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Notes

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Estrogen Receptor Variants in Normal Human Mammary Tissue

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Background: **Several estrogen receptor (ER) variant messenger RNAs (mRNAs) have been identified previously in human breast cancer biopsy samples and cell lines. The relative levels of certain ER variant mRNAs have been observed to increase with breast tumor progression. In vitro assays of the function of polypeptides encoded by some of these variant mRNAs have led to speculation that ER variants may be involved in the progression from hormone dependence to independence in breast cancer. *Purpose:* We set out to establish if ER variant mRNAs are present in normal human breast tissues and, if so, to compare levels of these variants between normal and neoplastic human breast tissues. *Methods:* Four human breast tissue samples from reduction mammoplasties and five samples from tissue adjacent to breast tumors were analyzed. The tissue samples were confirmed to be normal (i.e., not malignant) by histopathologic analysis. RNA was extracted immediately from adjacent frozen sections. Human breast tumor specimens originally resected from 19 patients were acquired from a tumor bank and processed in the same way as the normal tissue samples. The RNAs were then reverse transcribed and subsequently amplified with the use of the polymerase chain reaction (PCR). PCR primer sets were designed to detect several different exon-deleted ER variants and a truncated ER variant (i.e., clone 4). A semiquantitative PCR-based method was used to determine the relative expression of exon 5- and exon 7-deleted variants to wild-type ER mRNAs in the nine normal breast tissues and in 19 ER-positive breast tumor tissues. The Mann–Whitney rank sum test (two-sided) was used to determine *P* values. *Results:* ER variant mRNAs corresponding to the clone 4 ER truncated variant and to variants deleted in either exon 2, exon 3, exons 2-3, exon 5, or exon 7 were detected in all normal samples. The results were confirmed by restriction enzyme analyses and sequencing of the PCR products. The expression of exon 5-deleted ER variant relative to the wild-type ER mRNA was significantly lower ($P<.001$) in normal tissue than in tumor tissue. A similar trend was noted for expression of the exon 7-deleted ER variant mRNA; however, the difference did not achieve statistical significance ($P = .476$). *Conclusion:* Several ER variant mRNAs are present in normal human breast tissue, but the level of expression of some of these variants may be lower in normal tissue than in tumor tissue. *Implication:* These data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further investigation of the role of variant ER expression in development and progression of human breast cancer appears warranted. [J Natl Cancer Inst 1996; 88:284-90]**

The estrogen receptor (ER), which belongs to the superfamily of steroid–thyroid–retinoic acid receptors (*1*), is an important regulator of growth and dif-

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See "Notes" section following "References."

ferentiation of the mammary gland. The receptor can be divided into several structural and functional domains (A-F) (*2*). In the absence of hormone, the receptor is thought to be associated with a protein complex, including heat-shock proteins such as hsp90 (*3*). On binding of ligand, the ER dissociates from this complex, dimerizes, and binds to specific sequences (estrogen receptor element or ERE) located in the 5′ flanking region of ER-responsive genes. Such interactions alter the transcription of estrogen-responsive genes. Region E of the receptor is implicated in ligand binding, dimerization, and *trans*-activating functions (TAF-2). The DNA-binding domain is located in the C region; another cell- and promoter-specific *trans*-activating function (TAF-1) lies within the A/B regions. Region D is involved in the nuclear localization of the receptor.

Several ER variant messenger RNAs (mRNAs) have been identified previously in human breast cancer biopsy samples and cell lines (*4-7*). While it is unclear if these mRNAs are translated in vivo, some of the predicted ER-like proteins, lacking some domains, exhibit altered functions or may interfere with wild-type (WT) ER function when recombinantly expressed. Exon 3- and exon 7-deleted variants were shown to act as dominant-negative regulators of WT ER (*8,9*). In contrast, exon 5-deleted ER has ligand independent *trans*-activating activity in a yeast-expression system (*10*). It has been shown that the relative levels of some of these ER variants were increased during tumor progression. Exon 5-deleted ER variant mRNA expression was found to be higher in ER-negative/progesterone receptor (PR)-positive tumors than in ER-positive/PR-positive tumors (*6,7*). Furthermore, exon 7-deleted variant mRNA levels were shown to be higher in ER-positive/PR-negative than in ER-positive/PR-positive tumors (*9*). Additional data from our laboratory suggest that elevated expression of another ER variant, called clone 4 truncated ER variant (*11*), is correlated with parameters of poor prognosis and endocrine insensitivity (*12*). It has thus been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer (*13*). However, the expression of ER variants in normal human breast tissue remains unknown. A recent study (*14*) shows the detection of several exon-deleted ER mRNAs in a single normal breast tissue sample. The investigators did not exclude the eventual development of breast cancer in the individual from whom the sample was derived. Moreover, no data were provided addressing relative expression of ER variants between normal and tumorous breast tissues. It was, therefore, important to establish definitively the existence of these ER variants in multiple normal breast tissue samples as well as to determine their relative level of expression between normal and tumorous breast tissues.

The aim of this study was to determine if multiple ER variant mRNAs can be detected in normal human breast tissue and, where possible, to compare the level of expression in normal tissues with that observed in tumor tissues.

Materials and Methods

Human Breast Tissues and Cell Lines

Normal breast tissues were obtained from reduction mammoplasty surgical specimens collected at the Necker Hospital, Paris, France (four case patients) and from normal tissues adjacent to tumors in mastectomy specimens obtained through the Manitoba Breast Tumor Bank, University of Manitoba, Winnipeg, Canada (five case patients). Human breast tumor specimens were also obtained from the Manitoba Breast Tumor Bank (19 case patients). In all case patients, the specimens had been rapidly frozen at –70 °C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case patient (normal and tumor) was processed to create formalin-fixed and paraffin-embedded tissue blocks that were matched and orientated relative to the frozen tissue. These blocks provided tissue for high-quality histologic sections for pathologic interpretation and assessment. The presence of normal ducts and lobules was confirmed in all normal tissue specimens as well as the absence of any atypical lesion. The 19 primary invasive ductal carcinomas that were selected from the Manitoba Breast Tumor Bank database were all associated with high ER levels (105-284 fmol/mg protein). Within this group, 10 tumors were PR positive, four were PR negative, and five were borderline positive (<15 fmol/mg protein) as determined by ligand-binding assay. Specific frozen tissue blocks were chosen in each case on the basis of additional criteria as assessed in histologic sections. These criteria included the following: a cellular content of more than 30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histologic preservation, and absence of necrosis. In all tumors, grading was performed with the use of the Nottingham grading system (*15*), and additional clinical and staging information (e.g., patient age, tumor size, and nodal status) was obtained from the Tumor Bank database. The age distribution of patients associated with the normal samples was similar to that of the tumor group (mean, 70.2 years of age; standard deviation, 13 years). For reduction mammoplasties, women were younger (mean, 20 years of age; standard deviation, 3 years).

Ishikawa cells, an endometrial adenocarcinoma cell line (*16*) initially established by H. Iwasaki (Tsukuba, Japan), were provided by E. Gurpide (Mount Sinai School of Medicine, New York, NY). The breast cancer cell line T-47D-5 (*17*) was provided by R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells are known to express different ER variant mRNAs (*17,18*; our unpublished data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, MD) do not express ER mRNAs and were used as a negative control in our experiments. Cells were grown and harvested, and cell pellets were stored at –70 °C as previously described (*19*).

Extraction of mRNA and Reverse Transcription

Total RNA was extracted from histologically defined regions within 20-μm frozen normal and tumor cryostat sections with the use of a small-scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) according to the manufacturer's instructions. The yield was quantitated by spectrophotometer in a 50-μL microcuvette. The average yield of total RNA per 20-μm section was 4 $\mu g/cm^2$ for tumor and 0.6 $\mu g/cm^2$ for normal tissues (± 20% variation with cellularity); the optical density ratios (260 nanometers/280 nanometers) of the RNA preparations were greater than 1.8.

Reverse transcription was performed in a final volume of 15 μL. RNA (600 ng) was reverse transcribed in the presence of 1 m*M* deoxyadenosine triphosphate (dATP), 1 m*M* deoxycytidine triphosphate (dCTP), 1 m*M* deoxyguanosine triphosphate (dGTP), 1 m*M* deoxythymidine triphosphate (dTTP), 5 m*M* dithiothreitol (Life Technologies, Inc., [GIBCO-BRL], Gaithersburg, MD), 1 U/μL ribonuclease inhibitor (Promega Corp., Madison, WI), 20 μ*M* random primers, 50 m*M* Tris–HCl (pH 7.5), 75 m*M* KCl, 3 m*M* $MgCl_2$, and 5 U/μL Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) for 10 minutes at 22 °C and 1 hour at 37 °C. After 5 minutes at 95 °C, 1 μL of the reaction mixture was taken for subsequent amplification with the use of the polymerase chain reaction (PCR).

Primers and PCR Conditions

Four sets of primers were used in this study (Fig. 1). The primer set that detected exon 5-deleted ER mRNA was called the D5 set and consisted of D5U primer (5′-CAGGGGTGAAGTGGGGTCTGCTG-3′; sense; located in WT ER exon 4; 1060-1082) and D5L primer (5′-ATGCGGAACCGAGATGATGT-AGC-3′; antisense; located in WT ER exon 6; 1520-1542). This primer set allows amplification of 483-base-pair (bp) and 344-bp fragments corresponding to WT ER and exon 5-deleted ER variant(s), respectively. The primer set designed to

detect exon 7-deleted ER mRNA was called the D7 primer set and consisted of D7U primer (5′-TCCTGATGATTGGTCTCGTCTGG-3′; sense; located in WT ER exon 5; 1389-1411) and D7L primer (5′-CAGGGATTATCTGAACCGTGTGG-3′; antisense; located in WT ER exon 8; 2035-2057). This primer set allows amplification of 668-bp, 534-bp, 484-bp, and 350-bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted, and exon 6-7-deleted ER variants, respectively. The primer set that detects exon 2-, exon 3-, and exon 2-3-deleted ER mRNAs was called D2/3 primer set and consisted of D2/3U primer (5′-TGCCCTACTAC-CTGGAGAA-3′; sense; located in WT ER exon 1; 615-633) and D2/3L primer (5′-TGTTCTTCT-TAGAGCGTTTGA-3′; antisense; located in WT ER exon 4; 1125-1145). This primer set allows amplification of 531-bp, 414-bp, 340-bp, and 222-bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted, and exon 2-3-deleted ER variants, respectively. Primers designed to specifically detect clone 4 truncated ER mRNA consisted of clone 4 U primer (5′-TGCCCTACTAC-CTGGAGAA-3′; sense; located in WT ER exon 1; 623-641) and clone 4 L primer (5′-GGCTCTGTTC-TGTTCCATT-3′; antisense; 941-959). This set allows amplification of a 337-bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER complementary DNA (cDNA) (*20*) and clone 4 cDNA (*11*).

PCR amplifications were performed with the use of 1 μL of reverse transcription mixture in a final volume of 50 μL, in the presence of 20 m*M* Tris–HCl (pH 8.4), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* dATP, 0.2 m*M* dTTP, 0.2 m*M* dGTP, 0.2 m*M* dCTP, 4 ng/μL of each primer, and 1 U of *Taq* DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 40 cycles (1 minute at 60 °C, 30 seconds at 72 °C, and 30 seconds at 94 °C) on a Thermocycler (The Perkin-Elmer Corp., Foster City, CA). PCR products were then separated on 2% agarose gels before staining with ethidium bromide (15 μg/mL).

Identification of PCR Products

PCR products were identified by restriction enzyme analysis and sequencing. Bands corresponding to the clone 4 and exon 7-deleted ER variants were excised from gels after staining with ethidium bromide. The corresponding DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with 70% ethanol in the presence of 100 m*M* CH_3COONa. PCR-amplified DNA product corresponding to clone 4 was digested for 18 hours at 37 °C with *Taq*I alone or *Taq*I plus *Kpn*I (5 U each per microgram of DNA). The PCR product corresponding to exon 7-deleted ER variant was digested under similar conditions with *Pst*I. Digestion products were separated on 2% agarose gels and their sizes were determined by reference to size markers (ΦX174 RF DNA/Hae III fragments; Life Technologies, Inc.). In parallel, approximately 50 ng of DNA was sequenced using clone 4 and D7 primer sets and dsDNA cycle sequencing system (Life Technologies, Inc.) according to the manufacturer's instructions.

For exon 5-, exon 2-, exon 3-, and exon 2-3-deleted ER variants, the PCR products were labeled with [α-^{32}P]dCTP (*see* below). One microliter of PCR product corresponding to an exon 5-deleted ER variant was digested with *Hin*dIII as described above. Similarly, PCR products corresponding to exon 2-, exon 3-, and exon 2-3-deleted ER variants were digested with *Taq*I alone or *Taq*I plus *Hha*I. Digestion products were separated on 6% polyacrylamide gels containing 7 *M* urea (PAGE). After electrophoresis, the gels were dried and exposed to Kodak XAR film at −70 °C with an intensifying screen, and the size of digestion products was determined by reference to size markers. In parallel, slices of gel corresponding to each labeled PCR product were excised from the dried gel and rehydrated overnight in 100 μL of sterile water. For each sample, five different PCR reactions were performed as described above with the use of 1 μL of this solution previously boiled for 10 minutes. PCR products corresponding to each set of five reactions were pooled, purified (Wizard PCR preps kit, Promega Corp.), cloned with the use of an Invitrogen TA Cloning kit, and sequenced as previously described (*11*).

Labeling of PCR Products

To label PCR products, a standard PCR reaction was performed in 10 μL supplemented with 10 n*M* [α-^{32}P]dCTP (ICN Pharmaceuticals, Inc., Irvine, CA). A 2-μL aliquot of the reaction was denaturated in 80% formamide buffer and subjected to PAGE. After electrophoresis, the gels were dried and autoradiographed for 6-18 hours.

Quantification and Statistical Analysis

The method used to quantitate exon-deleted variant mRNAs relative to WT ER mRNA is a modification of a method described by Daffada et al. (*21,22*). They showed that coamplification of WT ER and exon 5-deleted variant generates two DNA fragments whose ratio was constant with varying cycle numbers. This assay provides a semiquantitative reverse transcribed (RT)-PCR, whose internal control is the WT ER mRNA coamplified and in which relative expression of variant mRNA can be determined for individual samples. In our study, quantification of signals was carried out after excision of the band corresponding to variant and WT mRNA, followed by the addition of 5 mL scintillant (ICN Pharmaceuticals, Inc.), and counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The exon-deleted signal was expressed as a percentage of the WT ER signal. Preliminary studies showed that for each sample, the ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal remained constant (± 20%) and independent of the number of PCR cycles or initial cDNA input quantities. It should be noted that the percentage obtained reflects the relative ratio of variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels.

For each sample, at least three independent assays were performed and the mean determined. The ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal measured during these assays never varied by more than 20%. The statistical significance of differences in the relative levels of expression of exon 5- and exon 7-deleted ER mRNAs in normal breast versus breast tumor tissue was determined with the use of the Mann–Whitney rank sum test (two-sided).

Results

Detection of ER Variants in Normal Breast Tissues

Total RNA from nine normal breast tissue specimens from nine different women was analyzed by reverse transcription-PCR with the use of the oligonucleotide primer pairs described in the "Materials and Methods" section and shown in Fig. 1. Primers were designed to allow the detection of different ER variants previously observed in breast cancer tissues or cell lines: exon 3-deleted (*8*), exon 5-deleted (*6*), and exon 7-deleted (*9*) ER variants and clone 4 ER truncated variant (*11*). These variants were detected with the use of D2/3, D5, D7, and clone 4 primer sets, respectively.

In the first series of experiments, PCR products were stained with ethidium bromide after separation on 2% agarose gel (Fig. 2). With the use of the D7 primer set (Fig. 2, A), two bands that corresponded in size to WT ER (668 bp) and to exon 7-deleted ER variant (Del 7, 484 bp) were obtained. These bands comigrated with those observed in the positive controls: T-47D-5 breast and Ishikawa uterine cancer cell lines. To confirm the identity of exon 7-deleted ER variant, the lower band was purified and digested with different restriction enzymes (data not shown). Nucleotide sequence obtained by cycle sequencing revealed a perfect boundary between exon 6 and exon 8 ER WT sequences (data not shown). These data definitively confirmed the identity of the exon 7-deleted ER PCR product obtained. With the use of the clone 4 primer set, a band migrating with the expected size of 337 bp was obtained (Fig. 2, B). Identity of this band was confirmed by enzymatic digestion and cycle sequencing (data not shown).

With the use of the D2/3 or D5 primer sets followed by ethidium bromide staining, no exon 3- and exon 5-deleted ER variant mRNAs were detected in normal tissues (data not shown). A more sensitive technique consisting of incorporation of labeled nucleotide into the PCR reaction followed by separation of PCR

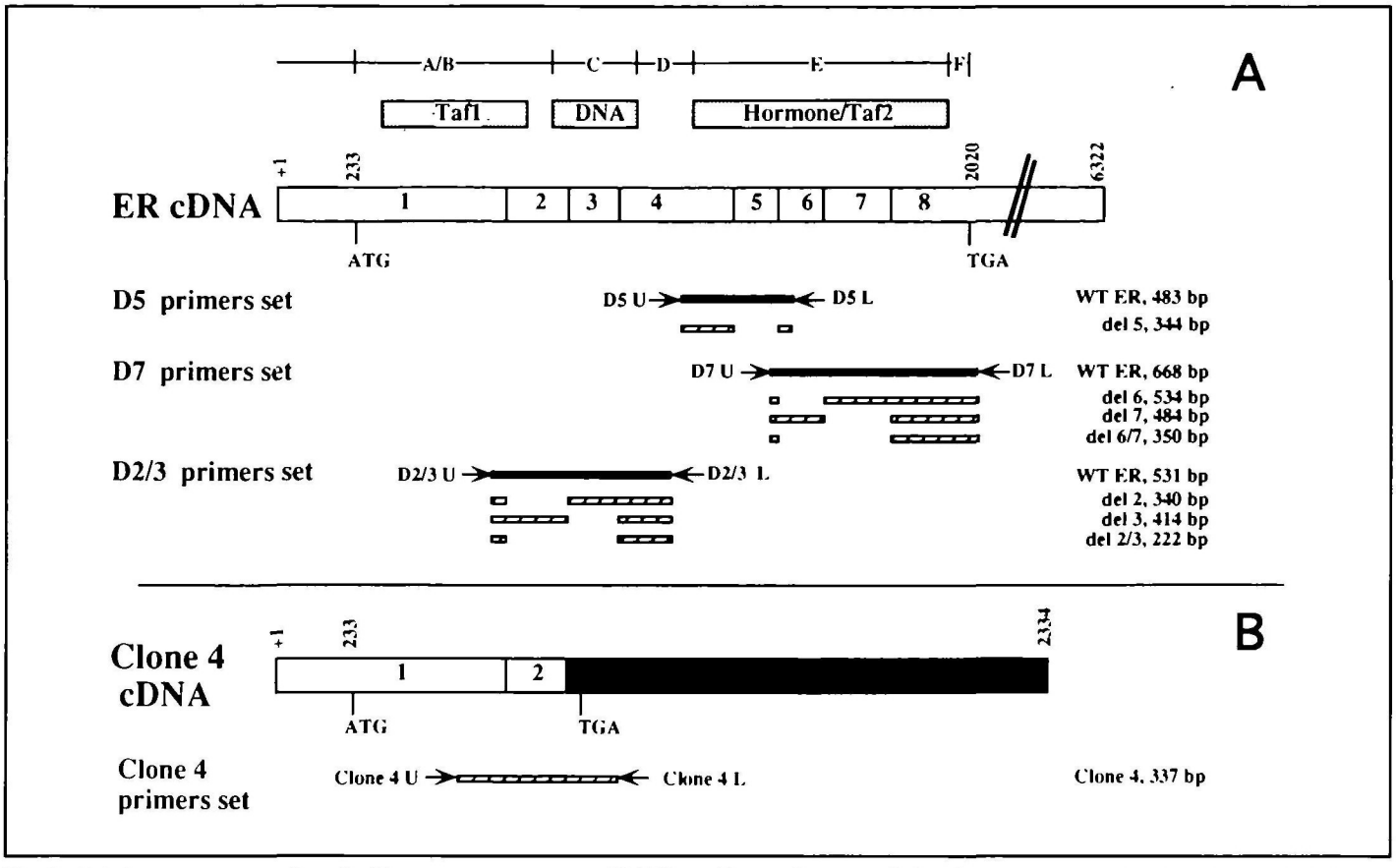

Fig. 1. A) Schematic representation of wild-type estrogen receptor (WT ER) complementary DNA (cDNA) and primers used to detect exon-deleted ER variants: ER cDNA contains eight different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in *trans*-activating function (TAF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another *trans*-activating function (TAF-2). D5 primer set allows amplification of 483-base-pair (bp) and 344-bp fragments corresponding to WT ER and exon 5-deleted ER variants, respectively. D7 primer set allows amplification of 668-bp, 534-bp, 484-bp, and 350-bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted, and exon 6-7-deleted ER variants, respectively. D2/3 primer set allows amplification of 531-bp, 414-bp, 340-bp, and 222-bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted, and exon 2-3-deleted ER variants, respectively. B) Schematic representation of clone 4 ER variant cDNA and primers used to detect this variant: clone 4 cDNA contains the first two exons of ER cDNA followed by sequences with similarity to line-1 sequences (*17*). Clone 4 primer set allows amplification of 337-bp fragment corresponding specifically to clone 4-truncated ER variant mRNA.

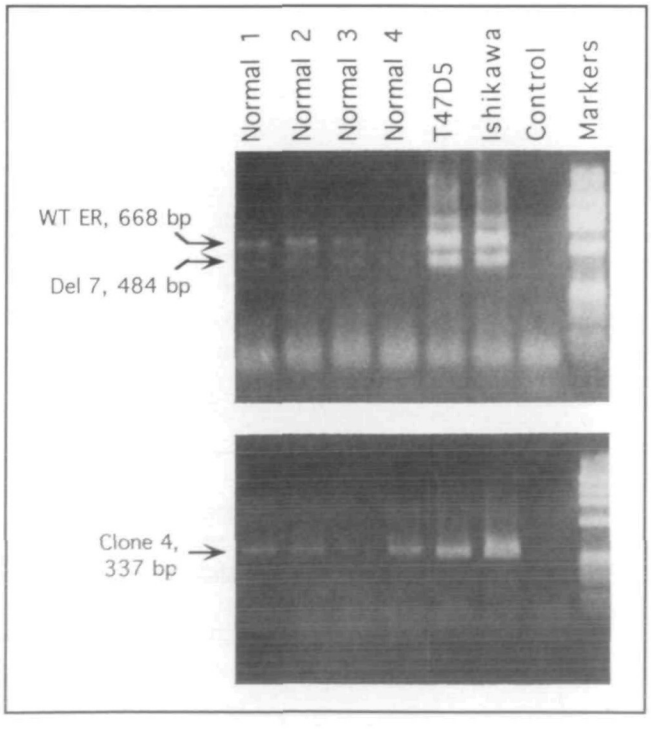

Fig. 2. Detection of exon 7-deleted and clone 4 ER variants in normal human breast: total RNA from normal human breast tissue samples (normal, 1-4), T-47D-5, and Ishikawa cancer cell lines, was reverse transcribed and polymerase chain reaction (PCR) amplified as described in the "Materials and Methods" section using the D7 primer set (A) and clone 4 primer set (B). PCR products were separated on 2% agarose gel before staining with ethidium bromide.

products on 6% denaturating polyacrylamide was subsequently used.

This technique, together with the D5 primer set, detected two bands corresponding in size to the WT ER mRNA (483 bp) and exon 5-deleted variant (344 bp) in all normal breast tissue samples (Fig. 3, A). Identity of the PCR products was confirmed following restriction enzyme digestion and sequencing (data not shown).

The D2/3 primer set and labeled PCR reactions resulted in the detection of four different PCR products in normal breast tissue samples. These products corresponded in size to WT ER (531 bp), exon 3-deleted (414 bp), exon 2-deleted (340 bp), and exon 2-3-deleted (222 bp) ER variant mRNAs (Fig. 3, C). Identity of these bands was confirmed with restriction enzyme digestion analysis and sequencing (data not shown).

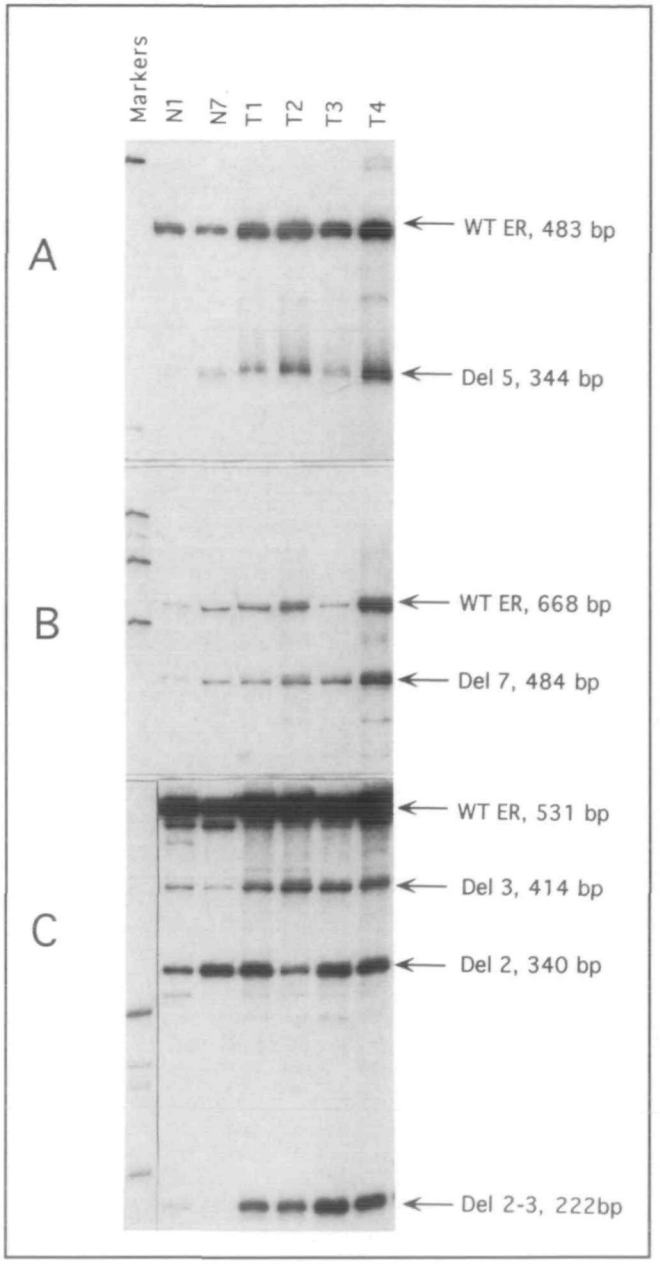

Fig. 3. Comparison of exon-deleted ER variants expression between normal and tumor breast tissues: total RNA from normal human breast tissue samples (N1-N2) and from breast tumors (T1-T4) was reverse transcribed and labeled polymerase chain reaction (PCR) amplification was performed as described in the "Materials and Methods" section with the use of the D5 primer set (A), D7 primer set (B), and D2/3 primer set (C). PCR products were separated on 6% polyacrylamide gel containing 7 *M* urea. After electrophoresis, the gel was dried and autoradiographed for 6-18 hours.

Comparison of Exon-Deleted ER Variant Expression in Normal and Tumor Tissues

The relative level of exon-deleted variant mRNA expression was compared in nine normal breast tissues and in 19 ER-positive breast cancer tissues. Expression relative to the WT ER mRNA was measured in each sample by incorporating a labeled nucleotide in the PCR products, which were then separated by PAGE (Fig. 3). Our preliminary studies confirmed the previous observation that amplification of WT and deleted variant transcripts occur with similar efficiency (*21,22*); therefore, the assay could be used to determine the relative levels of variant mRNA in individual samples. For exon 5- and exon 7-deleted variants, it was possible to express the signal measured as a percentage of the signal provided by the WT ER mRNA.

Because there was a substantial age difference between the patients who had reduction mammoplasty and the patients with breast cancer from whom normal breast tissue was taken, it was important to determine if the exon 5- and exon 7-deleted ER variant expression relative to WT ER in the normal breast tissues was identical irrespective of origin. No statistically significant difference was observed between these two subgroups of patients (data not shown).

The level of exon 5-deleted variant mRNA relative to the WT ER mRNA was found to be significantly less ($P<.001$) in normal (median, 21%) than in neoplastic breast cancer (median, 35%) tissues (Fig. 4).

Although a similar trend was observed for the exon 7-deleted variant between normal (median, 88%) and breast cancer (median, 107%) tissues, the difference failed to reach statistical significance ($P = .476$) (Fig. 4).

While expression of exon 2-, exon 3-, and exon 2-3-deleted variants was reproducibly observed in normal tissues, their relative expression changed from experiment to experiment, suggesting that the efficiency of reverse transcription-PCR varied when determination of relative expression of three different transcripts was attempted simultaneously. Fig. 3, C shows an experiment performed where the exon 2-3-deleted variant was not detected in normal sample N7. This could be explained by similarly low equivalent transcript levels of these variant mRNAs in normal tissues—the amplification occurring randomly on one variant rather than another. It was, therefore, not possible to quantify relative expression of these variants in normal tissues compared with that seen in tumor tissues.

Discussion

With the use of reverse transcription-PCR, it was possible to observe five different exon-deleted ER variant mRNAs and one truncated ER variant mRNA in each normal tissue studied. During the preparation of this manuscript, a paper describing the detection of exon 2-, exon 3-, and exon 7-deleted variant ER mRNAs in one normal human breast tissue sample was published (*14*). Our data confirm these observations and add new information concerning the expression of these variants in multiple normal human breast tissue samples. Furthermore, we have detected the expression of an exon 5-deleted ER transcript in multiple normal human tissue samples, and we have identified a previously unknown deletion variant (exon 2-3 deleted) in normal and breast cancer tissues. These two variants were not observed in the study by Pfeffer et al. (*14*). Our ability to detect these variants in normal tissues is probably due

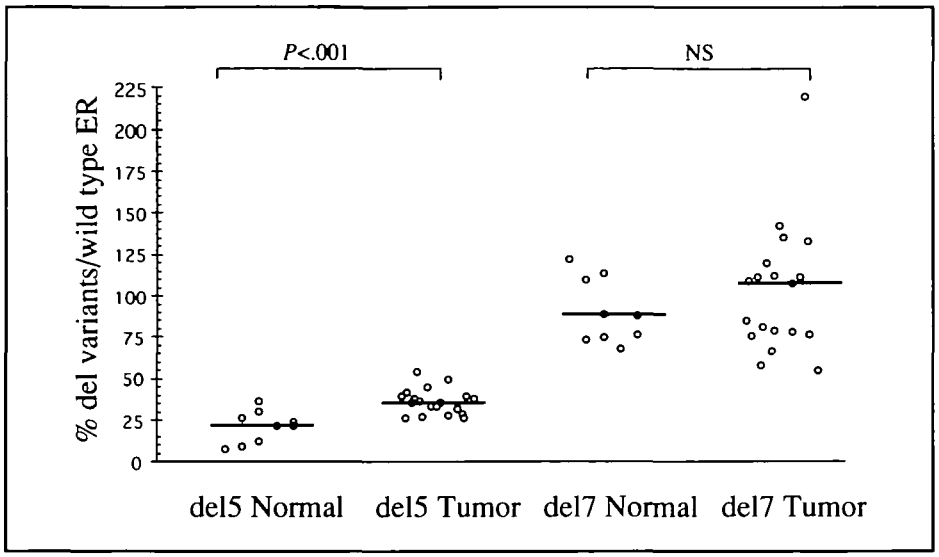

Fig. 4. Comparison of exon 5- (del5) and exon 7-deleted (del7) ER variant expression between normal and tumor breast tissues: total RNA from nine normal human breast tissue samples and from 19 human breast tumors was analyzed using the D5 primer set and D7 primer set as described in Fig. 3. Quantification of signals was carried out after excision of the band corresponding to variant and wild-type (WT) messenger RNA (mRNA), as described in the "Materials and Methods" section. The exon-deleted signal was expressed as a percentage of the WT ER signal. For each sample, at least three independent assays were performed and the mean was determined (circles). Differences in exon 5- and exon 7-deleted ER relative expression were then compared using the Mann–Whitney rank sum test (two-sided). Bar = median of each group. NS = nonsignificant.

to our use of a highly sensitive technique. The detection of ER variants in each of nine different normal tissue samples strongly suggests that the mechanisms generating these variants already exist in normal breast tissue. The primer sets we have used could potentially detect exon 6- and exons 6-7-deleted ER variants, but these were not observed. This suggests that the mechanisms used to generate deletion and truncated ER variants display some specificity and that generation of ER variants may have some role in normal ER regulation and/or function.

Many of these variants have been suggested to be involved in progression from estrogen dependence to independence in breast cancer (*7,23-25*). However, acquisition of hormone independence often occurs late in tumorigenesis; therefore, it was of interest to compare the expression of these ER variants between normal and cancer tissues with characteristics of good prognostic (i.e., ER positive) to gain further insight into their function and possible involvement in early tumorigenesis.

To have a representative group of ER-positive breast tumors, selection was made so that approximately one half of the speciments were ER positive/PR positive and the other half were ER positive/PR negative. While no significant difference in exon 5-deleted ER variant expression was observed between the two groups, the tumor group as a whole had significantly higher levels of exon 5-deleted variant expression relative to WT transcript than in normal breast tissues. Similarly, with the use of a new PCR-based quantitative method, we have recently demonstrated that clone 4 ER variant expression was increased in breast tumors compared with normal breast tissue (Leygue ER, Murphy LC, Watson PH: data submitted for publication). Taken together, these data suggest that the exon 5-deleted variant as well as clone 4 ER variant may have some role in early steps of tumorigenesis.

The absence of statistically significant differences between normal and breast tumor tissues with respect to the exon 7-deleted variant expression suggests that this variant may have a role in normal breast tissue that is not altered because of tumorigenesis. A similar finding, that altered expression of exon 5-deleted ER variant mRNA may occur in association with tumor progression while the same changes do not necessarily occur in the exon 7-deleted variant, has also been found in breast cancer cell lines that have developed tamoxifen resistance in vitro (*26*). This absence of difference may also be due to the small number of samples studied. Additional studies of larger numbers of normal breast and breast tumor tissue may be useful in clarifying this issue.

In conclusion, we have demonstrated that a range of ER variant mRNAs can be consistently detected in multiple independent samples of normal human breast tissues. Furthermore, by comparison between normal and neoplastic tissues, we have shown that the relative level of expression of specific variants in normal tissue can be altered and higher in tumor tissues. These data suggest that the mechanism(s) generating ER variant mRNAs already exist(s) in normal breast tissue and may be deregulated in breast cancer tissues; it is our speculation that such deregulation may contribute to progression in human breast cancer.

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Notes

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