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Expression of Estrogen Receptor β1, β2, and β5 Messenger RNAs in Human Breast Tissue[1]

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Abstract

A triple-primer PCR assay was developed, based on the coamplification of estrogen receptor (ER)-β1, -β2, and -β5 cDNAs, to investigate the relative expressions of the corresponding mRNAs in breast cancer lines and in 53 independent breast tumors. The expression of ER-β2 and ER-β5 mRNAs was higher than that of ER-β1 mRNA in both cancer cell lines and breast tumors. In breast tumors, increases in the ER-β2:ER-β1 and ER-β5:ER-β1 mRNA expression ratios were observed, which positively correlated with the level of tumor inflammation and tumor grade, respectively. A trend toward an increase of these ratios was also found in tumors, as compared to the normal adjacent breast tissue available for 13 cases. Our data suggest that changes in the relative expression of ER-β1, -β2, and -β5 mRNAs occur during breast tumorigenesis and tumor progression.

Introduction

Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression (1). Indeed, estrogens are thought to promote the growth of breast tumors through their mitogenic effects on breast cancer cells. The ability of antiestrogens such as tamoxifen or raloxifene to inhibit estrogenic action provides the basic rationale for the use of endocrine therapies. Estrogen action is believed to be mediated mainly through two ERs[3]: ER-α (2) and ER-β1 (3, 4). These two receptors, which are encoded by two different mRNAs containing eight exons each (5, 6), belong to the steroid/thyroid/retinoic acid receptor superfamily (7). ER-α and ER-β1 share the same structural and functional domain composition (8), defined as region A–F (Fig. 1*A*). The A–B regions contain the NH_2-terminal transactivation function (AF-1) of the receptors, whereas the C region of the molecule contains the DNA binding domain. The ligand binding domain and the second transactivation function (AF-2) are located within the E region of the receptors. The receptors, once bound to the ligand, are subject to conformational changes that result in complexes containing dimers of receptors/hormones that recognize estrogen-responsive elements located upstream of target genes. Interactions between ERs and accessory proteins ultimately lead to the modification of the transcription of these genes (9). The ER-ligand complexes can also interact with c-*fos*/c-*jun* complexes to modify the transcription of target genes through AP1 enhancer elements (10, 11). Differential activation of ER-α and ER-β1 by the antiestrogen 4-hydroxytamoxifen, determined by activation of estrogen response element-regulated reporter genes, and differential activation of AP1-regulated reporter genes by the two ERs have been observed (11, 12). Also, because heterodimerization of ER-α and ER-β1 has also been shown, putative cross-talk between the two signaling pathways is possible (4, 13).

Several variant forms of ER-α and ER-β1 mRNAs have been identified (for reviews see Refs. 14–17). Among them, exon-deleted variant mRNAs, which would encode ER-like proteins missing some of the functional domains of the wild-type receptors, could interfere with ER-α and/or ER-β1 signaling pathways. Indeed, exon 5- and exon 7-deleted ER-α variant proteins have been shown, *in vitro*, to exhibit a constitutive transcriptional (18) and a dominant negative activity (19) on ER-α, respectively. More recently, an ER-β2 variant, deleted of regions encoded by ER-β1 exon 8 sequences, has been shown to heterodimerize with both ER-β1 and ER-α and to inhibit ER-α DNA binding capability (20, 21). The ability of ER-α variants to potentially interfere with the ER-α signaling pathways raised the question of their possible involvement in mechanisms underlying breast tumorigenesis and tumor progression. Although much data have been published documenting the differential expression of ER-α variants at different stages of breast cancer progression (14), no studies have been performed comparing the relative expression of ER-β variant mRNAs in human breast tissue. We have developed a TP-PCR assay to evaluate the relative expression of ER-β1, -β2, -β4, and ER-β5 variant mRNAs. As shown in Fig. 1*A*, ER-β2, -β4, and -β5 variant mRNAs do not contain exon 8 ER-β1 sequences but share similar 3′ end sequences. This assay was used to evaluate the relative expression of ER-β1, -β2, and -β5 mRNA within breast tumors ($n = 53$) and, in some cases ($n = 13$), within adjacent normal breast tissue.

Materials and Methods

Human Breast Tissues and Tumor Cell Lines. Fifty-three cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As reported previously, all cases in the bank have been processed to provide paraffin-embedded tissue blocks and mirror-image frozen tissue blocks (22). Histopathological analysis was performed on H&E-stained sections from the paraffin tissue block to estimate, for each case, the proportions of tumor and normal epithelial cells, fibroblasts, and fat as well as to determine the levels of inflammation and Nottingham grade scores (23). The age of the patients ranged between 39 and 87 years ($n = 53$, median = 67 years). Tumors spanned a wide range of ER (from 0 to 159 fmol/mg protein, $n = 53$, median = 9 fmol/mg protein) and PR (ranging from 0 to 285 fmol/mg protein, $n = 53$, median = 10 fmol/mg protein) levels, as measured by ligand binding assay. These tumors also covered a wide spectrum of grades (Nottingham grading scores from 1 to 9, $n = 47$, median = 7). Inflammation levels were assessed for 51 cases by scoring the extent of lympho-histocystic infiltrates throughout the section using a semiquantitative scale from 0 (low to minimal infiltration) to 5 (marked infiltrate). For 13 cases, matched adjacent normal tissue blocks were also available. The characteristics of this subset of 13 tumors were as follows: ER status ranged from 0 to 159 fmol/mg protein (median = 3.5 fmol/mg protein), PR status ranged from 4.9 to 134 fmol/mg protein (median = 8.5), Nottingham grade scores ranged from 5 to 9 (median = 7), inflammation levels ranged from 1 to 5 (median = 3), and patients were between 39 and 75 years old (median age = 54 years).

MDA-MB-231, MDA-MB-468, ZR-75, BT-20, T-47D, and MCF-7 breast

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[3] The abbreviations used are: ER, estrogen receptor; TP-PCR, triple-primer PCR; PR, progesterone receptor.

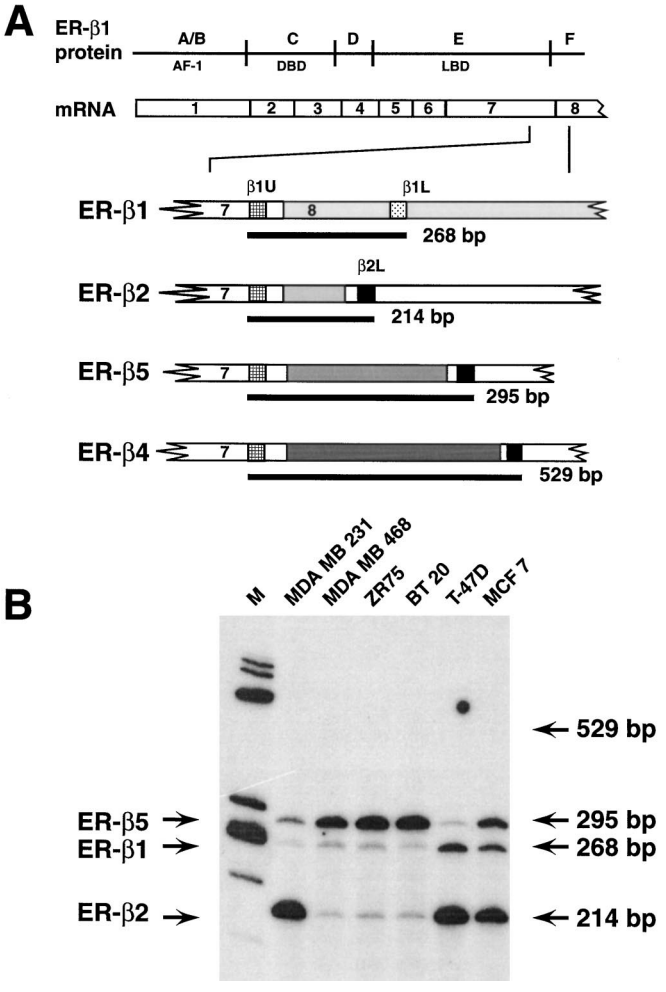

Fig. 1. Presentation of the TP-PCR assay and relative expression of ER-β1, -β2, -β4, and ER-β5 mRNAs in breast cancer cell lines. *A*, structural and functional domains (AF) of the ER-β1 protein (*AF-1*, transactivation function 1; *DBD* and *LBD*, DNA and ligand binding domains, respectively) are shown together with the corresponding exonic structure (*exons 1–8*) of the ER-β1 mRNA. Common sequences (□) and specific sequences (□) are depicted for each cDNA (β1, β2, β4, and β5). ER-β1U (▦), ER-β1L (▨), and ER-β2L (■) primer annealing sites are also represented. The sizes of the possible PCR products (*black bars*) obtained after TP-PCR are indicated. *B*, breast cancer cell line (MDA-MB-231, MDA-MB-468, ZR-75, BT-20 T-47D, and MCF-7) poly(A) mRNAs were reverse-transcribed, TP-PCR was performed, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." PCR products migrating at apparent sizes of 295, 268, and 214 bp have been subcloned, sequenced, and identified as corresponding to ER-β5, -β1, and -β2 mRNAs, respectively. *Lane M*, molecular size markers (φx174 RF DNA/Hae III fragments; Life Technologies, Inc.).

cancer cells were grown and poly(A) mRNA was obtained as described previously (24). Total RNA was extracted from frozen breast tissue sections using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions, and quantified spectrophotometrically. One μg of total RNA was reverse-transcribed in a final volume of 25 μl as described previously (25).

Primers and PCR Conditions. The primers used consisted of ER-β1U primer (5′-CGATGCTTTGGTTTGGGTGAT-3′; sense, located in exon 7, positions 1400–1420, GenBank accession no. AB006590), ER-β1L primer (5′-GCCCTCTTTGCTTTTACTGTC-3′; antisense, located in exon 8, positions 1667–1648, GenBank accession no. AB006590), and ER-β2L (5′-CTTTAGGCCACCGAGTTGATT-3′; antisense, located in ER-β2 extrasequences, positions 1933–1913, GenBank accession no. AF051428). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (25). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 4 ng/μl each primer (ER-β1U, ER-β1L, and ER-β2L), and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 60°C, 30 s at 72°C, and 30 s at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *glyceraldehyde-3-phosphate dehydrogenase* cDNA was performed in parallel, and PCR products, separated on agarose gels, were stained with ethidium bromide as described previously (25). Identity of PCR products was confirmed by subcloning and sequencing, as reported previously (25).

TP-PCR Validation. The first series of experiments, performed using cDNAs prepared from breast cancer cell line mRNA, showed that ER-β1, -β2, and -β5 cDNAs can be coamplified, and they led to the production of three PCR products that were subcloned and sequenced as described previously (25). Spiked cDNA preparations containing 1 fg of purified PCR products, corresponding to ER-β1 and -β5 mRNAs, were amplified together with increasing amounts of ER-β2 PCR product (0, 0.2, 0.4, 1, 4, and 8 fg) in a single PCR tube using the three primers (ER-β1U, ER-β1L, and ER-β2L), as described above. Similar experiments were performed using constant amounts of ER-β1 and ER-β2 or of ER-β2 and ER-β5, with increasing amounts of ER-β5 or ER-β1 PCR products, respectively. In parallel, preparations containing 1 fg of each PCR product alone were also amplified. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands and counting in a scintillation counter (Beckman). For each sample, ER-β1, -β2, and -β5 signals were expressed as a percentage of the sum of all signals measured (ER-β1 + ER-β2 + ER-β5 signals). Experiments have been performed in duplicate and the mean of the relative signals calculated. For each ER-β isoform, regression analyses between the relative signal obtained and the relative initial input (*i.e.*, ER-β isoform input expressed as a percentage of ER-β1 + ER-β2 + ER-β5 input) were performed using GraphPad Prism software.

Quantification and Statistical Analyses. To quantitate the relative expression of ER-β1, -β2, and -β5 mRNAs within each breast tissue sample, we used the TP-PCR described above. Quantification of ER-β1, -β2, and -β5 signals was carried out by excision of the bands and scintillation counting. For each sample, ER-β1, -β2, and -β5 signals were expressed as a percentage of the sum of all signals measured (ER-β1 + ER-β2 + ER-β5 signals). Three independent PCRs were performed and the mean of the relative expressions was calculated. Differences between ER-β1, -β2 and -β5 relative expression within the cohort studied were tested using the Wilcoxon signed rank test (two-tailed). Correlations with tumor characteristics were tested by calculation of the Spearman coefficient (*r*).

Results

Validation of TP-PCR as an Approach to Evaluate the Relative Expression of ER-β1, -β2, and -β5 mRNAs. We established previously that TP-PCR provided a reliable method to investigate the expression of a truncated mRNA relative to the wild-type mRNA expression within small breast tissue samples (25). In its initial design, the TP-PCR assay relied on the coamplification of one truncated and a wild-type cDNA molecule using three primers in the PCR. The upper primer recognized both sequences, whereas the two lower primers recognized the variant and the wild-type sequences, respectively. We have shown that the final ratio between the two coamplified products was linearly related to the initial cDNA input (25).

As shown Fig. 1*A*, ER-β1, -β2, -β4, and -β5 mRNAs all have exon 7 sequences but differ from each other in the following sequences. Interestingly, comparison of the sequences revealed that ER-β2, -β4, and -β5 mRNAs have sequence similarities within their 3′ extremities. Therefore, it was possible to use TP-PCR to investigate the relative expression of these variants. Three primers were designed (ER-β1U, ER-β1L, and ER-β2L) that recognized exon 7 sequences common to all transcripts, ER-β1 exon 8-specific sequences, and sequences shared by ER-β2, -β4, and ER-β5 mRNAs, respectively (Fig. 1*A*). As shown in Fig. 1*A*, the expected PCR products resulting from the coamplification of the corresponding cDNAs are different in size and can be easily distinguished on an acrylamide gel.

The assay was used initially to determine the expression of ER-β1, -β2, -β4, and -β5 mRNAs in several different human breast cancer cell lines (Fig. 1*B*). Three bands migrating at apparent sizes of 268, 214, and 295 bp were observed in all samples. Subcloning and

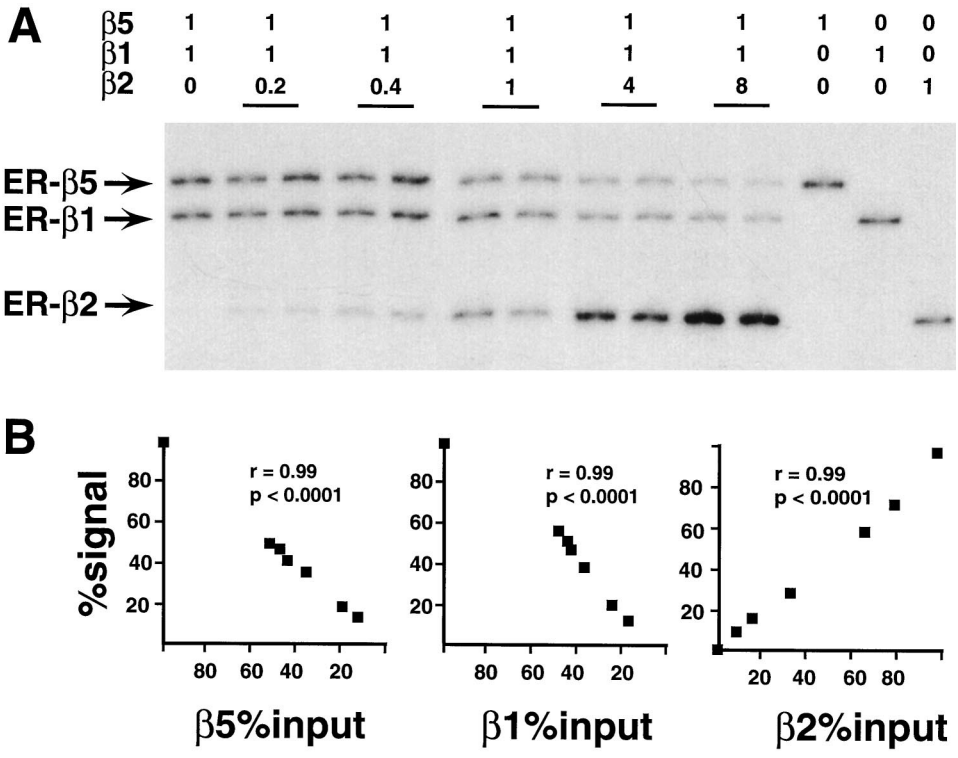

Fig. 2. TP-PCR validation. *A*, spiked cDNA preparations, containing various amounts [indicated *above* the autoradiogram] of ER-β5, -β1, and -β2 purified PCR products (β5, β1, and β2) were amplified by TP-PCR, and PCR products were separated on an acrylamide gel, as specified in "Materials and Methods." The autoradiogram shows the PCR products obtained. *B*, signals corresponding to ER-β5, -β1, and -β2 PCR products have been quantified in each lane, as described in "Materials and Methods." For each ER-β isoform, the relative signal observed (percentage signal, expressed as a percentage of the sum: β1 + β2 + β5 signals) is presented as a function of the initial relative cDNA input (percentage input, expressed as a percentage of the sum: β1 + β2 + β5 inputs). The regression coefficient (*r*) and *P* of the associations are also presented.

sequencing of these bands confirmed their identity as ER-β1, -β2, and -β5 cDNAs, respectively (data not shown). We were unable to detect a product of 529 bp, which would correspond to the ER-β4 PCR product. Interestingly, in all tumor cell lines, the ER-β1 signal was lower than the ER-β2 and/or ER-β5 signals (Fig. 1*B*).

Because TP-PCR performed using these primers produced three PCR products, instead of the two PCR products obtained in the original published validation studies (25), it was necessary to establish the quantitative relationship between the signals obtained and the initial target concentrations. To address this issue, spiked DNA preparations containing equal amounts of ER-β1 and ER-β5 PCR products and increasing amounts of ER-β2 PCR products were amplified (Fig. 2*A*). The relative signals of ER-β1, -β2, and -β5 have been measured and expressed as a percentage of the sum of the signals measured, as described in "Materials and Methods." As expected, in the absence of ER-β2, only two bands, corresponding to ER-β1 and ER-β5 PCR products, are observed. The relative signals of ER-β1 and ER-β5 decreased, whereas the ER-β2 relative signal increased linearly with increasing ER-β2 input. Indeed, for each ER-β isoform, regression analysis showed a linear correlation between the relative signal of the PCR product measured and its relative input (Fig. 2*B*). Similar results were obtained when experiments were performed using constant amounts of ER-β1 and ER-β2 with increasing amounts of ER-β5 PCR products or using constant amounts of ER-β2 and ER-β5 with increasing amounts of ER-β1 (data not shown). It should be noted that the amplification of similar amounts of the three molecules led to the production of three bands of similar intensities (Fig. 2*A*). It should also be stressed that the ER-β5:ER-β1 ratio was not affected by increasing amounts of ER-β2 and that the ER-β2:ER-β5 and ER-β5:ER-β2 ratios varied as a linear function of the initial ER-β2:ER-β5 and ER-β5:ER-β2 input ratios, respectively (data not shown). We concluded that the TP-PCR assay, performed under the described conditions, provided a reliable method with which to compare breast tissue samples for their relative expression of ER-β1, -β2, and -β5 mRNAs.

Comparison of the Relative Expression of ER-β1, -β2, and -β5 mRNAs in Breast Tumor Tissues. To determine whether alterations occur in the balance between ER-β1, -β2, and -β5 mRNAs during breast tumor progression, the relative expression of these transcripts was measured in primary breast tumor tissues from 53 different patients, using the TP-PCR assay described above. These tumors presented a wide spectrum of ER and PR statuses, as determined by ligand binding assay, and also spanned a wide range of grades and inflammation levels (for a more detailed description of the cohort characteristics, see "Materials and Methods"). Total RNA was extracted from frozen tissue sections and reverse-transcribed as described in "Materials and Methods." TP-PCR was then performed. Examples of the results obtained are shown in Fig. 3*A*. Three PCR products migrating at apparent sizes of 268, 214, and 295 bp were obtained. These PCR products were shown by cloning and sequencing to correspond to ER-β1, -β2, and ER-β5 cDNAs, respectively. As in our preliminary study performed in human breast cancer cell lines, no band of 529 bp was detected, which would correspond to ER-β4 PCR product. Amplification of the ubiquitously expressed *glyceraldehyde-3-phosphate dehydrogenase* cDNA, performed to check the integrity of each cDNA studied, revealed similar amounts of cDNA in all samples (data not shown). ER-β1, -β2, and -β5 signals obtained in three independent TP-PCRs were quantified as described in "Materials and Methods." For each sample, the percentage of each band relative to the sum of the signals obtained has been calculated. The medians of ER-β1, -β2, and -β5 relative expression within tumors, sorted according to their grade or to the level of inflammation, are presented in Fig. 3*B*. The ER-β1 relative signal was found to be significantly lower than ER-β2 (Wilcoxon sign rank test, $n = 53$, $P = 0.0002$) and ER-β5 (Wilcoxon sign rank test, $n = 53$, $P = 0.004$) signals. A trend toward a higher expression of ER-β2 as compared to ER-β5 was also observed but did not reach statistical significance (Wilcoxon sign rank test, $n = 53$, $P = 0.09$).

Possible associations between ER-β1, -β2, or -β5 signals and tumor characteristics were then investigated. ER-β1 relative expression was found (Fig. 3*B*) to be inversely related to the grade of the tumor ($n = 47$, Spearman $r = -0.33$, $P = 0.02$) and the level of inflammation ($n = 51$, Spearman $r = -0.28$, $P = 0.04$). No other associations were found between ER-β1 expression and ER status, PR status, or age of the patients. ER-β2 mRNA expression increased

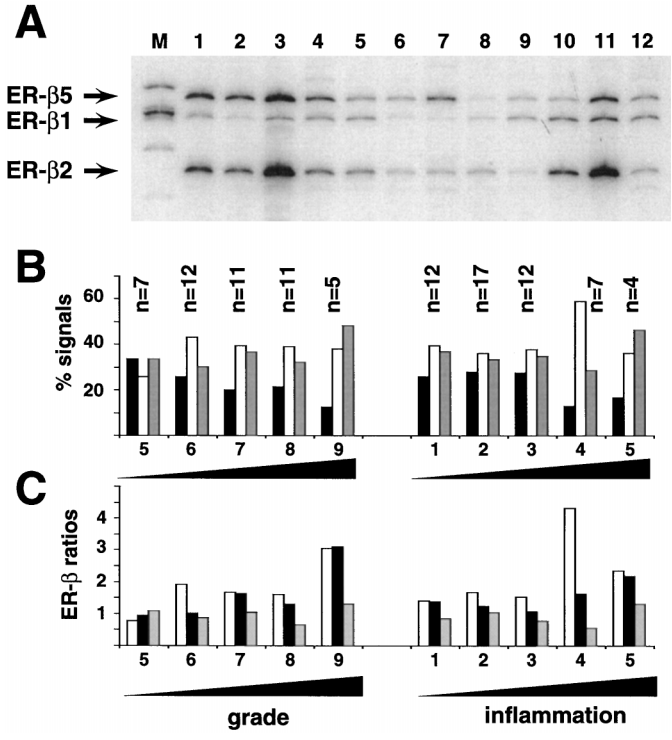

Fig. 3. TP-PCR analysis of the relative expression of ER-β1, -β2, and -β5 mRNAs within a cohort of 53 independent breast tumors. Total RNA was extracted from 53 breast tumors, reverse-transcribed, and analyzed by TP-PCR, as described in "Materials and Methods." PCR products were separated on acrylamide gels. *A*, autoradiogram showing the results obtained for 12 cases (*Lanes 1–12*). *Lane M*, molecular weight marker ϕx174 RF DNA/*Hae*III fragments. *B*, ER-β1, -β2, and -β5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in "Materials and Methods." Tumors have been sorted according to their Nottingham grade scores (*5–9*) or to their levels of inflammation (*1–5*). For each group, the number of patients (*n*) and the medians of the relative expression of ER-β1 (■), -β2 (□), and -β5 (▦) signals are indicated. *C*, for each group, the number of patients (*n*) and the median of ER-β2:ER-β1 (□), ER-β5:ER-β1 (■) and ER-β5:ER-β2 (▢) signal ratios are indicated.

significantly with the levels of inflammation ($n = 52$, Spearman $r = 0.28$, $P = 0.04$). No other associations were found between ER-β2 and ER-β5 and other characteristics.

Because the ratio between two signals was related to the respective proportion of the two corresponding cDNAs, we also addressed the question of the expression of ER-β2 and ER-β5 relative to ER-β1. The medians of the ER-β2:ER-β1, ER-β5:ER-β1, and ER-β5:ER-β2 ratios within tumors, sorted according to their grade or to the level of inflammation, are presented in Fig. 3*C*. ER-β5 and ER-β2 expression relative to ER-β1 were found positively associated with the tumor grade ($n = 47$; Spearman $r = 0.29$, $P = 0.04$; and Spearman $r = 0.28$, $P = 0.05$, respectively). In addition, one should note that ER-β2 expression relative to ER-β1 expression correlated ($n = 52$, Spearman $r = 0.34$, $P = 0.01$) with levels of inflammation. ER-β2 and ER-β5 expression relative to each other did not correlate with grade, degree of inflammation, or any other tumor characteristics. No correlations were found between the content of the tissue sections analyzed, *i.e.*, percentage of normal cells, tumor cells, fibroblasts, or fat, and ER-β1, -β2, and -β5 mRNA relative expression (data not shown).

ER-β1, -β2, and -β5 mRNA Expression within Matched Normal and Tumor Compartments. To determine whether changes in the expression of ER-β1, -β2, and -β5 mRNAs occur during breast tumorigenesis, we compared the relative expression of these transcripts between normal breast tissue and matched adjacent tumors. Normal adjacent breast tissue was available for 13 cases belonging to the cohort described earlier in the text. The characteristics of this tumor subset are detailed in "Materials and Methods." Total RNA was extracted, and following reverse transcription, TP-PCR was performed as described in "Materials and Methods." Typical results are shown in Fig. 4*A*. Quantification of the signals was performed as described above. Fig. 4*B* shows the relative expression within tumor and adjacent normal breast tissues of ER-β1 mRNA. A trend toward a lower ER-β1 signal (9 of 13 cases, Wilcoxon sign rank test, $P = 0.06$) in the tumor compartment compared to the normal adjacent components was observed. In contrast, trends toward higher expression of ER-β2 (Fig. 4*C*) and ER-β5 (Fig. 4*D*) mRNAs relative to ER-β1 mRNA were observed in tumor compartments (8 of 13 cases, Wilcoxon sign rank test, $P = 0.10$; and 9 of 13 cases, Wilcoxon sign rank test, $P = 0.06$, respectively).

Discussion

To evaluate the relative expression of ER-β1, -β2, and -β5 mRNAs within small frozen sections of human breast tissues, we have developed an assay based on the coamplification of the corresponding cDNAs in a single tube, using three primers in the PCR. The quantitative aspect of this assay was validated using preparations containing known amounts of target cDNA. The TP-PCR approach appeared to be a reliable approach to estimate not only the relative expression of each variant within the population of ER-β molecules measured (*i.e.*, ER-β1, -β2, and ER-β5 mRNAs) but also the proportion of each

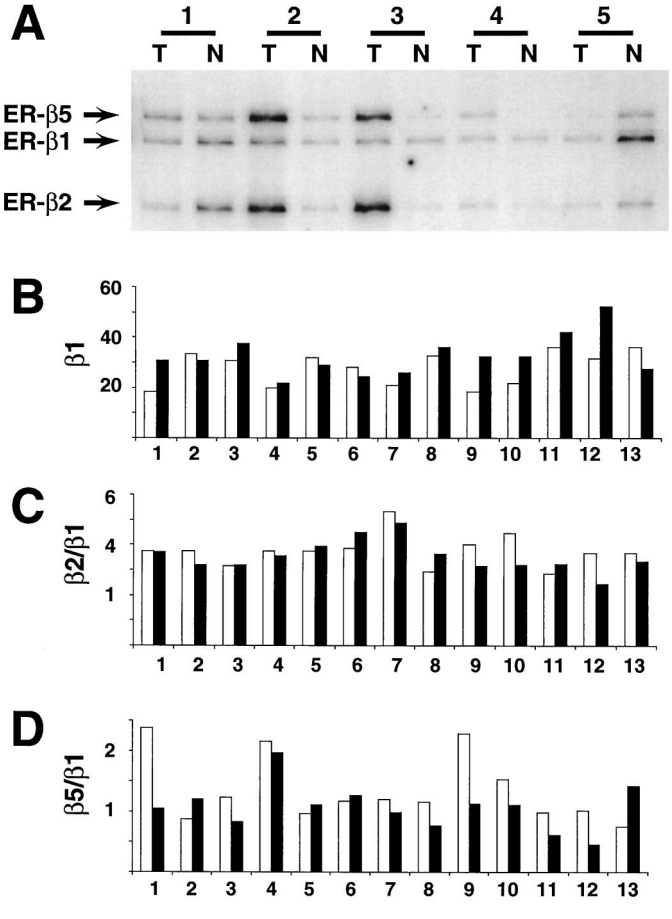

Fig. 4. TP-PCR analysis of the relative expression of ER-β1, -β2, and -β5 mRNAs within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from 13 breast tumors (*Lanes T*) and adjacent normal breast tissues (*Lanes N*), reverse transcribed and analyzed by TP-PCR as described in "Materials and Methods." PCR products were separated on acrylamide gels. *A*, autoradiogram showing the results obtained for five cases (*1–5*). *B*, ER-β1, -β2, and -β5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in "Materials and Methods." For each case (*1–13*), the relative percentages of ER-β1 in tumor (□) and normal (■) components are shown. *C*, for each case (*1–13*), the ER-β2:ER-β1 ratios in tumor (□) and normal (■) components are shown. *D*, for each case (*1–13*), the ER-β5:ER-β1 ratios in tumor (□) and normal (■) components are shown.

RNA relative to one another. One should note that the set of primers used would detect ER-β4 variant mRNA. However, this variant was not detected in any breast sample or tumor cell line studied. This might result from either a lower efficiency of amplification of this specific variant in our PCR conditions or a lower relative expression of ER-β4 mRNA as compared to ER-β1, -β2, and -β5. A low expression of ER-β4 mRNA would be consistent with data obtained on breast cancer cell lines by Moore *et al.* (20).

Our data show that ER-β1, -β2, and -β5 mRNAs are coexpressed in human breast cancer cells grown in culture. These data confirm the previous observation of Moore *et al.* (20). These authors however, did not address the question of the relative expression of these mRNAs. Striking differences in the pattern of expression of ER-β1, -β2, and -β5 mRNAs were found between breast cancer cell lines. If these differences in expression are conserved at the protein level, one might hypothesize that ER-β signaling pathways, which likely result from the balance between the different forms, vary in these cells. To date, multiple ER-β-like mRNAs that could encode different proteins that would be difficult to distinguish from each other by Western blot analysis have been described. For example, ER-β1 protein (5) and ER-β2 protein (20) have theoretical molecular masses of 54.2 kDa and 55.5 kDa, respectively. Most likely, antibodies specifically recognizing the different ER-β proteins would be the best approach to address the question of the relative expression of ER-β proteins within breast cancer cells. Higher ER-β2 and ER-β5 expression as compared to ER-β1 expression was observed in breast cancer cell lines. This suggests that the respective participation of ER-β2 and ER-β5 variants in ER-β signaling pathways within breast cancer cells might be as significant as or more significant than that of ER-β1.

As observed in breast cancer cell lines, ER-β1, -β2, and -β5 mRNAs were detected in human breast tumors. Consistent with the observations in breast cancer cell lines, ER-β2 and ER-β5 mRNAs were more highly expressed than ER-β1 mRNA in these tissues. However, even though this observation may result directly from the expression of different ER-β isoforms in breast cancer cells, it may also be a consequence of the heterogeneity of the cell populations expressing ER-β molecules and present in different proportions within the tumor sample analyzed. Indeed, because lymphocytes have previously been shown to express ER-β1, -β2, and -β5 mRNAs, one could speculate that infiltrating lymphocytes within the tumor might contribute to the higher level of ER-β2 mRNA expression in tumors with higher inflammation levels. Techniques such as *in situ* hybridization or immunocytochemistry, designed to distinguish between the different ER-β isoforms, are needed to address the question of the cellular origin of ER-β isoform expressions *in vivo*.

We observed an inverse relationship between the relative expression of ER-β1 mRNA and tumor grade. It has been shown that the Nottingham grade provides a useful marker of the length of disease free interval and overall survival (23). We have also observed a decrease of the relative expression of ER-β1 in tumor *versus* normal adjacent components. Taken together, these data suggest that changes in the relative expression of ER-β1, -β2, and -β5 mRNAs occur during breast tumorigenesis and tumor progression. Whether these changes are a cause or a consequence of tumorigenesis remains to be elucidated.

In conclusion, we have developed a TP-PCR assay allowing the investigation of the relative expression of ER-β1, -β2, and -β5 mRNA in human breast tissues. In these tissues, ER-β1 mRNA appeared to have the lowest level of expression when compared to the two other isoforms detected. We found that the relative expression of ER-β1 was inversely related to the grade of the tumor, suggesting that it could be used as a marker of tumor progression. Moreover, a lower relative expression of ER-β1 was observed in tumor *versus* adjacent normal breast tissues, suggesting that changes in the expression of ER-β isoforms occur during breast tumorigenesis. The cellular origin of the expression of ER-β1, -β2, and -β5 in breast tumor tissue *in vivo* remains to be determined, as does the putative role of the different ER-β variant forms in the mechanisms underlying tumorigenesis and tumor progression.

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