[CANCER RESEARCH 60, 530–533, February 1, 2000]

Advances in Brief

Differences in Estrogen Receptor α Variant Messenger RNAs between Normal Human Breast Tissue and Primary Breast Carcinomas[1]

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Abstract

We evaluated the differences in prevalence and functional activity of human estrogen receptor α (hER) variant mRNA between 21 normal breast tissues and 41 primary breast carcinomas using a functional assay in yeast for the hER. First, we found that the presence of wild-type hER, relative to the total amount of hER, differs markedly ($P < 0.0001$) between normal breast tissue (median, 85% wild-type hER) and breast tumors (median, 74% wild-type hER). Second, the hER variants with altered function that are present in normal breast tissue are mainly one-exon deleted splicing variants (median, 100%), whereas in breast tumors only half of all variants lack just one single exon (median, 50%; $P < 0.0001$). Our results suggest that hER-dependent estrogen responsiveness of breast tissue may change during tumor outgrowth, indicating that specific hER variants may play a role in breast cancer development or progression.

Introduction

Human estrogen receptor α (hER)[3] is a hormone-activated nuclear transcription factor that is an important regulator of growth and differentiation in estrogen-responsive cells (1–3). In the absence of estrogen, hER is thought to be associated in the cytoplasm with heat shock proteins such as HSP90 (2). Upon estrogen binding, hER dissociates from this complex and binds in the nucleus to a specific DNA sequence, the estrogen response element, located in the promoter region of target genes (1, 2, 4). By binding this estrogen response element, the receptor activates transcription of target genes in a hormone-dependent way. Tamoxifen, an antiestrogen widely used for the treatment of breast cancer, binds to hER in a manner similar to that of estrogen. However, unlike estrogen binding, tamoxifen binding represses the transcriptional activation of most of the target genes by hER, thereby inhibiting the growth of breast tumor cells. Clinical studies have shown that for postmenopausal patients with hER-positive breast tumors, adjuvant tamoxifen treatment is associated with significant improvement in both recurrence rate and overall survival (reviewed in Ref. 5). However, a major problem in treatment of these patients with tamoxifen is that ~40% of all patients with immunohistochemical hER-positive breast tumors are nonresponsive to tamoxifen, as was shown in patients with metastatic breast cancer (reviewed in Ref. 6).

Many hER splicing (related) variants have been identified in breast cancer specimens and breast cancer cell lines, whereas point mutations in hER are rare (reviewed in Ref. 7). The majority of these splicing variants lack one or more exons from the hER mRNA and have aberrant functional activity because they are either dominant negative, dominant active, or nonfunctional. Although their contribution to breast cancer development and, in particular, tamoxifen resistance is likely, their full significance in this respect is not yet clear because reports on the presence of hER variants in normal breast tissue are incomplete (8–12). These studies that have reported hER variants in normal tissue involved either only one specimen (8, 9) or a pool of normal breast tissue specimens (10) or used a method by which the presence of all hER variants among wild-type hER mRNA within one tissue specimen could not be evaluated (11, 12).

In this study we report the results of the hER-FASAY assay (13) that we used for analysis of the prevalence and functional activity of all variant receptors among wild-type hER in 21 normal human breast tissue specimens and 41 primary breast carcinomas. Our results show that hER variants are indeed prevalent in normal breast tissue but at a lower frequency and with a different molecular structure than in breast cancer.

Materials and Methods

Tissue Specimens. Normal breast tissue specimens were either normal-CBR or normal-TCB. Normal-CBR [14 specimens; mean age at time of surgery was 35 years (SD, 11 years)] were collected at the Academic Hospital of the University of Maastricht (Maastricht, the Netherlands). Normal-TCB [7 specimens; mean age at time of surgery was 57 years (SD, 14 years)] were collected at the Netherlands Cancer Institute (Amsterdam, the Netherlands). Normal-TCB tissue originated from the quadrant ipsilateral to the tumor. Primary breast carcinoma specimens [41 specimens; mean age at time of surgery was 64 years (SD, 6 years)] were obtained from the Netherlands Cancer Institute Tissue Bank. All specimens were placed in liquid nitrogen directly after surgery and stored at −70°C.

Histopathological Reviewing of Tissue Sections. Histopathological reviewing of all tissue specimens was performed on H&E-stained cryostat sections. H&E sections were reviewed by a pathologist and all normal breast tissue specimens were confirmed to be normal (*i.e.,* nonmalignant and containing normal ducts and lobules) and all primary breast carcinoma specimens were confirmed to be invasive carcinomas. The mean percentage of tumor cells in the breast cancer specimens was 89% (SD, 18%; as a percentage of all hER-expressing cells).

mRNA Isolation. mRNA was isolated from 5-μm cryostat sections (five sections per normal tissue specimen; three sections per breast tumor specimen) using Dynabeads oligo-d(T)$_{25}$ (Dynal, Oslo, Norway). The cryostat sections used for mRNA isolation originated from tissue consecutive to that used for histopathological analysis.

Reverse Transcription-PCR Amplification and hER-FASAY. Reverse transcription-PCR and hER-FASAY were performed as we have described (13) except for the use of a different sense primer, 5′-CCCGCCGGTTTCT-GAGCCTTCTGCCC (bp 227–252, with bp counting as in Ref. 14). Two independent hER-FASAY experiments were performed per tissue specimen (both starting from the same mRNA).

Received 10/18/99; accepted 12/10/99.

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[1] Supported by Grant NKB NKI 96-1268 from the Dutch Cancer Society.

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[3] The abbreviations used are: hER, human estrogen receptor α; hER-FASAY, functional analysis of separated alleles in yeast of hER; normal-CBR, normal breast tissue from cosmetic breast reduction; normal-TCB, normal breast tissue from tumor-containing breast; AF-2, transcription activation function 2; DB, DNA binding; DIG, digoxigenin; Δ22bpE7, mRNA splicing variant lacking the first 22 bp of exon 7; ΔE, mRNA splicing variant lacking the indicated exon; Δ31bpE1, mRNA splicing variant lacking the last 31 bp of exon 1; rt, room temperature.

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Automated Sequencing of hER Variants. hER cDNA from all yeast colonies showing a negative (DB) or dominant active (AF-2) phenotype in the hER-FASAYs of primary breast tumor specimens were analyzed by automated sequencing. hER plasmid isolation from yeast and DNA sequencing analysis was performed as described before (13). From each tumor specimen, all yeast colonies with AF-2 phenotype and a maximum of 10 colonies with DB phenotype were analyzed.

DIG Dot-Spot Analysis of hER Splicing Variants. hER cDNA from all yeast colonies showing an aberrant phenotype in the hER-FASAYs of normal-CBR and normal-TCB specimens was analyzed for exon deletions using DIG dot-spot analysis. With this assay, the hER variants were analyzed for deletion of exons 2, 3, 4, 5, or 7 using digoxigenin (DIG)-labeled probes. A probe for detection of exon 6 was not made because deletion of exon 6 in hER mRNA from breast carcinomas has never been found (DNA sequencing data in this paper and in Ref. 15). Specific probes for detection of each exon were synthesized by PCR (primer sequences available upon request) using 10 ng pMD11 containing full length hER cDNA, 10 pmol of each primer, and the PCR DIG probe synthesis mix (all DIG reagents were obtained from Boehringer Mannheim, Germany). Fragments were amplified and labeled in 20 cycles of 20 s at 92°C, 1 min at 60°C (probes exons 2, 3, and 4) or 55°C (probes exons 5 and 7), and 2 min at 72°C and then 7 min at 72°C and a cooling down to 15°C in a PTC-200 PCR apparatus (MJ Research, Waltham, MA). Probes vary in length from 70 to 110 bp.

hER cDNA present in the hER-FASAY yeast colonies was amplified by PCR using two hER-specific primers located in exons 1 and 8 [5′-CGGTCA-GACCGGCCTCCCCTAC, bp 511–532 (bp counting as in Ref. 14) and 5′-ATGAGGGTAAAATGCAGCAG, bp 2115–2134]. PCR was performed in a total volume of 100 μl containing 10 mM Tris-HCl (pH 8.8), 25 mM KCl, 3.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Pharmacia Biotech, Inc., Uppsala, Sweden), 50 pmol of each primer, 3% DMSO, 8.3 units *Taq* polymerase (Life Technologies, Inc.), and 0.83 units *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA). A matchhead of yeast and 3 μl Triton X-100 were added to the PCR mixture. The mixture was preheated for 10 min at 95°C and amplified in 40 cycles of 30 s at 92°C, 45 s at 64°C, and 3 min at 72°C and then 7 min at 72°C and a cooling down to 15°C in a PTC-200 PCR apparatus (MJ Research). PCR products were heated for 5 min at 100°C and 1 μl of each PCR product was spotted on five separate N^+ nylon membranes (Boehringer Mannheim). Membranes were dried and DNA was cross-linked to filters by exposure to UV light for 90 s. Filters were prehybridized in DIG Easy Hyb (30 min), hybridized in DIG Easy Hyb containing a DIG-labeled probe (2 h at 42°C), washed twice with 2 × SSC + 0.1% [5 min at rt] and twice with 0.5 × SSC + 0.1% SDS (15 min at 68°C), blocked with 1× blocking reagent (30 min at rt), hybridized with anti-DIG 1:10,000 in 1 × blocking reagent (30 min at rt), washed twice with 0.3% Tween 20, 0.1 M maleic acid, 0.15 M NaCl (pH 7.5; 15 min at rt), and soaked in detection reagent (2 min at rt). Finally, filters were soaked in CDP-star and diluted 1:100 in detection reagent. Hyperfilms (Amersham Corp., Arlington Heights, IL) were exposed to the filters for 10–20 min.

Analysis of hER Δ22bpE7 by PCR. Deletion of the first 22 bp of exon 7 of hER (Δ22bpE7) was determined by a triple-primer PCR, for which two primers were located 5′ and 3′ of the deletion and one primer was located within the location of the deletion (primer sequences available upon request). PCR was performed with 1 μl of the same PCR product that was used for DIG dot-spot analysis in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 25 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Pharmacia Biotech, Inc.), 50 pmol of each primer, 3% DMSO, and 5 units *Taq* polymerase (Life Technologies). DNA was amplified in 35 cycles of 20 s at 92°C, 1 min at 56°C, and 2 min at 72°C and then 7 min at 72°C and a cooling down to 15°C in a PTC-200 PCR apparatus (MJ Research).

Results

Breast Tissue Specimens Evaluated in This Study. Twenty-one normal breast tissue specimens and 41 primary breast carcinomas were analyzed for the prevalence and functional activity of hER mRNA variants. Fourteen specimens of normal breast tissue were obtained from cosmetic breast reduction surgery (normal-CBR) and 7 specimens of normal breast tissue originated from primary tumor-containing breast mastectomy (normal-TCB).

Functional Assay for hER. A functional assay in yeast, the hER-FASAY, was used to determine the prevalence and functional activity of hER in all breast tissue specimens. The hER-FASAY is a relatively fast and simple screening method for the presence and functional activity of hER (13). In one experiment, it allows for the determination of the relative abundance and functional activity of all variant and wild-type hER mRNA present in a tissue specimen. With the hER-FASAY, the functional activity of individual hER cDNA molecules is

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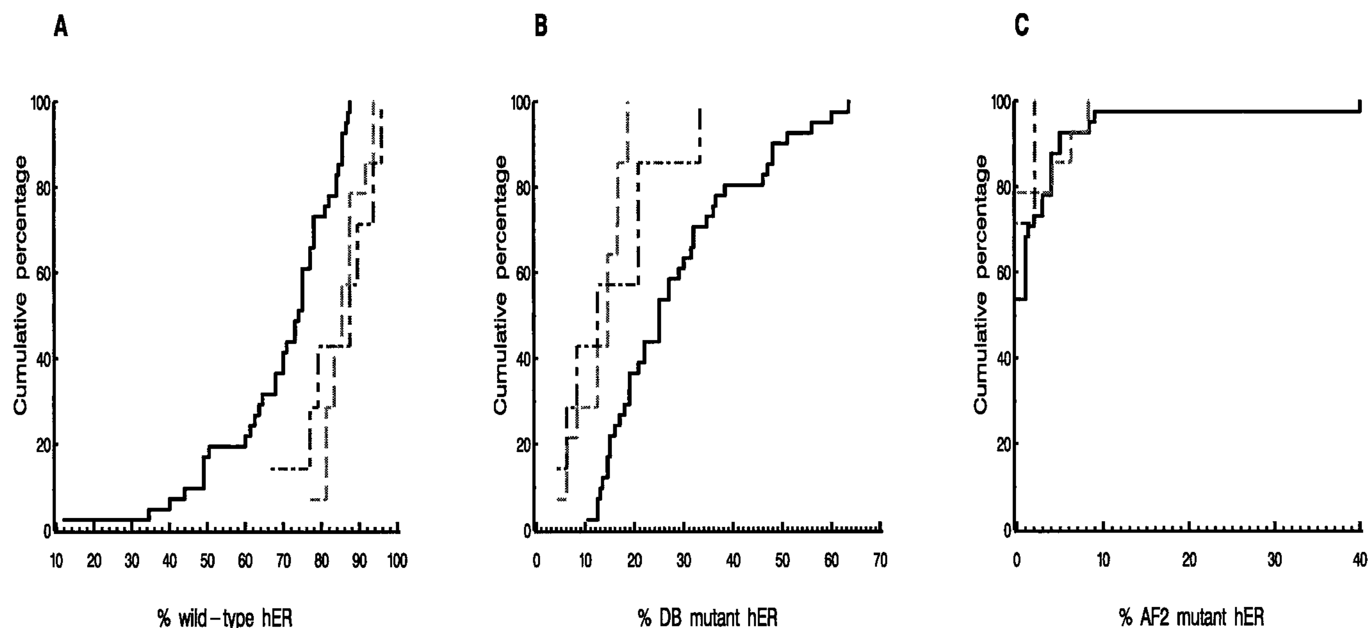

Fig. 1. Cumulative percentage plots from hER-FASAY results of 14 normal-CBR specimens (----), 7 normal-TCB specimens (-·-·-·), and 41 breast cancer specimens (———). On the *Y* axis of each figure, the cumulative percent of specimens with a specific amount of wild-type (*A*) or variant hER mRNA (*B* and *C*) is shown. For tumor specimens, one sample represents 2.4%; for normal-CBR specimens, one sample represents 7.1%, and for normal-TCB specimens, one sample represents 14.3%. *A*, Cumulative percentage plot of each tissue type for percent wild-type hER. *B*, Cumulative percentage plot of each tissue type for percent DB mutant hER. *C*, Cumulative percentage plot of each tissue type for percent AF-2 mutant hER.

tested in yeast by their ability to activate transcription of a reporter gene from an estrogen response element-containing GAL1 promoter. The assay can discriminate among wild-type hER, constitutively active hER [transcription activation function 2 (AF-2) mutant] and inactive hER [DNA binding (DB) mutant].

In Fig. 1 the hER-FASAY results of the three groups of tissue are shown. Fig. 1*A* shows that all of the 14 normal-CBR specimens have a percentage of wild-type hER ≥75%, whereas that is true for 6 of the 7 normal-TCB specimens and only 20 of the 41 breast cancer specimens. In the normal-CBR specimens the median percentage of hER that is wild-type is 85% (SD, 5%), and in the normal-TCB specimens this percentage is 88% (SD, 10%). In contrast, in breast tumors the median percentage of wild-type hER is 74% (SD, 17%; data not shown). In Fig. 1*B* one can see that all of the 14 normal-CBR specimens have a percentage of DB mutants ≤20%, whereas that is the case for 5 of the 7 normal-TCB specimens and only for 15 of the 41 breast cancer specimens. The percentage of AF-2 mutants is generally low in all three groups (Fig. 1*C*).

The differences in expression levels of wild-type and DB mutant hER mRNA (Fig. 1, *A* and *B*) between normal-CBR specimens and breast tumor specimens are significant. In normal tissue, the percentage of wild-type hER is proven to be generally higher than in breast cancer (normal-CBR *versus* breast cancer, $P < 0.0001$; Mann-Whitney test), whereas the percentage of DB mutants is lower (normal-CBR *versus* breast cancer, $P < 0.0001$; Mann-Whitney test). No differences are found regarding the percentage of AF-2 mutants (normal-CBR *versus* breast cancer, $P = 0.25$; Mann-Whitney test). Furthermore, although the number of normal-TCB specimens is too low to draw definite conclusions, comparison of the hER-FASAY data of normal-CBR specimens with those of normal-TCB specimens did not show any apparent difference.

Analysis of hER Variants. For 18 of the 41 primary breast carcinomas, the molecular structure of the hER functional variants that were detected by the hER-FASAY was determined by automated sequencing (data summarized in Fig. 2, black bars). In these carcinomas, only splicing variants and no point mutations were detected. Most frequently, splicing variants were observed which lack one or more complete exons. The only other splicing variant that is detected more than once in these breast carcinomas is Δ22bpE7, which is caused by the use of an alternative splicing acceptor site in exon 7. For detection of the exon-skipping splicing variants in the normal breast tissue specimens, we developed a "DIG dot-spot analysis" with exon-specific probes. Besides this, Δ22bpE7 was detected with a specific PCR test.

The exact nature of the hER variants detected with the hER-FASAY was determined for 12 of the normal-CBR specimens, 6 of the normal-TCB specimens, and 18 of the primary breast carcinoma specimens (Fig. 2, white and hatched bars respectively). These results are shown as the expression of a specific variant relative to the total amount of variant hER of each specimen. The variants present in normal-CBR specimens are mostly one-exon deleted splicing variants (median, 100%; SD, 11%; Fig. 2*A*), which in most cases are variants lacking exon 7 (median, 68%; SD, 28%; Fig. 2*B*). In contrast, in breast tumors a much more heterogeneous set of hER splicing variants is present. The median percentage of one-exon deletions in breast tumors is only 50% (SD, 22%; Fig. 2*A*) and the median percentage of variants lacking exon 7 is only 35% (SD, 21%; Fig. 2*B*). This difference in presence of one-exon deleted hER splicing variants between normal-CBR tissue and breast cancer cannot be attributed to chance ($P < 0.0001$; Mann-Whitney test on one-exon deleted splicing variants as a percentage of observed variants). Moreover, no apparent differences are seen when the hER variants found in normal-CBR specimens are compared with those found in normal-TCB specimens.

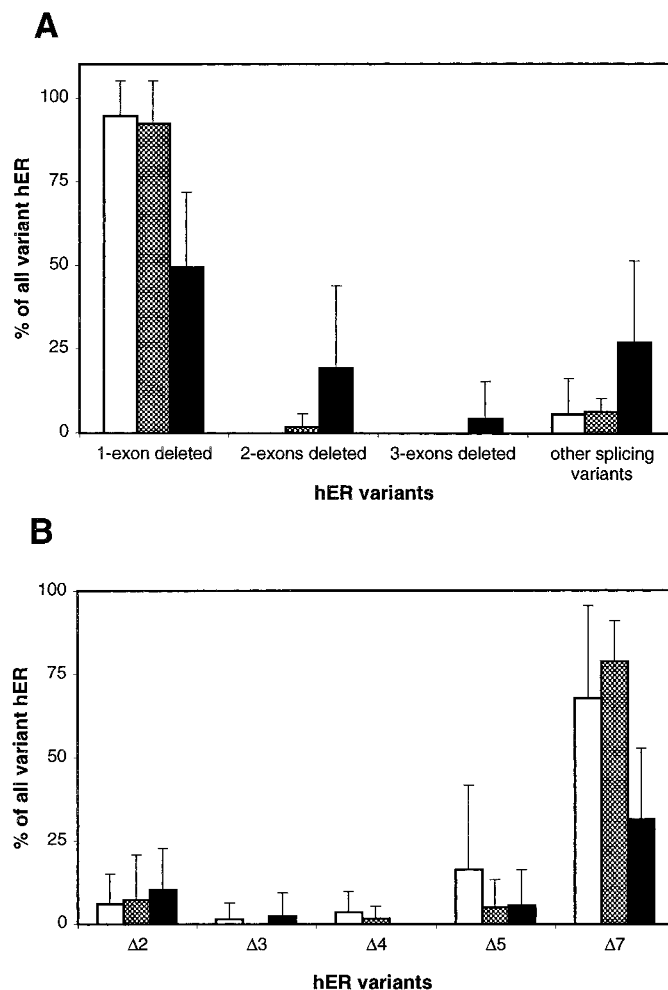

Fig. 2. Summary of the exact nature of hER mRNA variants detected in 12 normal-CBR specimens (□), 6 normal-TCB specimens (▩), and 18 breast cancer specimens (■), as determined by DIG dot-spot analysis and automated sequencing. The expression of a specific variant is shown relative to the total amount of hER variants of each specimen. Error bars indicate the SD. *A*, All hER variants detected in normal breast tissue and breast cancer. *B*, The category "1-exon deletions" (shown in *A*) is subdivided in the individual exon deletions. The category "2-exons deleted" (shown in *A*) consists of Δ31pbE1 + ΔE2; insertion of 6 bp between exons 2 and 3 + ΔE5; ΔE2 + ΔE3; ΔE2 + ΔE4; ΔE2 + ΔE7; ΔE2 + Δ22bpE7; ΔE3 + ΔE4; ΔE5 + ΔE7; and deletion of last 78 bp of exon 2 + ΔE4. In normal tissue, only ΔE2 +ΔE4 was found in this category. The category "3-exons deleted" (*A*) consists of Δ22bpE7 + ΔE4 + ΔE7; ΔE2 + ΔE3 + ΔE4; and ΔE2 + ΔE3 + ΔE7. The category "other splicing variants" (*A*) consists of Δ31pbE1; insertion of 172 bp between exons 3 and 4; insertion of 86 bp between exons 4 and 5; deletion of bp 1212–1348 (last part exon 5 and most of exon 6); and Δ22bpE7. In normal tissue, only Δ22bpE7 was evaluated for this category.

All hER variants with aberrant functional activity that were analyzed by DIG dot-spot analysis or PCR in this study turned out to be splicing variants lacking one or more complete exons or Δ22bpE7. The chance of missing a splicing-related variant that is combined with an exon deletion is small, a percentage that is on average 4% of all variants detected in breast carcinomas (data not shown).

Discussion

This paper is the first to show the quantity as well as the functional activity of all hER splicing variants that are present in specimens of normal breast tissue. Only such a complete investigation can determine the full significance of the presence of these variants in breast cancer. From the results of the hER-FASAY experiments presented here, we can conclude that on average primary breast cancer has a significantly lower amount of wild-type hER ($P < 0.0001$) and a significantly higher amount of DB mutants ($P < 0.0001$) present than

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normal breast tissue does. Also, the molecular structure of the variants is markedly different in that the variants present in normal-CBR specimens are mainly one-exon deleted splicing variants (median, 100%; SD, 11%), whereas in breast tumors only half of all variants lack just one single exon (SD, 22%; Fig. 2*A*; $P < 0.0001$). When the molecular structure of the hER variants present in each specimen is evaluated in more detail, even more differences are seen. In the normal-CBR group, 9 of the 12 specimens express only one-exon deleted hER variants, whereas that was the case in none of the 18 tested breast tumors (data not shown). The only additional variant that was seen in the remaining three normal-CBR specimens was hER Δ22bpE7. In contrast, in breast tumors two-exon deletions (nine tumors) and three-exon deletions (three tumors) were also observed (Fig. 2*A*).

The differences in the amounts and molecular structures of hER variants between normal tissue and breast cancer implicates that the hER-dependent estrogen responsiveness of breast tissue might change during tumor development. It also suggests that breast cells that contain high levels of hER variants (Fig. 1) or those containing variants lacking multiple exons (Fig. 2) may infer a selective advantage during breast cancer development or progression. Interestingly, despite the age differences and the presence or absence of cancer, there are no apparent differences in amounts or molecular structures of hER variants between normal-CBR and normal-TCB. Apparently, the higher amount of hER variants and the different molecular structure of the variants detected in breast cancer are specific for tumor cells and not for the complete breast with malignant disease.

hER ΔE7 is the predominant variant in normal breast tissue. Fig. 2*B* shows that the relative amount of this variant (*i.e.,* the presence of hER ΔE7 compared with the total amount of variant hER) is much higher in normal breast tissue than in tumors. Nevertheless, the absolute amount of hER ΔE7 (*i.e.,* the presence of hER ΔE7 compared with the total amount of variant and wild-type hER) is not found to be higher in normal-CBR (mean, 9.9%; SD, 5.8%) or normal-TCB (mean, 9.9%; SD 5.8%) than in breast tumors (data not shown) [mean 10.8%, SD 9.1%; data not shown; ANOVA, $P = 0.96$ (confidence interval, −5.1 to +6.8%)]. This may indicate that the presence of hER ΔE7 should be considered normal in human breast tissue and that this variant may even play a physiological role in normal breast tissue. hER ΔE7 has been shown to have a dominant negative function (*i.e.,* not only inactive itself but also preventing the function of wild-type hER) in yeast but was nonfunctional in HeLa cells (16, 17). Because the functional activity of hER ΔE7 in breast tissue is not yet clear, we can only speculate on its function in normal breast tissue. It recently was shown that during specific embryonic stages hER variants lacking exon 3 or 4 or both are expressed in the rat pituitary gland where they may be involved in pituitary gland development (18). Analogous to this finding we could hypothesize that hER ΔE7 may play a physiologically relevant role as a regulator of estrogen-dependent transcription in the mammary gland. On the other hand, hER ΔE7 expression also could be the remains of a development-specific expression of this hER variant, as is also seen for hER ΔE3, ΔE4, and ΔE3,4 in the adult rat pituitary gland (18).

Acknowledgments

We thank Erik Thunnissen for collecting the normal breast tissue specimens from cosmetic breast reductions, Hans Peterse for histopathological reviewing of all tissue sections, and Harry Bartelink for his helpful comments on the manuscript.

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