Estrogen Receptor β Protein in Human Breast Cancer: Correlation with Clinical Tumor Parameters

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ABSTRACT

The recent discovery of a second estrogen receptor (ER), designated ERβ, raises pressing questions about its role in estrogen regulation of human breast cancer cells and its significance for the prediction of recurrence and treatment responses in clinical breast cancer. Most of what we know about ERβ expression comes from studies examining a limited number of samples at the RNA level. We have now generated a monoclonal antibody useful for the detection of ERβ at the protein level in archival, formalin-fixed breast tumors and have examined its expression using immunohistochemistry in a pilot series of 242 breast cancer patients. Coexpression of ERβ and ERα was found in the majority of the tumors, with 76% of the tumors expressing ERα as determined by immunohistochemistry. ERα, but not ERβ, was strongly associated with progesterone receptor expression, suggesting that ERα is the predominant regulator of this estrogen-induced gene in breast tumors. Although ERα expression was positively correlated with low tumor grade, diploidy, and low S-phase fraction, all biological parameters of a good prognostic profile, ERβ trended toward an association only with aneuploidy; no association with tumor grade or S-phase fraction was seen for ERβ. We found that ERβ expression does cause false positive readings for ERα. These results suggest that ERβ expression is not a surrogate for ERα in clinical breast tumors and, as such, could be a useful biomarker in its own right.

INTRODUCTION

Many recent discoveries in the nuclear receptor field have contributed to our understanding of steroid hormone action and the mechanisms by which estrogens exert their effects in breast cancer cells. Until recently, estrogen action was thought to be mediated through a single ER, now called ERα (1). However, the identification of a second ER, called ERβ (2), casts uncertainty upon this understanding of estrogen action. ERα expression is a very useful clinical biomarker of breast tumor progression, and ERα and PR are now routinely used to estimate patient prognosis and select optimal therapies. Overall, approximately 50–60% of women with ER-positive, advanced breast cancer will receive some degree of benefit from standard endocrine treatment with the antiestrogen tamoxifen, whereas the majority of specimens, and we have investigated the relationships between ERβ, ERα, and clinical tumor parameters.

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MATERIALS AND METHODS

Tumor Samples. Two hundred and sixty-one human breast tumor specimens were included in this pilot study. Treatment histories and long-term follow-up for disease recurrence and death were not available for each patient. Breast tumor specimens were frozen in liquid nitrogen immediately after excision and sent to a central laboratory for steroid receptor assays and DNA flow cytometry. One paraffin-embedded, ERβ-positive breast tumor was used to evaluate whole tissue section staining with the ERβ antibody, and different areas of the slide were photographed to examine for intertumor heterogeneity of ERβ protein expression.

Steroid Receptor Assays. Tumor cytosols were prepared for LBA as described previously (10), using a standard multipoint dextran-coated charcoal assay incorporating 125I-estradiol and [H]-R5020 in a single assay, allowing for the simultaneous determination of both ER and PR status. Tumors with an ER or PR content of 3 fmol/mg protein or 5 fmol/mg protein, respectively,
were considered to be positive for receptor expression. The pulsed tissue that remained after LBA assay was stored at −70°C for future use.

Flow Cytometric Evaluation of SPF and DNA Ploidy Measurements. Flow cytometry was carried out as described previously (11). Briefly, approximately 100 mg of frozen pulsed tissue were homogenized, filtered, and centrifuged. Chicken red cells were added as an internal standard, and the cells were lysed and stained for DNA. DNA-stained nuclei were prepared and run on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Approximately 50,000 tumor events were acquired on a single-parameter 256-channel integrated fluorescence histogram. Frequency distributions of cells in G0/G1, SPF, and G2-M phases of the cell cycle were evaluated using a modeling program (MODFIT; Verity Software House, Inc., Topsham, ME). Debris was modeled as an exponential, and SPF was modeled as a single trapezoid. Proliferation status as determined with Ki67 staining has been described previously (12).

**Tumor Histological Grade.** Histological grading of the tumors was performed on complete sections (see below) using the Elston-modified Scarff-Bloom-Richardson system (13). This system utilizes a semiquantitative method to assess degree of differentiation (tubule formation), nuclear pleomorphism, and mitotic activity. Because our tissue sections were prepared from pulsed tissue, it was often not possible to count 10 individual and nonoverlapping fields. Therefore, mitotic activity was scored based on one field with maximum number of mitotic figures, an approach similar to the original Scarff-Bloom-Richardson system (14).

**Generation of a Monoclonal ERβ Antibody.** RNA from MCF-7 cells was used for reverse transcription-PCR of the NH2-terminal fragment (amino acid residues 1–146) of ERβ (15). This fragment was then cloned into the pET28a+ mammalian expression vector. Glutathione-S-transferase-fusion recombinant ERβ protein was purified on a histidine affinity column and used for immunization of female BALB/c mice. Spleen cells from mice producing immunoreactive antibody were fused to NS1 myeloma cells by standard hybridoma methods. Specific monoclonal antibody to ERβ as determined by Western blot analysis was purified and concentrated through protein G columns and used for the IHC and Western blot studies shown. The one monoclonal antibody useful for IHC was named 14C8, and it is now commercially available through GenTex (San Antonio, TX).

For Western blot analysis, MCF-7 total cell lysates were prepared as described previously (16). Briefly, a cell pellet was homogenized in a high-salt buffer [20 mM Tris·HCl (pH 7.5), 2 mM DTT, 0.4 mM KCI, and 20% glycerol] containing a mixture of protease inhibitors [10 μg/ml each of aprotinin, antipain, leupeptin, and pepstatin plus 0.3 mM phenylmethylsulfonyl fluoride (Sigma)]. Homogenates were then centrifuged at 100,000 × g for 1 h, and the supernatants stored at −80°C until use. Extract protein (50 μg) was separated by electrophoresis on 8% SDS-PAGE and transferred onto nylon membranes (Schleicher & Schuell, Keene, NH). The blots were first stained with Hemacolor S (Sigma, St. Louis, MO) to confirm uniform transfer of all samples and then incubated in blocking solution (5% nonfat dry milk in PBST). After brief washes with PBST, the filters were then reacted with the 14C8 monoclonal antibody at a dilution of 1:250 or with the NCL-ER-6F11 mouse monoclonal antibody at a dilution of 1:200 for 1 h at room temperature followed by extensive washes with PBST. Blots were then incubated with horseshadish peroxidase-conjugated secondary antibody (Amersham) for 1 h, washed with TBST, and developed using the enhanced chemiluminescence procedure (Amersham). As positive controls, 1 μl of in vitro-translated extract from two mammalian expression vectors (pSG5) containing the complete open reading frames of full-length ERβ1 and ERβ2 variant isoform expression cDNA clones (5) was used on the Western blots. As a negative control, in vitro-translated ERα (17) was also included.

**Preparation of TAs.** Tissue sections were initially prepared from the pulverized frozen tumor specimens left over from the LBA as described previously (18). TAs were assembled manually as described previously (5). Briefly, an H&E-stained slide from a complete tissue section was used as a guide to mark the area of maximum tumor cellularity on the corresponding formalin-fixed, paraffin-embedded donor block. Then, using a 3-mm dermal biopsy punch (Milex Instruments Inc.), a 3-mm core of tumor was punched out from the donor block. To create the TA recipient block, an empty paraffin block was used for reverse transcription-PCR of the NH2-terminal fragment (amino acid residues 1–146) of ERβ, was used for reverse transcription-PCR of the NH2-terminal fragment (amino acid residues 1–146) of ERβ. Specificity of ERβ1 and ERβ2 antibodies. Although the commercially available ERβ NCL-ER-6F11 antibody was generated to recombinant ERβ protein, because of the high degree of shared homology between the two ERs, we first had to demonstrate its specificity for the α-receptor subtype. Therefore, in vitro-translated ERα and full-length ERβ1 extract, along with total cellular extracts from MCF-7 breast carcinoma cells, were prepared and subjected to Western blot analysis with the NCL-ER-6F11 antibody (Fig. 1A). This antibody was indeed specific for the ERα form and did not cross-react with ERβ; a band corresponding to the M, 65,000–66,000 ERα subtype was the only form detected in MCF-7 cells with the NCL-ER-6F11 antibody.

We next analyzed a number of different monoclonal antibodies that we generated to a glutathione S-transferase-fusion protein of the NH2-terminal region of ERβ. Although all of these antibodies reacted with recombinant ERβ protein on Western blot analyses, only one, the 14C8 ERβ-specific antibody, demonstrated a strong nuclear signal in formalin-fixed material (data not shown). Because we have shown that ERβ variant isoforms exist in breast tumor cell lines (5), we included in vitro-translated ERβ1 and the COOH-terminal splicing variant ERβ2, along with ERα and extracts from MCF-7 cells, to examine the specificity of the 14C8 monoclonal ERβ antibody in immunoblot analysis (Fig. 1B). Our antibody reacted specifically with ERβ, but not with ERα, and detected full-length ERβ1 of approximately M, 58,000–60,000 in MCF-7 cells. Because our antibody was generated to a fusion protein of the NH2-terminal region of ERβ, it also detected the COOH-terminal-truncated ERβ2 isoform. This result suggests that our antibody is capable of detecting total ERβ expres-
ERβ EXPRESSION IN BREAST TUMORS

We next performed immunohistochemical analysis on a panel of whole tissue sections from primary breast tumors; representative photographs from one tumor are shown in Fig. 2. To look for heterogeneity of ERβ expression in the same tumor using whole sections, we examined three different areas of the same tumor and found the same pattern of staining throughout the tissue (compare Fig. 2, A–C). Thus, the expression of ERβ appears to be homogeneous. This finding suggests that the use of TAs would be representative of a given tumor sample, and in our experience, we have not seen significant heterogeneity of ERβ expression within a tumor to be concerned about sampling error with this antibody.

Validation of TAs. TAs offer the advantage of rapid staining and scoring of a large number of tumors. An obvious question, however, is whether the results obtained from TAs are indeed representative of results obtained from larger specimens as suggested by the homogeneity of staining obtained and shown in Fig. 2. To address this question, we arrayed 261 human breast tumors on 9 tissue blocks and stained for ERα using the NCL-ER-6F11 and ERβ antibodies. Representative IHC staining of four breast tumor specimens for ERα and ERβ is shown in Fig. 3. For comparison, ERα had previously been determined in larger sections from these same tumors using IHC with the NCL-ER-6F11 antibody (19). ERα results were obtained in the TAs from 237 of the 261 tumors (91%). Complete concordance of total IHC scores between TA and the previously stained, larger sections was observed in 46% of cases, and concordance within one IHC score was observed in 82% of cases. The Spearman correlation coefficient was 0.76. When results were classified as ER positive (ER+) or ER negative (ER−) using our previously defined criteria (total IHC score $\geq 2 = \text{ER+}$), only 12 cases (5%) were discordant (11 were ER+ on larger sections but ER− on TA; 1 was ER− on larger sections but ER+ on TA).

To put these results in perspective, we examined the inter-rater reliability of scoring the same slides from the larger sections. Complete concordance of total IHC scores from two independent pathologists was observed in 72% of cases, and concordance within one IHC score was observed in 99% of cases. The Spearman correlation coefficient was 0.92. Only two cases had different qualitative outcomes, and in each case, the results differed by only one score (IHC = 3 and 2, ER+ and ER−, respectively).

Therefore, we conclude that the scoring system is reproducible between observers, and although there is a slight loss of sensitivity, TAs yield results comparable with those obtained on larger specimens.

Distribution of ERβ Expression. Results for ERβ were obtained from 242 tumors on the TAs. Nuclear staining was observed in 184 (76%) of the cases. Although the rate of staining is very similar to that observed for ERα, the pattern is somewhat different (Table 1), with higher levels of ERα expression detected in the tumors. The Spearman correlation coefficients between ERβ and ERα were relatively modest ($r_sp = 0.33$ and 0.36, respectively, for TA and larger samples).

Relationships between ER Expression and Other Prognostic Factors. Spearman correlation coefficients between ERβ and ERα and other prognostic factors are displayed in Table 2. ERβ expression was positively correlated with ERα determined by IHC ($r_sp = 0.33$), but this relationship was not seen with ER LBAs ($r_sp = 0.09$). This result is consistent with the report by Dotzlaw et al. (20) showing that expression of ERβ RNA using reverse transcription-PCR amplification was not significantly correlated with ER by LBA in breast tumors. Similarly, ERβ expression was positively correlated with PR determined by IHC ($r_sp = 0.25$), but not with PR by LBA ($r_sp = 0.05$). The very strong, positive correlations between ERα by

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**Fig. 1.** Western blot comparison of ERα NCL-ER-6F11 antibody and our 14C8 ERβ antibody. Lysate (5 μl) from in vitro translation reactions primed with pSG5 expression plasmid containing either ERβ1 (β1) or ERβ2 (β2) and cell lysate (50–100 μg) prepared from the MCF-7 breast carcinoma cell line were electrophoresed on SDS-PAGE gels and transferred to nylon filters. The blots were incubated with the NCL-ER-6F11 antiserum diluted 1:500 (A) or ERβ antiserum diluted 1:200 (B). Binding of primary antibody was detected with horseradish peroxidase-conjugated secondary antibody.

**Fig. 2.** IHC of a representative breast tumor using a whole tissue section and our ERβ-specific 14C8 antibody. A–C represent three different areas of the same tumor staining homogeneously positive for ERβ protein expression.
IHC and ER by LBA, both in this study and in our previous larger study (19), suggest that the ER status of breast tumors as defined by the standard clinical dextran-coated charcoal assay fails to accurately reflect the levels of ERβ present in breast tumors.

No significant relationships were observed between ERβ expression and tumor grade, proliferation determined by Ki67 staining, SPF, or DNA ploidy. In contrast, ERα expression was significantly correlated with each of these prognostic factors, as we have previously described for ER by LBA (21), providing additional evidence that the samples in this pilot study are representative of clinical breast cancer. The different results for ERβ and ERα suggest that ERβ expression is not a surrogate for ERα, and is not correlated with the same clinical parameters as ERα. As such, ERβ could predict different biological features of breast tumors, but unfortunately, ERβ-specific functions or signaling pathways that may be clinically important for tumor progression have not yet been elucidated.

**ERα Expression Is the Major Determinant of PR Expression in Breast Tumors.** Although the study was relatively small, we next investigated the biological importance of ERα and ERβ coexpression and interactions with other prognostic factors. We defined tumors to be positive for either ER if its IHC scores were greater than 2. This corresponds to our published definition for ERα (19), which was based on correlations with clinical outcome, but is admittedly arbitrary for ERβ. Using these definitions, 9% of the tumors (21 of 234) were negative for both receptors, 14% (32 of 234) were positive only for ERα, 15% (36 of 234) were positive only for ERβ, and 62% (145 of 234) were positive for both receptors. Examples of all four categories are seen in Fig. 1. We found (Table 3) that receptor-negative tumors rarely (5% by LBA and 14% by IHC) expressed the PR. However, PR expression by IHC was more closely associated with ERα expression than ERβ expression: PR was expressed in 88% of ERα-positive/ERβ-negative tumors but in only 6% of the ERα-negative/ERβ-positive tumors (P < 0.0001). Similar results were found for PR by LBA.

**ERα Expression Is the Major Determinant of Some, but not All, Breast Cancer Prognostic Factors.** High tumor grade and high SPFs were significantly related to ERα expression, and the same strong relationships were observed when patients were further stratified by ERβ status (Table 3). For example, 24% of tumors with both receptors had high SPF, compared with 25% of tumors in the ERα-positive/ERβ-negative subset of tumors; conversely, 60% of tumors lacking both receptors had high SPF, compared with 67% of the ERα-negative/ERβ-positive subset. Based on these data, one could speculate that coexpression of ERβ may have little impact on the prognosis of patients with breast tumors, at least as assessed by established factors such as proliferation and tumor grade.

However, the relationships with DNA ploidy appear to be more complex. There was a trend (P = 0.12) for tumors with both receptors to be more aneuploid (59%), compared with ERα-positive/ERβ-negative tumors (44%). Similarly, the rate of aneuploidy was marginally higher (P = 0.20) in ERα-negative/ERβ-positive tumors (82%), compared with tumors that lack both receptors (65%), suggesting that ERβ-positive tumors might be more aggressive biologically, similar in characteristics to the receptor-negative breast tumors. Therefore, for ERα, 15% (36 of 234) were positive only for ERβ, and 62% (145 of 234) were positive for both receptors. Examples of all four categories are seen in Fig. 1. We found (Table 3) that receptor-negative tumors rarely (5% by LBA and 14% by IHC) expressed the PR. However, PR expression by IHC was more closely associated with ERα expression than ERβ expression: PR was expressed in 88% of ERα-positive/ERβ-negative tumors but in only 6% of the ERα-negative/ERβ-positive tumors (P < 0.0001). Similar results were found for PR by LBA.

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the specific measurement of ERβ, combined with the measurement of ERα by IHC, might provide useful clinical information in certain breast cancer patients, a possibility that will require a larger study with clinical follow-up information to validate.

**DISCUSSION**

ER and PR are measured in breast tumor specimens for prognostication of disease recurrence and prediction of treatment response. In guidelines published by the American Society of Clinical Oncology Tumor Marker Expert Panel (22), ER and PR were the only biomarkers recommended for routine use in the management of patients with breast cancer. The ER assay is most useful if the tumor is ER positive. Predicting the probability of response in ER-positive patients is more difficult. Overall, approximately 50–60% of women with ER-positive, advanced breast cancer will receive some degree of benefit from endocrine treatment (3). With the discovery of the second ER subtype, ERβ, it was reasonable to hope that an accurate measurement of the two forms might provide additional prognostic or predictive clinical information.

An accurate assessment of ERβ expression requires the availability of specific antibodies useful for routinely fixed clinical material. Only recently have antibodies to ERβ become available (23, 24), but unfortunately, most of these have not proven useful for IHC or worked only in frozen material (8). Although we had previously developed two antibodies to ERβ that were specific in Western blot analyses (5), neither of these antibodies worked in paraffin-embedded sections. Therefore, we developed a third new antibody, now called 14C8, which is capable of recognizing ERβ protein in archival samples. Because the antibody was prepared to the NH2-terminal region of the protein, it is capable of recognizing putative COOH-terminal-truncated forms as well as full-length ERβ, thus presumably detecting total translated ERβ protein, unlike many of the other commercially available ERβ antibodies (8).

We found that the majority of breast tumors coexpressed both receptors, which is in agreement with a number of other published studies. In a study of 60 tumors, Speirs et al. (7) hypothesized that breast tumors coexpressing both receptors, as opposed to those only expressing ERα, were more frequently associated with poor prognostic biomarkers, such as positive axillary nodes and higher tumor grade. This is certainly a viable hypothesis, given that the two receptors can form functional heterodimers on DNA (25) and that the heterodimer may be preferentially formed as opposed to homodimers (26). However, our results do not completely agree with this hypothesis. For instance, when comparing high tumor grade and high SPF with ER status, we found little difference between these two clinical parameters in tumors coexpressing both receptors versus those only expressing ERα. These results could lead us to hypothesize that ERβ expression might have little clinical impact on ERα function. However, we also found that tumors expressing both receptors tended to be more aneuploid, and tumors expressing only ERβ exhibited even slightly higher rates of aneuploidy. Our observed association between ERβ expression and tumor aneuploidy undoubtedly needs to be validated in a larger data set, but it does suggest an intriguing biological association for ERβ in breast tumors that has not been previously appreciated.

We know very little about the specific function of ERβ in the breast. Studies of ERβ knockout mice suggest that, although these mice have impaired ovarian function, breast development and function are not compromised (27). It has also been suggested that ERβ may function to inhibit the induction of PR by ERα, at least in the normal rodent mammary gland (28). However, this does not appear to be the case in human breast tumors, where there was no difference between the induction of PR in tumors expressing both receptors compared with those expressing only ERα. Our results strongly support the conclusion that ERα rather than ERβ is the predominant regulator of PR expression in clinical breast cancers, in agreement with reports of an inverse correlation of ERβ RNA expression and levels of the PR (20).

The failure of ERβ IHC measurements to correlate with ER or PR LBA is another relatively surprising finding from our study, especially because ERβ has a similar binding capacity for estradiol as ERα (2). Again, there are controversial data in the literature concerning this question, with one study also reporting no correlation between ERβ RNA and ER LBA (20), and one study reporting a good correlation between ERβ protein (using a COOH-terminal-specific ERβ antibody), and PR IHC (8). These discordant results highlight the necessity of developing reliable ERβ-specific antibodies useful for clinical studies. In addition, because there are a number of COOH-terminal-truncated forms of ERβ (9), it is uncertain what forms may be measured when using these different assays. It is possible that expression of these ERβ variant forms, especially in the ERα-negative/ERβ-positive subgroup of breast tumors, is indicative of a particular “bad” prognosis, equivalent to the truly ER-negative group.

Finally, RNA-based ERβ studies have also raised the possibility that the presence of ERβ in breast tumors may be a marker of endocrine therapy resistance. In a limited but provocative study of 17 breast cancer patients with treatment response follow-up, ERβ was significantly elevated in the tamoxifen-resistant group of tumors (29). This would be consistent with our observed inverse correlation with PR because PR is a known marker of endocrine responsiveness. We did not have endocrine response data on our pilot study of 242 breast tumors, but a predictive clinical study is currently under way in tumors archived in the Baylor Breast Tumor Bank.

In summary, our results suggest that ERα rather than ERβ expression is correlated with most, but perhaps not all, prognostic factors in breast cancer. Furthermore, we present data suggesting that ERβ is not just a surrogate for ERα in breast cancer prognosis and may have distinct but as yet unknown functions. Assessment of the ultimate clinical utility of ERβ IHC in breast cancer prognosis and its possible usefulness for the prediction of treatment response awaits its examination in large clinical studies.
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