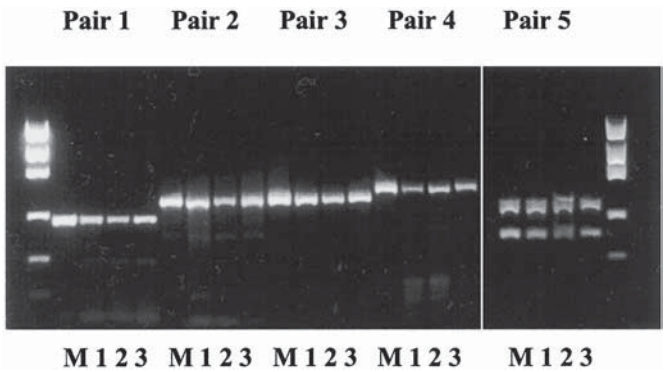


Fig. 2. PCR analyses of ER in normal human breast epithelial cells from three separate reduction mammoplasty specimens (1, 2, 3) and in MCF-7 (M).

To identify the existence of other variants that are in low abundance, seminested PCR was then performed. Figure 3 shows the approach taken to determine the presence of ER-spliced variants lacking any of the exons 1–8 that comprise the WT receptor transcript. Three separate seminested PCR combinations were used for this purpose. Primer combination 1 was used to determine the deletions in exons 1 and 2, primer combination 2 was used to determine the deletions in exons 3–6, and primer combination 3 was used to determine the deletions in exons 7 and 8.

Variants in Deletions in Exons 7 and 8

Using combination 3, two bands were detected: one of the expected size and the other smaller (Fig. 4A). Direct sequencing of the top band demonstrated that it was the WT receptor, whereas the sequence of the bottom band showed a complete deletion of exon 7 (Fig. 5).

Variants in Deletions in Exons 3–6

Using combination 2, several bands were seen: one of the expected size and the rest of which were smaller (Fig. 4B). Direct sequencing of one of the smaller bands demonstrated that it represented deletion of exon 4 (Fig. 5). There were variations among tissues in terms of the existence of these variants. It remains to be tested whether additional optimization of the reaction will elicit these variants in tissues that were negative under the standard condition utilized in this study.

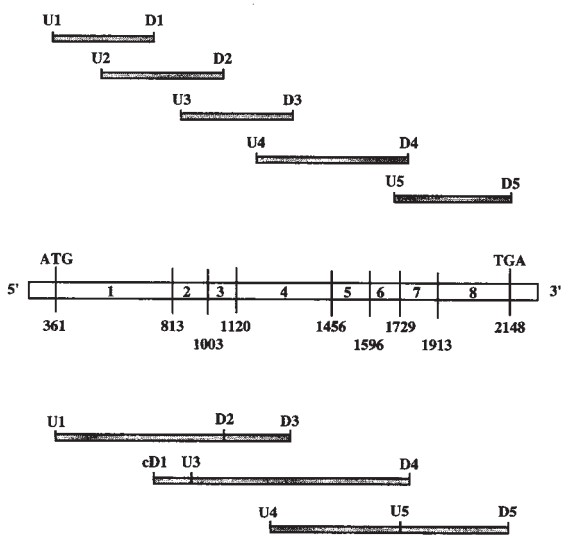
Variants in Deletions in Exons 1 and 2

Using combination 1, multiple bands were seen (Fig. 4C). One was of the expected size and the other two were smaller. Direct sequencing of the smallest band showed a precise deletion of exon 2. Another band located between the WT and exon 2–deleted variant was seen consistently. Based on its size, it could not represent a precise exon deletion but may represent a partial exon deletion.

A sequence variant in exon 8 was found in breast epithelial cells derived from five separate specimens (Fig. 6). There was a silent mutation in codon 594 (ACA to ACG [Thr]), which has been previously recognized as a polymorphic site *(1,2)*. Note that the published WT sequence of the ER cDNA from MCF-7 lists the less common allele ACA for codon 594 instead of ACG found in the majority of individuals *(1,2)*.

Discussion

Human breast cancers have been shown to express various ER variants resulting from different splicing events *(1–3)*. Although these variants have also been identified in normal human breast tissues *(4–6)*, there is great interest in the role of ER variants in human breast cancer pathogenesis. Altered expression of various ER variants in human breast cancer compared to normal human breast tissue has been reported *(2,3,11)*. There was an increased expression of the exon 5–deleted variant and decreased expression of the exon 3–deleted variant in human breast cancer compared to normal human breast *(11)*. However, both human breast cancer and normal human breast tissues comprise various cell types in varying proportions. Comparison of WT and



PRIMER COMBINATION	PRIMERS	POSITION	SEQUENCE 5' - 3'	ANALYZED EXONS
1	U1	351-370	GCCACGGACCATGACCATGA	1 & 2
	D2	1047-1028	AATGGTGCACTGGTTGGTGG	
	D3	1317-1298	CAAGGCACTGACCATCTGGT	
2	cD1	707-726	AGCTGTCGCCTTTCCTGCAG	3, 4, 5, 6
	U3	831-850	ACGCCAGGGTGGCAGAGAAA	
	D4	1756-1737	GGGTGCTGGACAGAAATGTG	
3	U4	1211-1230	GAGACATGAGAGCTGCCAAC	7 & 8
	U5	1684-1703	GAAGAGGAGTTTGTGTGCCT	
	D5	2167-2148	TGTGGGAGCCAGGGAGCTCT	

Fig. 3. Position and sequences of various primers to detect different exon deletion variants of ER mRNA in normal human breast epithelial cells.

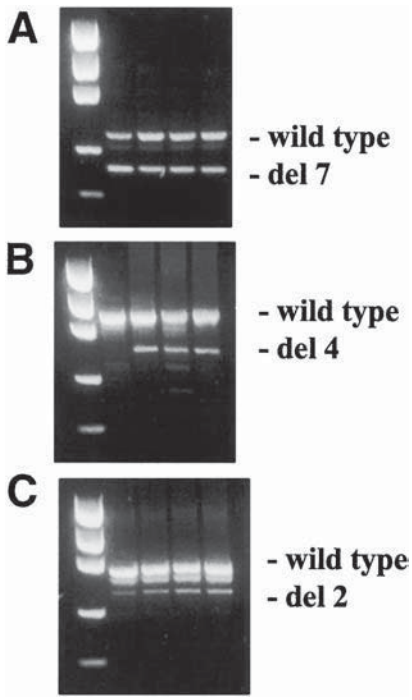


Fig. 4. Seminested PCR analyses of ER variants in normal human breast epithelial cells from four separate reduction mammoplasty specimens. **(A)** Presence of exon 7–deleted variant, **(B)** exon 4–deleted variant, and **(C)** exon 2–deleted variant.

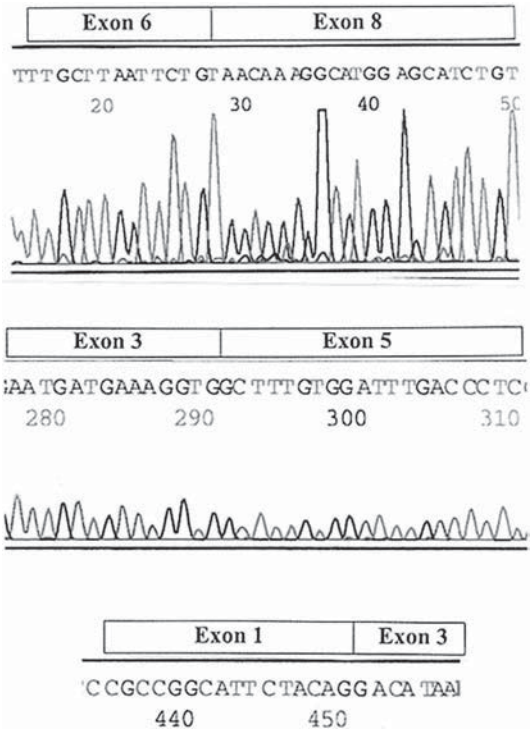


Fig. 5. Sequence analyses of ER variants with deletions of exons 2, 4, and 7. **(Top)** Sequence analysis of ER variant with deletion of exon 7, **(middle)** exon 4, and **(bottom)** exon 2.

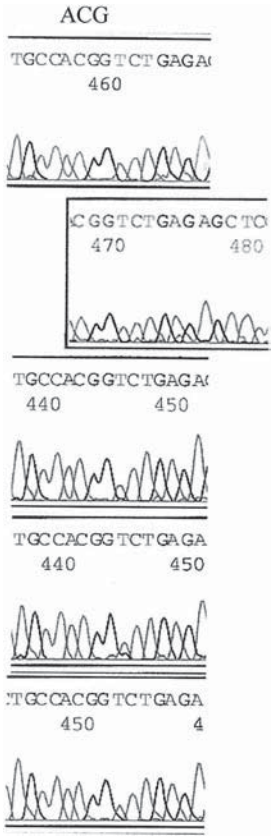


Fig. 6. Sequence analyses showing silent mutation in codon 594 (ACA to ACG [Thr]).

variant ER without regard for various cell types comprising the samples from which the total RNA were extracted may lead to erroneous conclusion. Previous studies on normal human breast tissues *(4,5)* have all utilized total RNA extracted from the entire breast tissues, which comprise other cell types besides the epithelial cells. In the present study, the breast epithelial cells were dissociated from reduction mammoplasty specimens and separated from other cell types before extracting the total RNA. We demonstrated that ER variants are expressed in human breast epithelial cells dissociated from reduction mammoplasty specimens. These variants, including ER with deletions of exons 2, 4, and 7, coexist with the WT receptors. Although our data represent a limited number of samples, they suggest that different ER splice variants, previously reported for human breast cancer and normal human tissues, are present in normal human breast epithelial cells dissociated from reduction mammoplasty specimens. These variants are therefore present in the epithelial compartment of the normal human breast. It remains to be seen whether the difference in the relative abundance of the WT to variant ER between breast cancer and normal breast tissue will still stand after normalizing for the epithelial content as well as after considering other factors such as age and hormonal environment.

The nonepithelial compartment from the same normal human breast tissue could not be isolated for analyses because it represents a heterogeneous mixture of different cell types including adipose cells, fibroblasts, myofibroblasts, endothelial cells, and various blood cells. However, other studies as well as our own *(12)* suggest widespread occurrence of both WT and variant receptors in many normal human tissues. This suggests that the mechanisms generating these variants exist in many normal human tissues and that these variants may have some role in the regulation of estrogen action via the ER as well as in tissue-specific action. Both tissue- and gender-specific expressions of ER variants have been reported *(2)*. A possible role of these variants in terms of modulating the action of estrogen has been reviewed *(3)*. The exon 5–deleted variant and the exon 7–deleted variant, both with deletion in domain E, which is the steroid binding domain, have been shown to compete with WT ER to inhibit ERE-dependent transactivation. Whether these variant mRNA are actually translated into proteins in the normal human breast is not known, but there is strong support for some variants to be translated into proteins in several tissues including the BT20 human breast cancer cell line and ovary. Therefore, it is possible that these variants may have a functional role in modulating the estrogen action in the normal human breast.

Future analyses of ER not only must take into account the variant expression relative to the WT receptor but also the various cell types comprising both breast cancer and normal breast tissue. This is especially true when comparing malignant and normal tissues as well as comparing different tissues and organs because all tissues and organs comprise various cell types in varying proportions. Additional consideration is an understanding of the factors that regulate the expression because the same cell under different hormonal and physiological states may express different amounts of the WT and variant receptors. Failure to account for various cell types as well as factors regulating expression may result in erroneous conclusions especially when using a sensitive PCR-based methodology. With these precautions, studies on both WT and variant ER in normal human breast are warranted in view of a possible relationship between the level of ER in normal cells and the risk of breast cancer *(16–18)*. It has been reported that the ER status in normal breast tissue may predict breast cancer risk presumably owing to enhanced estrogen responsiveness *(16,17)* and that the low ER expression in normal breast tissue may underlie the low breast cancer incidence in Japan *(18)*.

Materials and Methods

Tissue Sources

Human breast tissues consisting of reduction mammoplasty specimens from seven different patients were obtained from the Cooperative Human Tissue Network. Human breast epithelial cells dissociated by enzymatic dissociation of reduction mammoplasty specimens and enrichment using Percoll gradient have been previously utilized in both the primary cell culture system *(7,8)* and the in vivo system using nude mice *(9,10)*. A step-by-step procedure is described elsewhere *(12,13)*.

Reverse Transcriptase PCR

Total RNA was isolated from human breast epithelial cells using the SNAP Total RNA Isolation Kit (Invitrogen, Carlsbad, CA), and reverse transcription was performed using Thermoscript RT-PCR System (Life Technologies, Rockville, MD) following the manufacturer's protocol. Varying quantities of RNA were denatured at 65°C for 5 min with 10 pmol of gene-specific primers in a total volume adjusted to 10 μL with diethylpyrocarbonate (DEPC)-treated water. Nine microliters of reaction mix (containing 15 U of Thermoscript RT, 5X cDNA Synthesis Buffer, 0.1 *M* dithiothreitol, 10 m*M* dNTP Mix, 40 U of RNase Out, DEPC-treated water) was added to make a total volume of 19 μL. Reverse transcription was performed at 50°C for 50 min and terminated by incubating at 85°C for 5 min. Finally, any residual RNA was digested by treating with 1 μL of *Escherichia coli* RNase H (40 U) and incubating at 37°C for 20 min. Samples were stored at 4°C or used for PCR immediately.

PCR amplification was performed on cDNA preparations using the HotStarTaq Master Mix Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

PCR was carried out in a final volume of 50 μL comprising 25 μL of HotStarTaq Master Mix (2.5 U of HotStarTaq DNA Polymerase, 1X PCR Buffer, 200 μ*M* of each dNTP), 10 pmol of each primer, and 5 μL of cDNA preparation. Using different pairs of primers (Fig. 3), PCR reactions were carried out in a Perkin-Elmer 4800 Thermocycler. Reactions were activated at 95°C for 15 min and run for 38 cycles of denaturation (94°C, 30 s), annealing (58°C, 50 s), and extension (72°C , 1 min). The PCR products were subjected to electrophoresis on a 2.0% agarose gel, stained with Gelstar (FMC Bioproducts, Rockland, ME), and photographed.

ER mapping was performed on three reduction mammoplasty specimens (patient ages: 23, 25, and 33 yr) using primers previously reported *(14)*. They are reproduced here for convenience:

1. Primer U1(351-370): 5'-GCC ACG GAC CAT GAC CAT GA-3'.
2. Primer D1 (726-707): 5'-CTG CAG GAA AGG CGA CAG CT-3'.
3. Primer U2 (565-584): 5'-AAC GCG CAG GTC TAC GGT CA-3'.
4. Primer D2 (1047-1028): 5'-AAT GGT GCA CTG GTT GGT GG-3'.
5. Primer U3 (831-850): 5'-ACG CCA GGG TGG CAG AGA AA-3'.
6. Primer D3:(1317-1298) 5'-CAA GGC ACT GAC CAT CTG GT-3'.
7. Primer U4 (1211-1230): 5'-GAG ACA TGA GAG CTG CCA AC-3'.
8. Primer D4 (1756-1737): 5'-GGG TGC TGG ACA GAA ATG TG-3'.
9. Primer U5 (1684-1703): 5'-GAA GAG GAG TTT GTG TGC CT-3'.
10. Primer D5 (2167-2148): 5'-TGT GGG AGC CAG GGA GCT CT-3'.

Seminested PCR was performed on four separate reduction mammoplasty specimens (ages unknown). For seminested PCR, a second round of PCR was performed using the products from the first round. First-round amplification products were diluted by mixing 1 μL with 99 μL of sterile nuclease-free water. An internal primer (Fig. 3) in combination with one of the external primers from the first round was used for seminested amplification on 10 μL of the 1 : 100 dilution of first-round PCR product. The conditions for the second round amplification were the same as those for the first round amplification. All primers used in this study have previously been utilized except for primer cD1 (*see* Fig. 3), which is a complement of primer D1 in the original reference *(14)*.

Sequencing

The PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Direct sequencing of the PCR products was performed using BigDye Terminator Ready Reaction Mix (Perkin-Elmer) and Applied Biosystems 377 Sequencer (Perkin-Elmer) by the DNA Sequencing Facility of the University of California, Berkeley.

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