Estrogen Receptor α – Negative Breast Cancer Tissues Express Significant Levels of Estrogen-Independent Transcription Factors, ERβ1 and ERβ5: Potential Molecular Targets for Chemoprevention

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Abstract We have investigated the expression of two estrogen receptor β (ERβ) isoforms, ERβ1 and ERβ5, which activate gene transcription independent of estrogen or growth factors, in ERα-negative breast cancer tissues. We report here, for the first time, that ERα-negative tissues express significant levels of ERβ1 and ERβ5, and their expression levels are not different from levels in ERα-positive tumors. However, significant differences exist between the two racial groups, African American and Caucasian, in that the patients from the former group express higher levels of ERβ1 and ERβ5 but not ERα. These two transcription factors could be potential molecular targets for designing chemopreventive drugs to treat ERα-negative breast cancers.

It is now well accepted that unopposed stimulation of breast epithelial cells by the natural hormone, estrogen, plays a major role in the advancement of breast cancers. Although the exact mechanism(s) by which estrogen causes breast cancer progression are not known, several studies have established that increased gene transcription by estrogen-activated transcription factor, the estrogen receptor α (ERα), leads to genetic/cellular aberrations and the genesis and progression of breast cancer. Because endogenous estrogens directly affect the growth of breast cancer cells, estrogen deprivation either by inhibiting its biosynthesis or blocking estrogen-mediated gene transcription through ERα is the primary line of therapy for all ERα-positive cancers. Clinical studies have shown that only ERα-positive tumors but not ERα-negative tumors respond to the above two therapies. The ERα-negative patients do not have the benefits of relatively safe and effective targeted endocrine therapies, because their cancers are considered to be estrogen independent.

In an effort to develop alternate endocrine therapies for ERα-negative breast cancer patients, we investigated whether ERβ isoforms, ERβ1 and ERβ5, which can activate the same genes as the ERα, independent of estrogen (1), are expressed in these tissues. The rational for our study is that once we establish the expression of ERβ in ERα-negative tissues, a novel line of ERβ-targeted drugs could be designed to treat ERα-negative tumors similar to ERα blockers for ERα-positive tumors. We studied the ERβ isoform expression at mRNA levels by quantitative real-time PCR and at protein levels by Western blotting and immunohistochemistry. We also compared the expression of these isoform mRNA levels with ERα-positive tissues. We report here for the first time that ERα-negative breast cancer tissues have significant levels of ERβ gene expression, and ERβ5 is the most abundantly expressed isoform. We also report here that African American patient tumors express significantly higher levels of ERβ isoforms compared with Caucasian patient tumors. We expect that our results on ERβ isoform expression in ERα-negative breast cancers will have clinical implications in designing a new line of ERβ-targeted molecular therapies to treat these cancers.

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Materials and Methods

HotStartTaq PCR core kits, Omniscript reverse transcriptase, and MinElute gel extraction kits were from Qiagen, Inc. (Valencia, CA). Taqman Universal PCR Master Mix, RNase inhibitor, and random hexamers were from Applied Biosystems (Foster City, CA). All the primers used in the current study were synthesized by Life Technologies Bethesda Research Laboratories (Carlsbad, CA), and 5′ FAM- and 3′ TAMARA-labeled oligonucleotide probes described here were synthesized at Applied Biosystems. The bp numbering for ERα and ERβ primers and probes described here were based on the sequences published by Green et al. (2) and Ogawa et al. (3), respectively. PCR quality water and Tris-EDTA buffer were from Bio Whittaker (Rockville, MD). Polyclonal antibodies against ERβ (H-150) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and monoclonal antibodies against ERβ were obtained from Genetex (San Antonio, TX).

Protease inhibitor cocktail containing AEBSF, EDTA, Bestatin, E-64 leupeptin, and aprotinin was from Sigma (St. Louis, MO). Horseradish peroxidase–conjugated goat anti-rabbit IgG and protein molecular weight standards were from Bio-Rad (Hercules, CA). Enhanced chemi-luminescence reagents were from Amersham (Piscataway, NJ).

Breast tumor samples. Breast tumor tissues were obtained from the Breast Center, Baylor College of Medicine Breast Tumor Bank (Houston, TX) and Howard University Hospital. Fresh tumor tissues were collected immediately after surgery and stored at −80°C until use. Fresh tumor tissue samples for research were routinely harvested immediately adjacent to the histologic/diagnostic sections and considered to be representative of the tissue used for diagnosis. All the samples were examined by a pathologist and tissues containing >80% cancer cells were excised and used for research. ERα status in the tissues collected from Howard University Hospital was determined immunohistochemically using monoclonal antibodies against NH_2-terminal portion of the molecule at Oncotech Laboratories. The tumor tissues were considered positive for ERα if >5% of cancer cells showed positive for nuclear staining. ERα status in tumor tissues collected from Baylor College of Medicine Breast Center Tumor Bank was determined by ligand binding assay (4). The tissues were diagnosed as ERα positive if the cancer tissue extract showed >3 fmol ER/mg total tissue extract. A total of 60 ERα-negative (20 from Caucasian and 40 from African American patients) and 74 ERα-positive (34 from Caucasian and 40 from African-American patients) cancer tissues were included in the current study. Tumor collection procedures were approved by the Institutional Review Boards of both institutions.

RNA extraction and cDNA synthesis. Total RNA was extracted from frozen breast tissues using the Trizol reagent (Life Technologies Bethesda Research Laboratories) as previously described (5). RNA integrity was verified by both electrophoresis in 1.5% agarose gels and amplification of the constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The total RNAs were reverse transcribed using Omniscript reverse transcriptase as previously described (5, 6).

Table 1. Primers and probes for quantitation of various ERs by real-time PCR

Wild type (ERβ1)
- Sense, 5′-TTTGGGTGATTGCCAAGAGC-3′ (position, exon 7, 1,411-1,430 bp)
- Antisense, 5′-AGCACGTGGGCATTCAGC-3′ (position, exon 8, 1,580-1,597 bp)
- Probe, FAM 5′-CCTCCCAGCAGCAATCCATGCG-3′ TAMARA (position, exon 7, 1,438-1,460 bp)

ERβ5
- Sense, 5′-TTTGGGTGATTGCCAAGAGC-3′
- Antisense, 5′-CACTTTTCCCAAATCACTTCACC-3′ (position, exon 8, 243-265 bp)
- Probe, FAM 5′-CCTCCCAGCAGCAATCCATGCG-3′ TAMARA

ERα
- Sense, 5′-CAAGCCCGCTCATGATCAA-3′ (position, exon 4, 1,110-1,128 bp)
- Antisense, 5′-CTGATCATGGAGGGTCAAATCCAC-3′ (position, exon 5, 1,358-1,338 bp)
- Probe, FAM 5′-AGAACAGCCTGGCCTTGTCCCTG-3′ TAMARA (position, exon 4, 1,140-1,162 bp)

GAPDH
- Sense, 5′-TTCCAGGAGCGAGATCCCT-3′ (position, 304-322 bp)
- Antisense, 5′-GGCTGTTGTCATACTTCTCATGG-3′ (position, 483-505 bp)
- Probe, FAM 5′-TGCTGGCGCTGAGTACGTCGTG-3′ TAMARA (position, 342-363 bp)

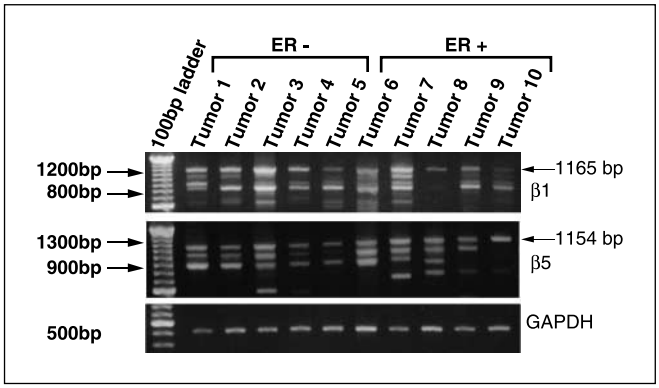

Fig. 1. Amplification of ERβ1 and ERβ5 transcripts in ERα-negative breast cancer tissues by RT-PCR. To show the presence of ERβ1 and ERβ5 in ERα-negative breast cancer tissues, cDNAs from these tissues were amplified using a sense primer in exon 1 and isoform-specific antisense primers as described in Materials and Methods. ERβ1- and ERβ5-specific primer pairs amplified 1,165-bp and 1,154-bp products respectively. The PCR products were cloned, sequenced, and identified as coding sequences of ERβ1 and ERβ5. Expression of ERβ isoform in ERα-negative cancer tissues. Representative products from five tumor cDNAs. PCR products from five ERα-positive breast cancer tissues for comparative purposes. Primers also amplified several lower molecular products presumably exon deletion variants.

Conventional PCR and identification of PCR products. Conventional PCRs were done in an automatic thermal cycler (MJ Research, Waltham, MA) as previously described (7). To amplify ERβ1, ERβ4, and ERβ5 sequences, a sense primer in exon 1, 5′-CGCTAGAACACACCTTACCTG-3′ (position, exon 1, 335-355 bp; ref. 3) and isoform-specific antisense primers, 5′-AGCACGTGGGCATTCAGC-3′ (position, exon 8, 1,481-1,499 bp; ref. 3), 5′-GTCTGGGTTTTATATCGTCTGC-3′ (position, exon 8, 1,612-1,632 bp; ref. 1), and 5′-CACTTTTCCCAAATCACTTCACCCT-3′ (position, exon 8, 1,464-1,489 bp; ref. 1) respectively, were applied. All the base pair numbering is given with reference to the translational start site. The PCR amplified products (8.0 μL) were separated by electrophoresis in 1% Nu Sieve agarose gels in Tris/acetic acid/EDTA buffer and detected by ethidium bromide staining. PCR products were purified by gel extraction, cloned into pCR2.1-TOPO vector and identified by sequence analysis as previously described (8).

Absolute quantification of ERβ1, ERβ5, and ERα mRNA copy numbers by quantitative real-time PCR. Absolute quantification of ERβ isoform and ERα mRNA copy numbers was done by quantitative real-time PCR in ABI Prism GeneAmp 7900HT Sequence Detection System at a modified 50% ramp rate as previously described (9, 10). A typical real-time PCR reaction mixture contained cDNA prepared from reverse transcription of 100 ng of tumor total RNA, 0.04 μmol/L sense and antisense primers, 0.05 μmol/L 5′FAM- and 3′TAMARA-labeled oligonucleotide probe, and 1× Taqman Universal PCR mix in a total volume of 25 μL. The PCR conditions were initial hold at 50°C for 2 minutes followed by denaturation for 10 minutes at 95°C and denaturation for 15 seconds at 95°C in the subsequent cycles and annealing and extension for 1.5 minutes at 60°C for 40 cycles. The primer pairs and probes for the quantification of ERα, ERβ1, and ERβ5 by real-time PCR are listed in Table 1. Absolute quantification of every isoform was achieved compared with a standard graph that was simultaneously generated using 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 copies of its reverse-transcribed cRNA (9, 10). All the samples were amplified in triplicate and real-time PCRs were repeated four times for every isoform and normalized to the copy numbers of the housekeeping gene, *GAPDH*, as previously described (9, 10).

Immunohistochemical staining. The presence of ERβ protein was also studied in formalin-fixed, paraffin-embedded breast cancer tissues by immunohistochemistry using monoclonal antibodies against ERβ protein. Briefly, slides were deparaffinized in two changes of toluene for 5 minutes each and gradually rehydrated through five changes of graded ethanol (100%, 90%, 70%, 50%, 30%, and distilled water, 2 minutes each). Antigens were unmasked by steam treating the slides in 10 mmol/L citrate buffer (pH 6.0) for 25 minutes. Tissue sections were incubated with blocking buffer (supplied with the antibody) and then with mouse anti-ERβ (1:100 dilution) overnight. The slides were rinsed and incubated with EnVision peroxidase conjugated secondary antibody (DakoCytomation, Mississauga, Ontario, Canada) for 30 minutes. The slides were washed and incubated with peroxidase substrate (3,3′-diaminobenzidine liquid chromogen, from DakoCytomation) for 5 minutes. Finally, the slides were washed and stained with hematoxylin, mounted, and visualized under Leica DMRXA microscope. All slides and micrographs for the above marker were evaluated for the presence of ERβ. A total of 20 tissue samples from each of the ERα-negative and ERα-positive tissues were stained for ERβ in duplicate by the above procedure. ERβ staining was compared between ERα-positive and ERα-negative tissues by scoring nuclear staining intensity and the proportion of positively stained nuclei, as described by Harvey et al. (11). Slides were scored independently by two pathologists, and mean scores were compared between ERα-positive and ERα-negative tissues.

Table 2. Expression of ERβ1, ERβ5, and ERα mRNA (copies/10^{10} copies of GAPDH) in breast cancer tissues from African-American patients

No	ERα by IHC	ERβ1	ERβ5	ERα
1	–	1×10^{6}	2×10^{7}	NA
2	–	4×10^{5}	1×10^{5}	NA
3	–	6×10^{5}	6×10^{5}	NA
4	–	4×10^{5}	2×10^{5}	NA
5	–	9×10^{4}	3×10^{5}	NA
6	–	1×10^{7}	1×10^{7}	NA
7	–	8×10^{4}	2×10^{5}	NA
8	–	7×10^{4}	8×10^{5}	NA
9	–	1×10^{6}	5×10^{8}	NA
10	–	2×10^{5}	3×10^{5}	NA
11	–	6×10^{5}	1×10^{6}	NA
12	–	7×10^{5}	5×10^{6}	NA
13	–	4×10^{5}	4×10^{6}	NA
14	–	4×10^{5}	3×10^{6}	NA
15	–	8×10^{4}	2×10^{5}	NA
16	–	2×10^{5}	2×10^{6}	NA
17	–	4×10^{4}	6×10^{5}	NA
18	–	4×10^{7}	3×10^{7}	NA
19	–	2×10^{5}	3×10^{6}	NA
20	–	4×10^{5}	3×10^{6}	NA
21	–	1×10^{6}	9×10^{6}	NA
22	–	4×10^{5}	1×10^{6}	NA
23	–	6×10^{5}	1×10^{6}	NA
24	–	7×10^{4}	2×10^{6}	NA
25	–	5×10^{4}	4×10^{5}	NA
26	–	1×10^{4}	3×10^{6}	NA
27	–	6×10^{5}	9×10^{5}	NA
28	–	3×10^{4}	5×10^{6}	NA
29	–	3×10^{5}	1×10^{6}	NA
30	–	9×10^{5}	7×10^{6}	NA
31	–	9×10^{4}	4×10^{6}	NA
32	–	8×10^{5}	2×10^{6}	NA
33	–	3×10^{5}	3×10^{7}	NA
34	–	4×10^{6}	4×10^{7}	NA
35	–	2×10^{4}	5×10^{6}	NA
36	–	3×10^{5}	2×10^{5}	NA
37	–	3×10^{4}	2×10^{5}	NA
38	–	2×10^{5}	3×10^{5}	NA
39	–	5×10^{4}	3×10^{5}	NA
40	–	8×10^{5}	5×10^{6}	NA
41	+	1×10^{5}	3×10^{6}	5×10^{6}

Table 2. Expression of ERβ1, ERβ5, and ERα mRNA (copies/10^{10} copies of GAPDH) in breast cancer tissues from African-American patients (Cont'd)

No	ERα by IHC	ERβ1	ERβ5	ERα
42	+	7×10^{5}	3×10^{6}	2×10^{7}
43	+	4×10^{4}	4×10^{6}	2×10^{8}
44	+	3×10^{5}	8×10^{5}	6×10^{6}
45	+	4×10^{5}	1×10^{6}	4×10^{7}
46	+	5×10^{6}	5×10^{6}	4×10^{8}
47	+	8×10^{7}	5×10^{10}	5×10^{9}
48	+	3×10^{4}	3×10^{6}	2×10^{7}
49	+	5×10^{4}	1×10^{6}	6×10^{7}
50	+	2×10^{5}	3×10^{6}	5×10^{6}
51	+	3×10^{5}	3×10^{6}	4×10^{7}
52	+	2×10^{5}	1×10^{6}	3×10^{7}
53	+	7×10^{4}	8×10^{5}	1×10^{6}
54	+	4×10^{5}	2×10^{6}	2×10^{8}
55	+	1×10^{6}	4×10^{6}	3×10^{7}
56	+	6×10^{5}	3×10^{6}	6×10^{6}
57	+	5×10^{5}	2×10^{6}	1×10^{7}
58	+	3×10^{6}	2×10^{7}	1×10^{7}
59	+	1×10^{6}	3×10^{6}	6×10^{7}
60	+	3×10^{4}	6×10^{4}	4×10^{7}
61	+	3×10^{5}	2×10^{6}	1×10^{8}
62	+	8×10^{4}	1×10^{6}	4×10^{7}
63	+	5×10^{5}	1×10^{6}	6×10^{7}
64	+	5×10^{4}	8×10^{5}	6×10^{7}
65	+	5×10^{7}	8×10^{8}	4×10^{8}
66	+	9×10^{6}	5×10^{6}	4×10^{7}
67	+	4×10^{4}	1×10^{7}	8×10^{7}
68	+	5×10^{4}	3×10^{6}	2×10^{7}
69	+	5×10^{6}	1×10^{7}	1×10^{8}
70	+	5×10^{5}	3×10^{7}	3×10^{7}
71	+	3×10^{4}	2×10^{5}	6×10^{6}
72	+	7×10^{6}	1×10^{7}	4×10^{7}
73	+	4×10^{5}	2×10^{6}	1×10^{6}
74	+	3×10^{4}	7×10^{5}	4×10^{7}
75	+	4×10^{5}	7×10^{6}	2×10^{6}
76	+	4×10^{5}	2×10^{6}	3×10^{7}
77	+	7×10^{4}	1×10^{6}	5×10^{7}
78	+	2×10^{5}	1×10^{5}	2×10^{7}
79	+	5×10^{4}	1×10^{6}	6×10^{7}
80	+	4×10^{5}	3×10^{6}	3×10^{7}

Abbreviations: NA, not applicable; IHC, immunohistochemistry.

Table 3. Expression of ERβ1, ERβ5, and ERα mRNA (copies/10^{10} copies of GAPDH) in breast cancer tissues from Caucasian patients

No	ERα by IHC	ERβ1	ERβ5	ERα
1	–	9×10^4	2×10^5	NA
2	–	2×10^4	1×10^5	NA
3	–	8×10^4	2×10^6	NA
4	–	2×10^5	1×10^6	NA
5	–	4×10^4	2×10^6	NA
6	–	5×10^4	3×10^6	NA
7	–	4×10^4	6×10^5	NA
8	–	2×10^4	5×10^5	NA
9	–	7×10^4	3×10^5	NA
10	–	5×10^4	2×10^5	NA
11	–	6×10^4	6×10^5	NA
12	–	3×10^4	6×10^5	NA
13	–	3×10^4	2×10^6	NA
14	–	5×10^4	6×10^5	NA
15	–	2×10^4	5×10^5	NA
16	–	7×10^4	5×10^4	NA
17	–	5×10^4	3×10^5	NA
18	–	8×10^4	7×10^5	NA
19	–	2×10^5	2×10^6	NA
20	–	3×10^4	3×10^4	NA
21	+	8×10^4	1×10^7	2×10^7
22	+	2×10^4	1×10^6	9×10^5
23	+	2×10^4	3×10^5	5×10^7
24	+	9×10^4	1×10^5	3×10^7
25	+	1×10^4	1×10^6	4×10^7
26	+	5×10^4	6×10^5	2×10^7
27	+	1×10^4	2×10^5	6×10^5
28	+	7×10^4	1×10^5	4×10^6
29	+	4×10^4	2×10^5	4×10^8
30	+	1×10^4	4×10^5	2×10^7
31	+	2×10^5	1×10^6	2×10^7
32	+	2×10^5	1×10^6	1×10^7
33	+	1×10^4	5×10^5	1×10^7
34	+	8×10^4	6×10^5	1×10^8
35	+	8×10^4	8×10^5	1×10^7
36	+	1×10^4	1×10^5	4×10^5
37	+	4×10^4	4×10^5	4×10^6
38	+	8×10^4	5×10^5	4×10^7
39	+	7×10^4	2×10^5	1×10^6
40	+	8×10^4	5×10^5	4×10^7
41	+	8×10^4	1×10^6	4×10^8
42	+	7×10^4	4×10^6	6×10^7
43	+	6×10^4	9×10^5	2×10^8
44	+	3×10^4	8×10^5	5×10^7
45	+	1×10^5	1×10^6	4×10^7
46	+	1×10^4	3×10^5	1×10^6
47	+	1×10^4	3×10^5	3×10^7
48	+	4×10^4	1×10^5	3×10^7
49	+	6×10^4	2×10^6	3×10^6
50	+	8×10^4	8×10^5	2×10^8
51	+	3×10^4	9×10^5	2×10^7
52	+	4×10^4	2×10^6	1×10^8
53	+	1×10^5	8×10^5	3×10^8
54	+	4×10^4	1×10^5	5×10^7

Abbreviations: NA, not applicable; IHC, immunohistochemistry.

Protein extraction, electrophoresis, Western blotting, and other methods. For extracting total proteins from tumor samples, ~10 mg of fresh frozen tumor tissues were homogenized for 5 minutes at 4°C using 100 μL of 10 mmol/L Tris-HCl buffer (pH 7.6) containing 150 mmol/L NaCl, 1% Triton 100-X, and 1% sodium deoxycholate using a T line laboratory stirrer. The extracts were centrifuged at 15,000 × *g* for 30 minutes at 4°C and the supernatant was stored at −80°C. The presence of ERβ protein(s) in 20 μL (20 ERα negative and 20 ERα positive) extracts were tested by Western blotting using a dilution of 1:200 anti-ERβ polyclonal antibodies (H-150). A 20-μL tumor extract was also probed for the expression of a housekeeping gene, *β-actin*, using a dilution of 1:100 anti-actin polyclonal antibodies from Santa Cruz Biotechnology. Protein gel electrophoresis and Western blotting were done as described previously (12, 13). SDS-PAGE (15%) was conducted in a Bio-Rad slab gel apparatus as described by Laemmli (14). Proteins were transblotted to nitrocellulose membranes as described by Towbin et al. (15). Blocking and antibody treatments were done as described (12, 13). The antigen-antibody complexes were detected using a 1:7,500 dilution of the horseradish peroxidase–conjugated goat anti-rabbit IgG and development with the enhanced chemiluminescence detection system.

Statistical analysis. The expression of ERβ isoforms was compared between ERα-positive and ERα-negative tumors and between two racial groups using Wilcoxon rank sum test (two sided). The association between the expression of every ERβ isoform with grade, stage, nodal status, histologic type, menopausal status, and progesterone receptor status was also tested using Wilcoxon rank sum test (nonparametric ANOVA). Test results were considered significant if $P \leq 0.05$.

Results

ERα-negative breast cancer tissues have significant levels of ERβ gene expression. We first tested the expression of ERβ1, ERβ4, and ERβ5 in ERα-negative tissues by conventional reverse transcription-PCR (RT-PCR) using a sense primer in exon 1 and isoform-specific antisense primers. The ERβ1- and ERβ5-specific primer pairs generated expected PCR products of 1,165 and 1,154 bp, which were identified by sequence analyses as ERβ1 and ERβ5, respectively (Fig. 1). However, the ERβ4-specific primer pair did not generate any product, indicating the absence of this isoform in breast cancer tissues, consistent with previous observations (10).

We next quantitatively determined ERβ1 and ERβ5 expressions at mRNA levels using molecular assays developed by us (10) based on reverse transcriptase quantitative real-time PCR and isoform-specific primers and probes (Table 1). Using the quantitative methods, we were able to precisely quantify the exact copy numbers of each isoform mRNAs with respect to the mRNA copy numbers of the housekeeping gene, *GAPDH*. For comparative purposes,

we also determined the expression levels of the above receptors and wild-type ERα in ERα-positive tumor tissues by real-time PCR. The expression levels of ERβ1 and ERβ5 in ERα-negative and ERα-positive tissues are shown in Tables 2 and 3. The mean values and SD are presented in Table 4 and shown as histograms in Fig. 2. ERα-negative tissues expressed significant levels of ERβ1 and ERβ5, and their expression levels are not statistically different from ERα-positive tissues.

In addition to the mRNA levels, we also established the presence of ERβ protein(s) in ERα-negative patient tumors, by Western blotting the tumor extracts and immunohistochemistry of formalin-fixed, paraffin-embedded samples. The expression of ERβ protein(s) in eight representative ERα-negative and seven ERα-positive tumor tissues by Western blotting is shown in Fig. 3. Two closely spaced bands of M_r 55 to 58 kDa were visualized when the Western blots were probed with polyclonal antibodies specific to ERβ protein. All the 20 samples tested from each group were positive and gave similar pattern by this procedure. To determine any differences in the protein levels between ERα-positive and ERα-negative tissues, the ERβ protein bands in the Western blots were scanned, normalized to the housekeeping gene, *βactin*, and the normalized values were compared between ERα-negative and ERα-positive tissues. By this procedure, we did not find any significant differences in the levels of ERβ proteins in ERα-positive and ERα-negative samples.

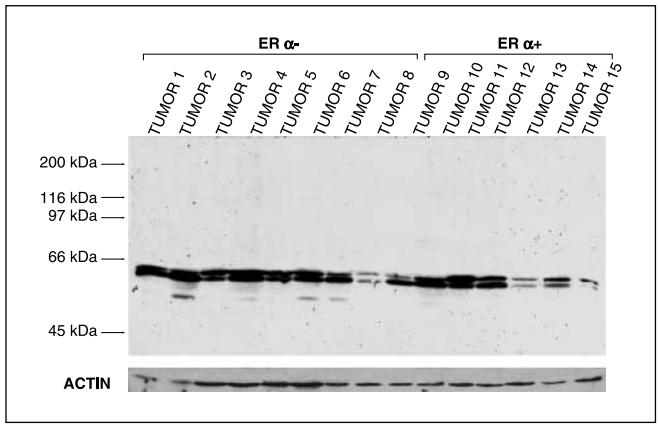

Fig. 3. Expression of ERβ proteins in ERα-negative breast cancer tissues by Western blotting. To show the presence of ERβ proteins, the cancer tissues were homogenized and proteins extracted as described in Materials and Methods. The tumor extracts (20 μL) were separated by SDS-PAGE and immunoblotted using polyclonal antibodies against ERβ. Two closely spaced bands of M_r 55 to 58 kDa were observed showing the presence of ERβ protein(s) in these tissues. ERβ protein expression in ERα-positive cancer tissues for comparative purposes. No significant differences were observed in ERβ protein expression levels ERα-positive or ERα-negative tumor tissues.

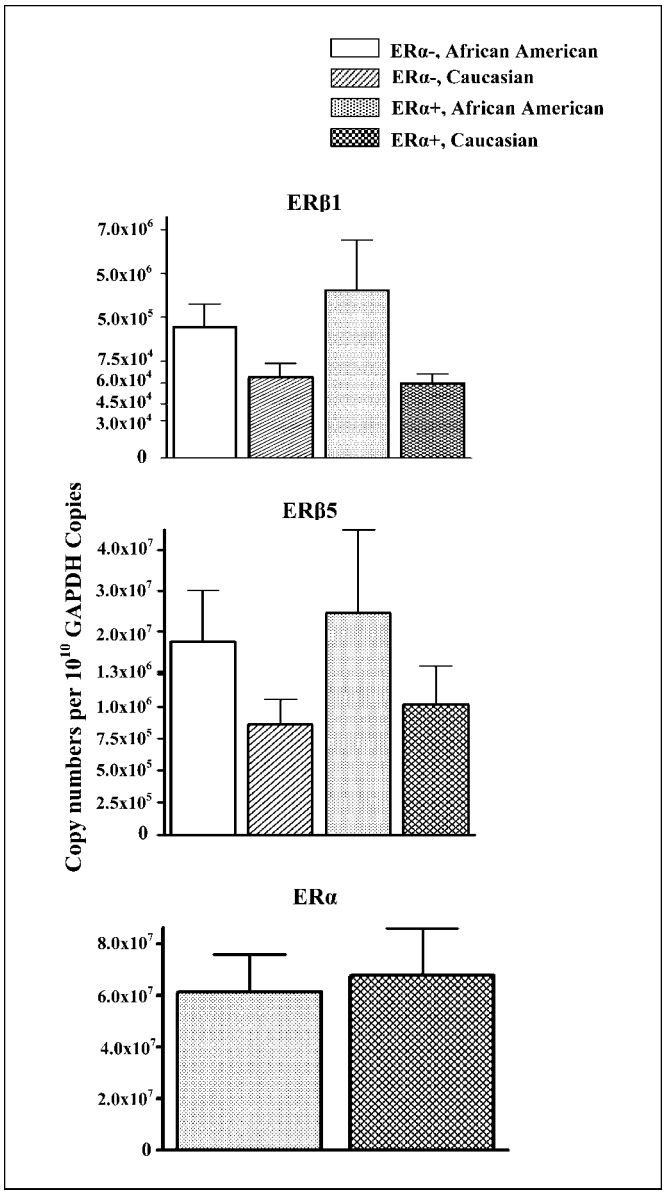

Fig. 2. Expression levels of ERβ1 and ERβ5 transcripts in ERα-negative and positive tissues from African-American and Caucasian patient tumors. Columns, mean expression levels of these two receptors in the two racial groups; bars, SD. The expression level of ERα is also shown in the positive tissues.

By immunohistochemistry, we observed strong nuclear staining when probed with monoclonal antibodies obtained from Genetex (Fig. 4) in ERα-negative tissues. The polyclonal antibodies, although they detected ERβ protein(s) in Western blots, were not suitable for immunohistochemistry. All 20 tissues tested from each group were positive for ERβ by immunohistochemistry procedure. For comparative purposes, the ERβ protein expression in ERα-positive tissues by immunohistochemistry is also shown in Fig. 4.

Expression levels of ERβ isoforms are different in breast tumors of Caucasian and African-American patient groups. To test whether ERβ gene expression in the two racial groups are similar, we compared the data obtained on mRNA levels of ERβ isoforms by quantitative real-time PCR (Tables 2 and 3) using statistical procedures described in Materials and Methods. Statistical analyses of data showed that African-American patient tumors expressed significantly higher levels of these receptor mRNAs in both types of tissues compared with Caucasian patient tumors. (ERα-negative tissues, $P = 0.0048$ for ERβ1 and $P = 0.0213$ for ERβ5; ERα-positive tumors, $P = 0.0004$ for ERβ1 and $P = 0.0002$ for ERβ5; all by two-sided Wilcoxon rank sum tests). Interestingly, ERα mRNA levels in ERα-positive tissues were not significantly different in the two racial groups (Tables 2 and 3 and Table 4; Fig. 2). However, the expression levels of the above two receptor mRNAs were not associated with tumor grade, stage of the cancer, histologic

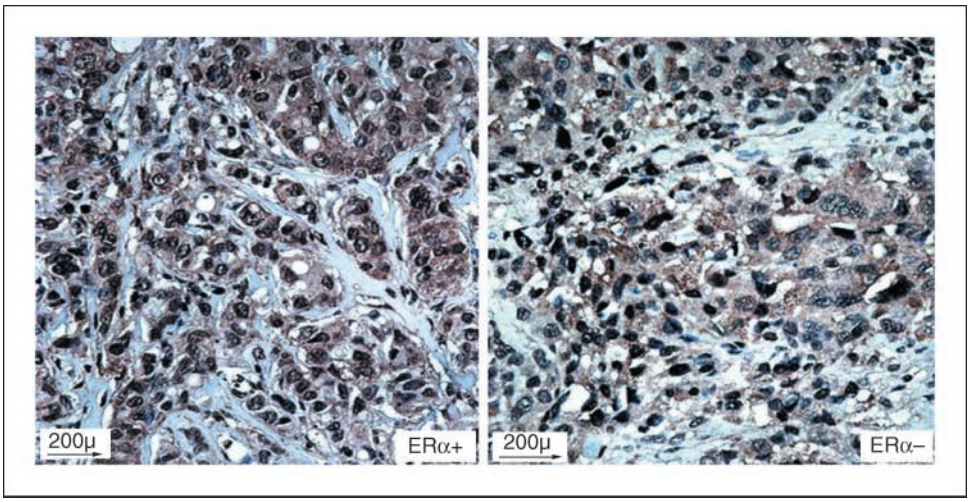

Fig. 4. ERβ protein expression in ERα-negative breast cancer tissues by immunohistochemistry. To show ERβ protein expression in the ERα-negative tissues, paraffin sections from these tissues were immunostained using monoclonal antibodies against ERβ as described in Materials and Methods. Strong staining could be visualized in the nuclei of ductal epithelial cells. Representative of immunohistochemically stained ERα-negative tissues. A representative from ERα-positive tissues for comparative purposes.

type, menopausal status, progesterone receptor, or nodal status either in the ERα-positive or ERα-negative tumors (data not shown).

Discussion

It is now widely accepted that aberrant expression of growth-promoting genes by the transcription factor, the ERα, signaled through estrogen or growth factors, promotes survival and progression of breast cancer cells. When the ERα expression is lost, it is assumed that breast cancer cells gain the ability to progress in the absence of estrogen. Although the mechanism(s) by which the cancer-promoting genes are expressed in the absence of ERα are not known, it is presumably by other transcription factors that have the ability to activate their expression independent of estrogen. One group of molecules that can activate the same genes as ERα in the absence of estrogen or growth factors includes ERβ isoforms, ERβ1 and ERβ5 (1, 16, 17). The isoform ERβ5 was recently cloned by our group and shown to have thrice higher estrogen-independent transcriptional activity than ERβ1 (1).

In the cells where both ERα and ERβ isoforms are expressed, the primary function of ERβs seems to regulate the degree of estrogen action by negatively modulating ERα, and the estrogen-independent transcriptional activity of ERβ isoforms is inhibited by ERα (1, 16–18). In the absence of inhibiting ERα, as in the case of ERα-negative breast cancer tissues, ERβ1 and ERβ5 could contribute to tumor progression by activating the transcription of cancer-promoting genes, independent of estrogen or growth factors. However, there were no reports to show whether ERα-negative breast cancer tissues express significant levels of ERβ isoforms.

A number of groups investigated the expression of ERβ in breast cancer tissues by RT-PCR and immunohistochemical methods. All the reports to date established that breast cancer tissues express ERβ mRNA and protein, although at levels lower than the normal breast tissues (19–25). However, most of the studies conducted thus far focused on ERα-positive tissues, and there is little information on ERβ expression in ERα-negative tumors. Jenson et al. (26) studied the expression of ERβ by immunohistochemistry in 11 ERα-negative tumor tissues and reported its presence in seven tissues. Shaw et al. (27) studied in six ERα-negative tissues by RT PCR and 17 tissues by immunohistochemistry. They reported the presence of ERβ mRNA in 3 of 6 and protein in 7 of 17 tissues studied. However, none of the above studies distinguished between ERβ1 and ERβ5 or reported quantitative differences between ERα-positive and ERα-negative tumors.

In the current study, we investigated the expression levels of ERβ1 and ERβ5 isoforms in ERα-negative tissues at mRNA using isoform-specific molecular assays and protein levels by immunohistochemical and Western blotting methods. The rational for our studies is that once we establish the presence of ERβ isoforms in ERα-negative tissues, these molecules

Table 4. Expression levels of ERβ1 and ERβ5 (mean and SD) in ERα-negative and ERα-positive breast cancer tissues (copies/10^{10} of GAPDH)

Isoform	ERα negative		ERα positive	
	African-American	Caucasian	African-American	Caucasian
ERβ1	5×10^5 and 1×10^5	6×10^4 and 1×10^4	1×10^6 and 3×10^5	6×10^4 and 8×10^3
ERβ5	2×10^7 and 1×10^7	9×10^5 and 2×10^5	3×10^7 and 2×10^7	1×10^6 and 3×10^5
ERα	NA	NA	6×10^7 and 1×10^7	7×10^7 and 2×10^7

Abbreviation: NA, not applicable.

could be targeted for molecular therapy similar to ERα-blocking drugs for ERα-positive tumors. Inhibiting the estrogen-independent gene activation by ERβs could slow or completely block the progression of ERα-negative cancers. The data presented here established that ERα-negative tissues have significant levels of ERβ gene expression. The data presented above also established for the first time that ERα-negative tissues express ERβ isoforms at levels similar to ERα-positive tissues, and ERβ5, which was recently been characterized by us (1), is the most abundant isoform. These observations show that the ERβ expression is independent of ERα gene expression. Although the expression of ERβ1 is far less than ERα levels, ERβ5 levels are comparable with ERα levels of the ERα-positive tissues (Tables 2, 3 and 4).

Although all tumor tissues analyzed expressed both ERβ1 and ERβ5 isoforms, a wide variation was observed in between tissues as seen in Table 2. However, the variation in the expression levels of neither ERβ1 nor ERβ5 significantly correlated with tumor characteristics. Similar observations were made by Fuqua et al. (21). They reported the presence of ERβ in 76% of 242 tissues by immunohistochemistry, but the presence did not correlate with tumor grade or S-phase fraction. The negativity observed in 24% of tumors by Fuqua et al. and others (21, 26, 27) was probably due to low levels of ERβ1, which could not be detected by immunohistochemistry and lack of interaction of antibodies with ERβ5.

When we compared the levels of ERβ isoforms in tumors from African-American patients with Caucasian patients, the tumors from African-American patients showed significantly higher levels of these two receptors. This trend is seen both in ERα-positive and ERα-negative tissues (Fig. 2; Table 4). The higher levels of ERβ isoforms, particularly the most abundant estrogen-independent transcription factor, ERβ5, may contribute, in part, to poor survival observed in African-American patients (28). Given the success of ERα-blocking drugs for inhibiting tumor growth of ERα-positive tumors, the drugs that can block ERβ1 or ERβ5 or both could be potential targeted therapies for treating ERα-negative tissues. The ERβ-targeted therapies could particularly benefit African-American patients, because these patients express comparatively higher levels of ERβ isoforms and bear disproportionately higher percentage of ERα-negative tumors (29, 30).

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