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Estrogen-related Receptor α and Estrogen-related Receptor γ Associate with Unfavorable and Favorable Biomarkers, Respectively, in Human Breast Cancer[1]

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ABSTRACT

The importance of estrogen-related receptors (ERRs) in human breast cancer was assessed by comparing their mRNA profiles with established clinicopathological indicators and mRNA profiles of estrogen receptors (ERs) and ErbB family members. Using real-time quantitative PCR assays, mRNA levels of ERα, ERβ, epidermal growth factor receptor, ErbB2, ErbB3, ErbB4, ERRα, ERRβ, and ERRγ were determined in unselected primary breast tumors ($n = 38$) and normal mammary epithelial cells enriched from reduction mammoplasties ($n = 9$). ERRα showed potential as a biomarker of unfavorable clinical outcome and, possibly, hormonal insensitivity. ERRα mRNA was expressed at levels greater than or similar to ERα mRNA in 24% of unselected breast tumors, and generally at higher levels than ERα in the progesterone receptor (PgR)-negative tumor subgroup (1-way ANOVA with repeated measures, $P = 0.030$). Increased ERRα levels associated with ER-negative (Fisher's exact, $P = 0.003$) and PgR-negative tumor status (Fisher's exact, $P = 0.006$; Kruskal-Wallis ANOVA, $P = 0.021$). ERRα levels also correlated with expression of ErbB2 (Spearman's rho, $P = 0.005$), an indicator of aggressive tumor behavior. Thus, ERRα was the most abundant nuclear receptor in a subset of tumors that tended to lack functional ERα and expressed ErbB2 at high levels. Consequently, ERRα may potentiate constitutive transcription of estrogen response element-containing genes independently of ERα and antiestrogens in ErbB2-positive tumors. ERRβ's potential as a biomarker remains unclear; it showed a direct relationship with ERβ (Spearman's rho, $P = 0.0002$) and an inverse correlation with S-phase fraction (Spearman's rho, $P = 0.026$). Unlike ERRα, ERRγ showed potential as a biomarker of favorable clinical course and, possibly, hormonal sensitivity. ERRγ was overexpressed in 75% of the tumors, resulting in the median ERRγ level being elevated in breast tumors compared with normal mammary epithelial cells (Kruskal-Wallis ANOVA, $P = 0.001$). ERRγ overexpression associated with hormonally responsive ER- and PgR-positive status (Fisher's exact, $P = 0.054$ and $P = 0.045$, respectively). Additionally, ERRγ expression correlated with levels of ErbB4 (Spearman's rho, $P = 0.052$), a likely indicator of preferred clinical course, and associated with diploid-typed tumors (Fisher's exact, $P = 0.042$). Hence, ERRα and ERRγ status may be predictive of sensitivity to hormonal blockade therapy, and ERRα status may also be predictive of ErbB2-based therapy such as Herceptin. Moreover, ERRα and ERRγ are candidate targets for therapeutic development.

INTRODUCTION

Breast cancer afflicts one in eight women in the United States over their lifetime (1). ERα[3] [NR3A1, (2)] mediates estrogen responsiveness (3) and plays crucial roles in the etiology of breast cancer (4). It has been developed into the single most important genetic biomarker and target for breast cancer therapy. ERα is present at detectable levels by LB and immunohistochemical assays in ~75% of clinical breast cancers. Selection of patients with ERα-positive breast tumors increases endocrine-based therapy response rates from about one-third on unselected patients to about one-half in patients with ERα-positive tumors (5). Because expression of PgR is dependent on ERα activity, further selection of patients with ERα- and PgR-positive tumors enhances the breast cancer hormonal therapy response rate to nearly 80% (5). Although ERβ [NR3A2 (2)] also mediates responses to estrogens (3), its roles in breast cancer are not as well understood. Reports have shown that ERβ is frequently coexpressed with ERα (6), but that increased levels of ERβ are also linked with PgR-negative status (7), proliferation markers in the absence of ERα (8), and other indicators of high tumor aggressiveness (9).

Members of the ErbB family of transmembrane tyrosine kinase receptors have been implicated in the pathogenesis of breast cancer. The members include EGFR (also HER1; ErbB1), ErbB2 (HER2; Neu), ErbB3 (HER3) and ErbB4 (HER4; Ref. 10). ErbB members stimulate signal transduction pathways that involve MAPK. In response to initial binding of EGF-like peptide hormones, ErbB members form homodimers and heterodimers in various combinations to recruit distinct effector proteins (10). Although ErbB2 has not been demonstrated to interact directly with peptide hormones, it serves as a common regulatory heterodimer subunit with other ligand-bound ErbB members (11). Unlike the other ErbB members, ErbB3 lacks intrinsic kinase activity and, therefore, is required to heterodimerize with other ErbB members to participate in signaling (11).

Independent overexpression of either EGFR (12) or ErbB2 (13) associates with ER-negative tumor status, indicates aggressive tumor behavior, and predicts poor prognosis. In addition, patients whose tumors coexpress both *EGFR* and *ErbB2* exhibit a worse outcome than patients with tumors that overexpress only one of these genes (14). Overexpression of ErbB2, most often caused by gene amplification, occurs in ~15–30% of all breast cancers (13, 15). The phosphorylated form of ErbB2, indicative of this transmembrane kinase being in an activated state, may serve as an additional marker of poor prognosis (16, 17). Some (18–20), but not all (21), reports have implicated ErbB2 in the development of resistance to antiestrogens.

ErbB2 has been targeted for development of the successful clinical agent Herceptin (trastuzumab), a recombinant humanized monoclonal antibody directed against this receptor's ectodomain (22). Herceptin has been shown to be a suitable option as a first-line single-agent therapy (23) but will likely prove most beneficial as an adjuvant (24). In the near future, Herceptin will also likely be evaluated in combination with the small molecule EGFR tyrosine kinase inhibitor ZD1829 (Iressa) because it blocks transphosphorylation of ErbB2 via heterodimerization with EGFR in intact cells and inhibits the growth of breast cancer cells overexpressing both EGFR and ErbB2 (25, 26).

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[3] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase; ERR, estrogen-related receptor; ERE, ER response element; SERM, selective ER modulator; LB, ligand binding; MEC, mammary epithelial cell; Q-PCR, quantitative-PCR

The utility of ErbB3 and ErbB4 status for predicting clinical course is not as clear. ErbB3 has been observed at higher levels in breast tumors than in normal tissues, showing associations with unfavorable prognostic indicators including ErbB2 expression (27) and lymph node-positive status (28). However, it also associates with ERα-positive status, a favorable marker of hormonal sensitivity (29). ErbB4 associates with positive indicators including ERα-positive status (17, 29), more differentiated histotypes (30), and a more favorable outcome (14). Possibly, ErbB4 opposes the negative effects of ErbB2 (14, 17).

Despite the utility of ERs and ErbB members as indicators of clinical course, there remains a great need to identify additional breast cancer biomarkers. A family of potential candidate biomarkers includes the orphan nuclear receptors ERRα (31–33), ERRβ (31, 34), and ERRγ (34, 35) [NR3B1, NR3B2, and NR3B3, respectively (2)]. These orphan receptors share significant amino acid sequence identity with ERα and ERβ. They also exhibit biochemical and transcriptional activities that are similar to, yet distinct from, the ERs. Each of the ERRs has been demonstrated to bind and activate transcription via consensus palindromic EREs (36–40) as well as ERR response elements (33, 35, 37, 38, 41) composed of an ERE half-site with a 5′ extension of 3 bp. However, whereas ERs are ligand-activated transcription factors, the ERRs do not bind natural estrogens (31, 42). Instead, the ERRs likely serve as constitutive regulators, interacting with transcriptional coactivators *in vitro* in the absence of ligands (39, 41, 43) with bulky amino acid side chains in the LB pocket substituting for ligand-induced interactions (43, 44). Nevertheless, the ERRs still bind the synthetic estrogen diethylstilbestrol, but as an antagonist because it also disrupts coactivator interactions with ERRs (42). Similarly, the SERM 4-hydroxytamoxifen selectively antagonizes ERRγ in cell-based assays (40, 43, 45). Additionally, two organochlorine pesticides, toxaphene and chlordane, antagonize ERRα (46).

The transcriptional activity of each ERR depends on the promoter, the particular cell line, and the presence of ERs. For example, whereas ERRα stimulates ERE-dependent transcription in the absence of ERα in HeLa cells, it down-modulates estradiol-stimulated transcription in ERα-positive human mammary carcinoma MCF-7 cells via an active mechanism of repression (36). ERRs can also modulate transcription of at least some genes that are estrogen responsive and/or implicated in breast cancer such as *pS2* (47), *aromatase* (48), *osteopontin* (49), and *lactoferrin* (37, 50). Thus, the ERRs likely play important roles in at least some breast cancers by modulating, or substituting for, ER-dependent activities.

We sought to assess the potential utility of ERRs as novel breast cancer biomarkers in the context of ER and ErbB family members and established clinicopathological parameters. Hence, mRNA levels of ERs (ERα, ERβ), ErbB members (EGFR, ErbB2, ErbB3, ErbB4), and ERRs (ERRα, ERRβ, ERRγ) were characterized using real-time Q-PCR assays in a panel of 38 unselected primary breast cancers and 9 normal MEC preparations from mammaplastic reductions. These mRNA profiles were compared with established clinical biomarkers. Our findings indicate that ERRα and ERRγ may well be useful as negative and positive markers, respectively, of clinical course and in selection of appropriate therapies.

MATERIALS AND METHODS

Tissue Sources. Random primary breast cancer samples were obtained from the National Breast Cancer Tissue Resource Specialized Programs of Research Excellence (SPORE) at Baylor College of Medicine (Houston, TX) in the form of frozen pulverized specimens. Records of previously determined clinicopathological tumor biomarkers were maintained at the SPORE, including ER-LB and PgR-LB protein levels measured by the LB assay, and S-phase fraction and DNA ploidy determined by flow cytometry. The mRNA profiling studies were conducted in a blinded manner regarding these previously determined biomarkers. The percentage of tumor cells present in these tissue specimens was not determined. However, the vast majority of tumor samples from this tissue bank that had been prepared similarly contained at least 50% tumor cells by histological examination (51).

As a basis of comparison, mammary gland tissues were also profiled for mRNA expression. Because bulk mammary gland contains overwhelming amounts of adipose, it was necessary to enrich these samples for epithelial cells before the isolation of RNA. Hence, mammary tissues from reduction mammoplasties were processed through collagenase digestion and differential centrifugation and filtration steps (52). These enriched MECs were kindly provided by Dr. Stephen Ethier (University of Michigan-Ann Arbor, Ann Arbor, MI) and Dr. Michael N. Gould (University of Wisconsin-Madison, Madison, WI). Primary cultures of MECs obtained from reduction mammoplasties have been shown to consist of cells at different stages of differentiation and of multiple lineages including luminal and basal epithelial (myoepithelial) cells (52, 53). The normal MECs used here were not expanded in culture to minimize possible changes in RNA profiles that might occur with passage. Nevertheless, the range of expression of some of the RNAs (*i.e.*, EGFR, ErbB2, and ERRα) in these preparations of normal MECs was large, reflecting heterogeneity of mammary cell types present within these particular specimens. The use of human tissues was approved by the University of Wisconsin's Human Subjects Committee.

Real-Time Q-PCR Assays. The mRNA abundances of ER, ErbB, and ERR family members were determined by real-time Q-PCR assays. Amplification of PCR products was continuously monitored by fluorescence of SYBR Green I specifically complexed with double-stranded, but not single-stranded DNA (54).

Total RNA was isolated from tissues using the Total RNeasy kit (Qiagen; Valencia, CA), treated with RNase-free DNase I (Ambion, Austin, TX), and again purified with the Total RNeasy kit. cDNA was synthesized by incubation of 10 μg total RNA with SuperScript II reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA) and 50 nmol each of oligo$(dT)_{15}$ VN (where V = A, G, or C, and N = any nucleotide) and random hexamers as primers in a total reaction volume of 100 μl at 45°C for 1 h. Because the quality of the mRNA purified from the tumors likely varied considerably, differences in mRNA integrity were compensated for by careful quantitation by trace radiolabel incorporation of the amount of cDNA synthesized from each sample followed by the use of the same amount of cDNA in each Q-PCR assay. In brief, cDNA synthesis reactions were performed in parallel in the presence of a trace amount of [α-^{32}P]dCTP. Incorporated and total amounts of radiolabel were measured in triplicate by trichloroacetic acid precipitation and scintillation counting. Calculation of the total mass of cDNA synthesized was based on the molar amount of nucleotide present in the reaction converted to mass and multiplied by the ratio of incorporated:total radiolabel. Q-PCR assays involving tissue samples used 1 ng cDNA as template and were performed in triplicate.

PCR primer sets were designed to promote efficient amplification by yielding products smaller than 150 bp in length. The products they generated were verified for specificity by sequence analysis. The PCR primer set sequences used here and amplicon sizes were as follows: ERα forward primer 5′-GGAGGGCAGGGGTGAA-3′ and reverse primer 5′-GGCCAGGCTGTTCTTCTTAG-3′, 100-bp amplicon; ERβ forward primer 5′-TTCCCAGCAATGTCACTAACTT-3′ and reverse primer 5′-TTGAGGTTCCGCATACAGA-3′, 137-bp amplicon; EGFR forward primer 5′-GTGACCGTTTGGGAGTTGATGA-3′ and reverse primer 5′-GGCTGAGGGAGGCGTTCTC-3′, 104-bp amplicon; ErbB2 forward primer 5′-GGGAAGAATGGGGTCGTCAAA-3′ and reverse primer 5′-CTCCTCCCTGGGGTGTCAAGT-3′, 82-bp amplicon; ErbB3 forward primer 5′-GTGGCACTCAGGGAGCATTTA-3′ and reverse primer 5′-TCTGGGACTGGGGAAAAGG-3′, 106-bp amplicon; ErbB4 forward primer 5′-TGCCCTACAGAGCCCCAACTA-3′ and reverse primer 5′-GCTTGCGTAGGGTGCCATTAC-3′, 105-bp amplicon; ERRα forward primer 5′-AAAGTGCTGGCCCATTTCTAT-3′ and reverse primer 5′-CCTTGCCTCAGTCCATCAT-3′, 100-bp amplicon; ERRβ forward primer 5′-TGCCCTACGACGACAA-3′ and reverse primer 5′-ACTCCTCCTTCTCCACCTT-3′, 144-bp amplicon; and ERRγ forward primer 5′-GGCCATCAGAACGGACTTG-3′ and reverse primer 5′-GCCCACTACCTCCCAGGATA-3′, 67-bp amplicon. PCR

primer sequences were designed using Oligo 5.0 software (National Biosciences; Plymouth, MN) and synthesized at the University of Wisconsin-Biotechnology Center (Madison, WI).

Transcript copy numbers were determined by generating standard curves with serially diluted single-stranded PCR products, which were produced by linear amplification using only the primer corresponding to the noncoding DNA strand. The amount of each template required for the standard curves was determined by trace incorporation of [α-^{32}P]dCTP during the PCR amplification process. The mass of PCR product synthesized was converted to copy number based on the size of the amplicon. All of the standard curves covered eight orders-of-magnitude and were assayed in triplicate.

Q-PCR assays were performed in a total volume of 20 μl with 1 ng cDNA. SYBR Green I (Molecular Probes; Eugene, OR) was diluted in anhydrous DMSO at 1:2,500, then added to the enzyme reaction buffer to obtain a final concentration of 1:50,000 SYBR green I and 5% DMSO. To normalize fluorescence intensity between samples, the enzyme reaction buffer contained 180 nM passive reference dye ROX (Molecular Probes). The final concentrations of the remaining constituents were as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 50 μM each dNTP, 500 nM each forward and reverse primer, and 0.025 units/μl HotStar *Taq* DNA polymerase (Qiagen). The thermal cycling parameters were 1 cycle of 95°C for 10 min and 40 cycles of 96°C denaturation for 15 s followed by 60°C annealing/extension for 1 min. Q-PCR assays were performed with an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA).

ER and PgR by LB Assays. ER and PgR content of the breast tumors were previously determined in a central laboratory. The standard multipoint dextran-coated charcoal assay was modified as described previously (55) to incorporate ^{125}I-labeled estradiol and ^{3}H-labeled R5020 in a single assay, allowing for the simultaneous determination of both ER and PgR. ER-LB levels greater than or equal to 3 fmol/mg protein were considered positive, and PgR-LB levels greater than or equal to 5 fmol/mg protein were considered positive.

DNA Ploidy and S Phase Fraction by Flow Cytometry. Flow cytometry was performed as described previously to determine DNA ploidy and S-phase fraction (55, 56). S-phase fractions were estimated using the MODFIT program (Verity House Software, Inc., Topsham, ME). S-phase fractions less than 6% were considered low. S-phase fractions greater than 10% were considered high. Values between 6 and 10% were considered intermediate.

Statistics. Changes in the median level of a single mRNA species between tissue groups were tested by the nonparametric Kruskal-Wallis ANOVA (Figs. 1–3). Associations between aberrant mRNA levels and clinicopathological biomarkers in the breast tumors were evaluated by Fisher's exact tests (Table 1). To analyze aberrant tumor expression relative to MECs, high and low expression in the breast tumors was defined as mRNA levels above or below, respectively, the range of expression in the normal MECs. Similarly, very high and very low expression in the tumors was defined as 10-fold above or below, respectively, the range of expression in normal MECs. Additionally, to analyze aberrant tumor expression relative to other tumors, typical expression was defined as being within a SD and atypical expression as greater than a SD away from the mean tumor level. Differences in expression between ERα and ERRα mRNA levels within the same tissue sample were assessed by 1-way ANOVA with repeated measures on $\log_2$-transformed data (Fig. 4). To discern whether ERα and ERRα were expressed at approximately equivalent levels within tumors, the ratio of their levels was stratified according to those found in normal MECs; ratios within a SD of the average ratio in normal MECs were defined as equivalent. Pairwise relationships among gene expression levels and clinicopathological factors were tested by the nonparametric rank correlation method, Spearman's rho analysis (Table 2). Spearman rank correlations involving ER-LB assays, PgR-LB assays, S-phase fraction, and DNA ploidy used raw values on continuous scales instead of simple status assessments. All of the analyses described above were performed using SAS version 8.2 from SAS Institute, Inc. (Cary, NC).

RESULTS AND DISCUSSION

Statistical Considerations. The sample size in this study was modest: 38 tumors and 9 normal MEC preparations. Hence, some important differences or relationships could have remained undetected. On the other hand, statistically significant results observed with this modest sample size may indicate truly important relationships and differences. Notably, gene expression was accurately measured, even when at low levels, because of the use of real-time Q-PCR, thereby allowing much finer stratification of tissue samples than would have been possible by less quantitative methods (*e.g.*, immunohistochemistry or LB assays). Consequently, these more refined stratifications allowed improved statistical considerations given the modest sample size.

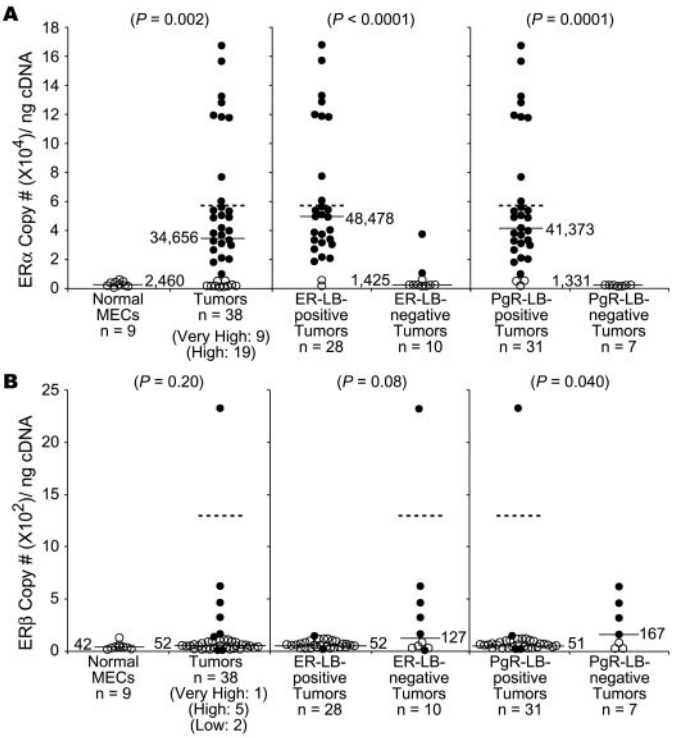

Fig. 1. ER family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. ERα levels (*A*) and ERβ levels (*B*). *Numbered solid horizontal bars*, the median level within each group. *Dashed horizontal bars in the tumor groups*, the level 10-fold above the upper limit of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

To comprehensively evaluate three potentially novel biomarkers in the context of six previously studied genes implicated in breast cancer, a large number of pairwise comparisons were made. Thus, some of the associations reported here could be attributable to chance alone. Nevertheless, this exploratory analysis of the involvement of ERRα, ERRβ, and ERRγ in human breast cancer generates hypotheses, the validity of which can be tested in subsequent, more-extensive studies.

ERα mRNA Levels. ERα exhibited significantly higher mRNA levels than the other evaluated nuclear receptors in approximately three-fourths of the tumors (compare Fig. 1*A* with Fig. 1*B* and Fig. 3). The median ERα mRNA level was 14-fold higher in breast carcinomas compared with normal MECs (Kruskal-Wallis ANOVA, $P = 0.002$; Fig. 1*A*) and expressed at high or very high levels in 74% (28 of 38) of the breast tumors (Fig. 1*A*). These results exemplify the critical role ERα plays in the majority of breast cancers. The median ERα mRNA level was 34-fold greater in ER-LB-positive and 31-fold greater in PgR-LB-positive tumors relative to negative tumors (Kruskal-Wallis ANOVA, $P < 0.0001$ and $P = 0.0001$, respectively; Fig. 1*A*). Tumors that overexpressed ERα mRNA segregated with ER-LB- and PgR-LB-positive status (Fisher's exact, $P < 0.0001$ and $P < 0.0001$, respectively; Table 1). Furthermore, ERα mRNA levels strongly correlated with ER-LB ($\rho_s = 0.86$, $P < 0.0001$; Table 2) and

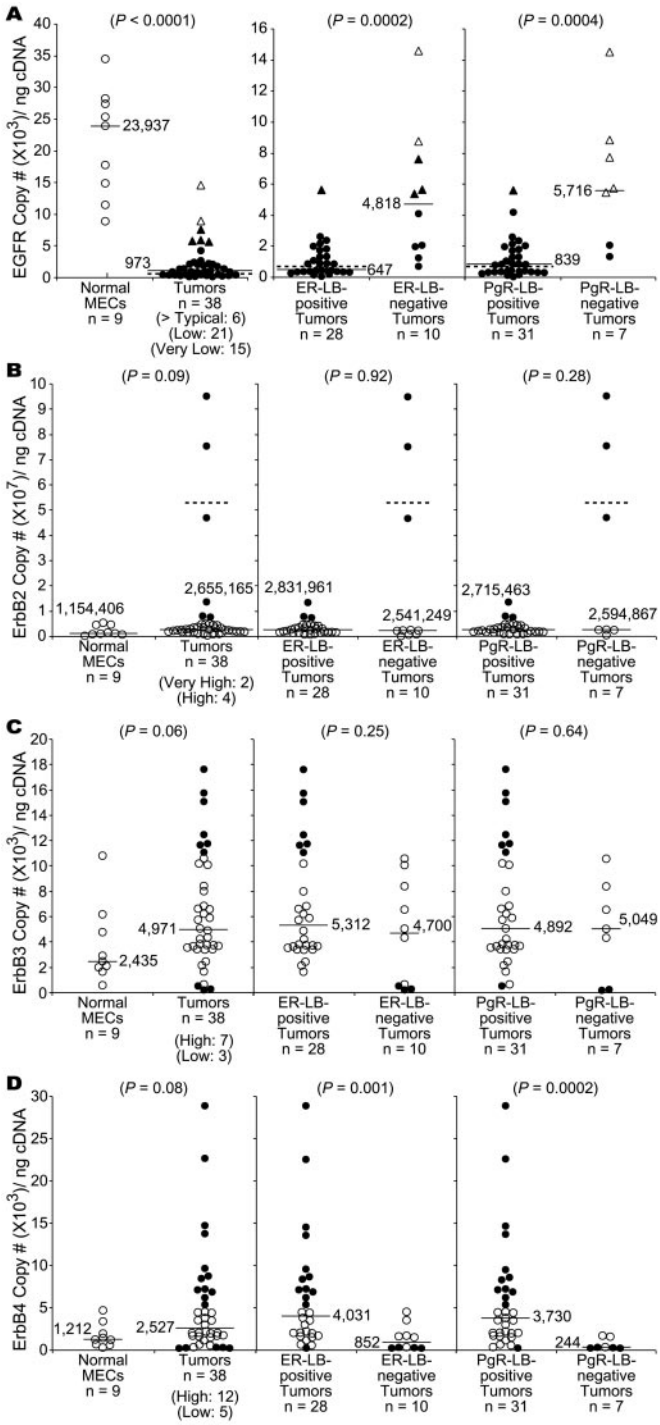

Fig. 2. ErbB family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. EGFR levels (*A*), ErbB2 levels (*B*), ErbB3 levels (*C*), and ErbB4 levels (*D*). Different scales are used within *A*. *Numbered solid horizontal bars*, the median level within each group. *Dashed horizontal bars in the tumor groups*, the level 10-fold above or below the upper or lower limit, respectively, of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. *Triangles in A*, tumors expressing EGFR mRNA at levels greater or less than one SD surrounding the mean for the tumor group. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

PgR-LB protein levels ($\rho_s = 0.68$, $P < 0.0001$; Table 2) in the tumors as evaluated using the raw LB values over a continuous scale. These expected relationships validated the real-time Q-PCR assays and conformed well with established findings of others regarding both typical percentage of ER-LB-positive tumors and elevated levels of ERα in these tumors (5).

ERβ mRNA Levels. ERβ mRNA levels were high or very high in 16% (6 of 38) of tumors and low in 5% (2 of 38) of tumors (Fig. 1*B*). The median level of ERβ mRNA expression was approximately 3.2-fold higher in PgR-LB-negative tumors compared with positive tumors (Kruskal-Wallis ANOVA, $P = 0.040$; Fig. 1*B*). Dotzlaw *et al.* (7) have also reported increased ERβ expression in PgR-negative tumors. Also, tumors that overexpressed ERβ associated with ER-LB-negative and PgR-LB-negative status (Fisher's exact, $P = 0.002$ and $P = 0.005$, respectively; Table 1). Thus, increased ERβ levels inversely related with functional ERα status and may, therefore, have reflected improper estrogen responsiveness as has been suggested by others (7–9).

EGFR mRNA Levels. The median EGFR mRNA level was ~1/25 in breast tumors relative to normal MECs (Kruskal-Wallis ANOVA,

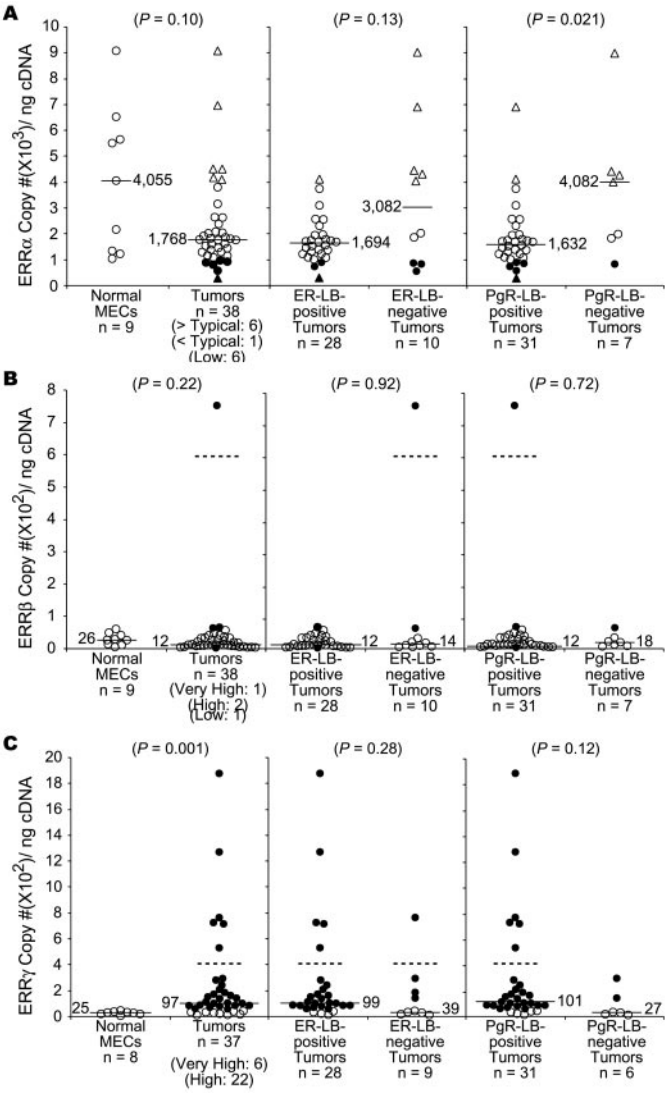

Fig. 3. ERR family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. ERRα levels (*A*), ERRβ levels (*B*), and ERRγ levels (*C*). *Numbered solid horizontal bars*, the median level within each group. *Dashed horizontal bars in the tumor groups*, the level 10-fold above or below the upper or lower limit, respectively, of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. *Triangles in A*, tumors expressing ERRα mRNA at levels greater or less than one SD surrounding the mean for the tumor group. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

Table 1 *Fisher's exact tests for association between aberrant gene expression and clinicopathological features*

mRNA levels	ER-LB status			PgR-LB status			S-phase fraction				DNA ploidy		
	Pos[a]	Neg	*P*	Pos	Neg	*P*	Low	Int	High	*P*	Di	Aneu	*P*
ERα													
Normal	2	8		3	7		5	1	4		6	4	
High	17	2		19	0		11	3	4		10	9	
Very high	9	0	<0.0001	9	0	<0.0001	5	1	3	0.88	4	5	0.91
ERβ													
Low	1	1		2	0		1	0	0		2	0	
Normal	26	4		27	3		18	4	8		15	15	
High	1	4		1	4		1	1	3		2	3	
Very high	0	1	0.002	1	0	0.005	1	0	0	0.53	1	0	0.66
EGFR[b]													
Typical	27	5		30	2		18	5	9		16	16	
> typical	1	5	0.003	1	5	0.0002	3	0	2	1.00	4	2	0.66
EGFR													
Very low	14	1		15	0		8	1	6		7	8	
Low	14	7		16	5		12	4	4		12	9	
Normal	0	2	0.012	0	2	0.003	1	0	1	0.51	1	1	0.86
ErbB2													
Normal	25	7		28	4		18	4	9		18	14	
High	3	1		3	1		3	0	1		1	3	
Very high	0	2	0.11	0	2	0.029	0	1	1	0.35	1	1	0.65
ErbB3													
Low	0	3		1	2		3	0	0		3	0	
Normal	21	7		23	5		16	2	9		15	13	
High	7	0	0.005	7	0	0.060	2	3	2	0.10	2	5	0.19
ErbB4													
Low	1	4		1	4		2	1	2		3	2	
Normal	15	6		18	3		12	2	6		11	10	
High	12	0	0.002	12	0	0.002	7	2	3	0.86	6	6	1.00
ERRα[b]													
< typical	1	0		1	0		1	0	1		1	0	
Typical	26	5		28	3		18	4	7		16	15	
> typical	1	5	0.003	2	4	0.006	2	1	3	0.54	3	3	1.00
ERRα													
Low	3	3		5	1		4	0	1		5	1	
Normal	25	7	0.31	26	6	1.00	17	5	10	0.66	15	17	0.18
ERRβ													
Low	5	0		5	0		1	1	2		1	4	
Normal	22	8		24	6		18	4	7		18	12	
High	1	1		1	1		1	0	1		0	2	
Very high	0	1	0.12	1	0	0.38	1	0	0	0.61	1	0	0.069
ERRγ													
Normal	4	5		5	4		3	2	3		4	5	
High	19	3		20	2		12	3	7		10	12	
Very high	5	1	0.054	6	0	0.045	6	0	0	0.21	6	0	0.042

[a] Pos, positive; Neg, negative; Int, intermediate; Di, diploid; Aneu, aneuploid.
[b] Expression levels relative to other tumors, not MECs.

$P < 0.0001$; Fig. 2*A*), with 55% (21 of 38) of tumors showing low and 39% (15 of 38) showing very low expression (Fig. 2*A*, *solid symbols*). However, when compared within the tumors as a class, 16% (6 of 38) showed elevated or greater than typical levels of EGFR expression (Fig. 2*A*, *triangles*) in agreement with other reports (12). EGFR exhibited a strongly significant inverse relationship with ERα expression in breast tumors. The median EGFR mRNA level was ~7.4-fold higher in ER-LB-negative and 6.8-fold higher in PgR-LB-negative *versus* positive tumors (Kruskal-Wallis ANOVA, $P = 0.0002$ and $P = 0.0004$, respectively; Fig. 2*A*). Also, tumors exhibiting greater than typical EGFR levels associated with ER-LB-negative and PgR-LB-negative status (Fisher's exact, $P = 0.003$ and $P = 0.0002$,

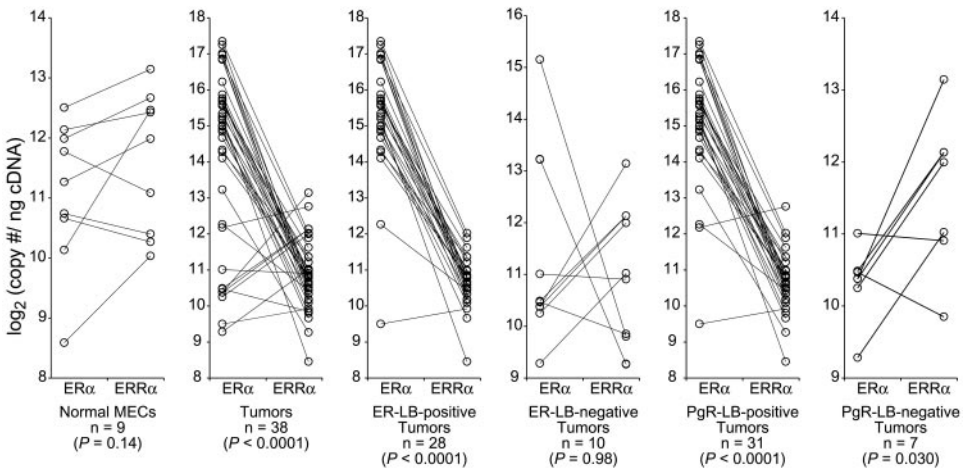

Fig. 4. ERα and ERRα mRNA levels within the same tissue sample. Significance was assessed by 1-way ANOVA with repeated measures on $\log_2$-transformed values.

Table 2 *Spearman's rank correlation coefficients (ρ_s) for pairwise comparisons in breast tumors and normal MECs*[a,b]

Breast Tumors	PgR-LB	S phase	Ploidy	ERα	ERβ	EGFR	ErbB2	ErbB3	ErbB4	ERRα	ERRβ	ERRγ
ER-LB	**0.74*** **< 0.0001** **39**	-.070 0.68 38	0.09 0.59 39	**0.86*** **<0.0001** **38**	-0.11 0.51 38	**-0.76*** **<0.0001** **38**	-0.01 0.97 38	0.17 0.30 38	**0.53*** **0.001** **38**	-0.23 0.16 38	0.11 0.51 38	0.19 0.27 37
PgR-LB		-0.13 0.43 38	0.07 0.68 39	**0.68*** **<0.0001** **38**	-0.22 0.18 38	**-0.63*** **<0.0001** **38**	0.05 0.76 38	0.22 0.19 38	**0.44*** **0.006** **38**	-0.15 0.39 38	0.12 0.47 38	0.08 0.63 37
S-phase			**0.75*** **<0.0001** **39**	-0.13 0.43 37	0.01 0.95 37	-0.09 0.60 37	0.08 0.63 37	**0.35*** **0.034** **37**	-0.18 0.29 37	0.19 0.26 37	**-0.37*** **0.026** **37**	-0.31 0.07 36
Ploidy				0.06 0.71 38	0.05 0.77 38	-0.24 0.15 38	0.13 0.45 38	0.19 0.25 38	-0.04 0.82 38	0.19 0.25 38	-0.19 0.26 38	-0.27 0.11 37
ERα					-0.15 0.39 38	**-0.54** **0.001** **38**	0.17 0.31 38	**0.42*** **0.009** **38**	**0.74*** **<0.0001** **38**	-0.13 0.44 38	0.07 0.66 38	0.20 0.24 37
ERβ				0.27 0.49 9		0.08 0.64 38	0.24 0.14 38	-0.15 0.36 38	-0.16 0.34 38	**0.35*** **0.032** **38**	**0.58*** **0.0002** **38**	-0.14 0.42 37
EGFR				**0.73*** **0.025** **9**	0.03 0.93 9		0.27 0.19 38	0.09 0.57 38	-0.30 0.07 38	0.19 0.25 38	-0.13 0.44 38	-0.17 0.31 37
ErbB2				**0.82*** **0.007** **9**	0.25 0.52 9	**0.83*** **0.002** **9**		**0.54*** **0.0004** **38**	0.04 0.80 38	**0.45*** **0.005** **38**	-0.10 0.54 38	0.10 0.55 37
ErbB3				0.52 0.15 9	0.15 0.70 9	0.48 0.19 9	**0.70*** **0.036** **9**		**0.42*** **0.009** **38**	**0.33*** **0.047** **38**	-0.28 0.09 38	0.11 0.51 37
ErbB4				0.35 0.36 9	0.27 0.49 9	-0.15 0.70 9	0.20 0.61 9	0.60 0.09 9		-0.15 0.36 38	-0.10 0.56 38	**0.32*** **0.052** **37**
ERRα				**0.70*** **0.036** **9**	0.33 0.38 9	**0.90*** **0.0009** **9**	**0.93*** **0.0002** **9**	0.57 0.11 9	0.05 0.90 9		0.30 0.07 38	0.02 0.92 37
ERRβ				0.23 0.55 9	0.27 0.49 9	0.50 0.17 9	0.58 0.10 9	0.28 0.46 9	0.00 1.00 9	**0.77*** **0.016** **9**		-0.08 0.62 37
ERRγ				0.48 0.23 8	0.33 0.42 8	0.14 0.74 8	0.64 0.09 8	**0.81*** **0.015** **8**	**0.76*** **0.028** **8**	0.38 0.35 8	0.17 0.69 8	

Normal MECs

[a] For each comparison: line 1, ρ_s; line 2, P; line 3, sample size.
[b] Numbers in bold type, Spearman coefficient significance at $P \leq 0.05$.

respectively; Table 1). Furthermore, EGFR mRNA levels inversely correlated with ERα mRNA levels ($\rho_s = -0.54$, $P = 0.001$; Table 2) as well as with ER-LB protein amounts ($\rho_s = -0.76$, $P < 0.0001$; Table 2) and PgR-LB protein amounts ($\rho_s = -0.63$, $P < 0.0001$; Table 2) over a continuous scale in tumors, and directly correlated with ERα mRNA levels in normal MECs ($\rho_s = 0.73$, $P = 0.025$; Table 2). These data indicate that EGFR and ERα were coregulated in the normal MECs, but, in accordance with previous reports (12), inversely regulated in the tumors, indicative of a negative feedback regulatory loop.

ErbB2 mRNA Levels. ErbB2 was the dominant transmembrane receptor because it was observed at markedly higher levels than the other ErbB members in every tissue subgroup (compare Fig. 2*B* with Fig. 2, *A*, *C*, and *D*). This finding is consistent with ErbB2 acting as the dominant heterodimerization subunit (11) and highlights its importance in mammary tissues. The median ErbB2 level showed a nonstatistically significant 2.3-fold increase in expression in the breast tumors compared with the normal MECs (Fig. 2*B*). However, in agreement with reports of others (13, 15), ErbB2 expression was significantly increased in 16% (6 of 38) of tumors, with 11% displaying high and 5% displaying very high ErbB2 levels. The maximum level of ErbB2 expression was 18-fold higher in the tumors compared with the maximum level in the normal MECs. Overexpression of ErbB2 associated with PgR-LB-negative status (Fisher's exact test, $P = 0.029$; Table 1) and, thereby, inversely associated with ERα functionality in the tumors, as has been demonstrated previously (13). On the other hand, ErbB2 mRNA levels directly correlated with both ERα mRNA levels ($\rho_s = 0.82$, $P = 0.007$; Table 2) and EGFR mRNA levels ($\rho_s = 0.83$, $P = 0.002$; Table 2) in the normal MECs. Thus, in a manner similar to that with EGFR, ErbB2 likely participated in similar functions along with ERα in the normal MECs, yet in functions distinct from ERα in a subset of the tumors.

ErbB3 mRNA Levels. The median ErbB3 mRNA level showed a nonsignificant 2.0-fold increase in breast tumors compared with normal MECs (Fig. 2*C*). High expression of ErbB3 was observed in 18% (7 of 38) of the tumors, whereas low ErbB3 expression was observed in 8% (3 of 38) of the tumors. ErbB3 overexpression associated with ER-LB-positive tumor status (Fisher's exact test, $P = 0.005$; Table 1). Furthermore, ErbB3 levels correlated with ERα mRNA levels in the tumors ($\rho_s = 0.42$, $P = 0.009$; Table 2), indicating that ErbB3 may

have participated in ERα-mediated activities in this tissue type. A similar relationship between ErbB3 and ERα has been previously described (29). ErbB3 expression also correlated with ErbB2 expression in the tumors ($\rho_s = 0.54$, $P = 0.0004$; Table 2) and normal MECs ($\rho_s = 0.70$, $P = 0.036$; Table 2), consistent with a prior report (27) and suggesting that these ErbB members form heterodimers in both tissue types. Moreover, ErbB3 correlated with S-phase fraction ($\rho_s = 0.35$, $P = 0.034$; Table 2), an established clinical indicator of tumor aggressiveness. Hence, ErbB3 may have similar yet distinct roles with both ErbB2 and ERα in tumor cell proliferation.

ErbB4 mRNA Levels. ErbB4 mRNA was present at high levels in 32% (12 of 38) of the tumors and at low levels in 13% (5 of 38) of them. Interestingly, ErbB4 mRNA levels were elevated 4.7-fold in the ER-LB-positive and 15-fold in the PgR-LB-positive tumors relative to the LB-negative tumors (Kruskal-Wallis ANOVA, $P = 0.001$ and $P = 0.0002$, respectively; Fig. 2*D*), and overexpression of ErbB4 associated with ER-LB-positive and PgR-LB-positive status (Fisher's exact test, $P = 0.002$ and $P = 0.002$, respectively; Table 1). Furthermore, ErbB4 levels correlated with ERα mRNA levels ($\rho_s = 0.74$, $P < 0.0001$; Table 2) as well as with ER-LB ($\rho_s = 0.53$, $P = 0.001$; Table 2) and PgR-LB protein levels ($\rho_s = 0.44$, $P = 0.006$; Table 2) over a continuous scale in the tumors. Therefore, in accordance with a similar finding of Knowlden *et al.* (29), ErbB4 shared a strong relationship with ERα functionality in tumors. Levels of ErbB4 and ErbB3 correlated in tumors ($\rho_s = 0.42$, $P = 0.009$; Table 2), indicating that ErbB4 and ErbB3 likely shared some functions, potentially via the formation of heterodimers. Because the relationships observed between ErbB4 and ERα were stronger and more extensive than the ones observed between ErbB3 and ERα, the latter may have been the indirect result of heterodimerization between ErbB4 and ErbB3. These findings are consistent with reports showing that ErbB4 likely serves as a favorable biomarker (14, 17, 29, 30).

ERRα mRNA Levels. The median ERRα mRNA level in the breast tumors was nonsignificantly 44% of the median level observed in normal MECs, although 16% (6 of 38) of tumors did contain significantly lower levels of ERRα (Fig. 3*A*, *solid symbols*). However, when ERRα levels were compared within the tumor group, ERRα levels were significantly greater than typical in 16% (6 of 38) of the samples, whereas only 3% (1 of 38) of the samples showed significantly lower than typical levels (Fig. 3*A*, *triangles*). Quite importantly, most of these ERRα-elevated tumors were also ER-LB-negative and PgR-LB-negative (Fisher's exact test, $P = 0.003$ and $P = 0.006$, respectively; Table 1), with the median ERRα mRNA level being significantly 2.5-fold higher in the PgR-LB-negative compared with the PgR-LB-positive tumors (Kruskal-Wallis ANOVA, $P = 0.021$; Fig. 3*A*). Thus, as with ERβ, EGFR and ErbB2, higher levels of ERRα occurred in the absence of functional ERα in the tumors. ERRα levels correlated with ERβ levels in tumors ($\rho_s = 0.35$, $P = 0.032$; Table 2), and with ERα levels in normal MECs ($\rho_s = 0.70$, $P = 0.036$; Table 2). ERRα also correlated with ErbB3 in tumors ($\rho_s = 0.33$, $P = 0.047$; Table 2), and with EGFR in normal MECs ($\rho_s = 0.90$, $P = 0.0009$; Table 2). Additionally, ERRα displayed correlations with ErbB2 in both the tumors ($\rho_s = 0.45$, $P = 0.005$; Table 2) and normal MECs ($\rho_s = 0.93$, $P = 0.0002$; Table 2). Hence, ERRα may have functioned together with ErbB2 in both normal and tumor mammary cells. It may have also acted together with ERα and EGFR in normal MECs, and with ERβ and ErbB3 apart from ERα in tumors. These correlations could be indicative of irregular estrogen responsiveness in the pathogenesis of breast cancer.

After ERα, ERRα was the next most abundant nuclear receptor, showing greater levels of expression than ERβ, ERRβ, and ERRγ in every tissue subgroup (compare Fig. 3*A* with Figs. 1 and 3, *B–C*). The distributions of ERα and ERRα expression were compared within the same tissue sample as paired variables by 1-way ANOVA with repeated measures (Fig. 4). ERα and ERRα were expressed at similar levels in normal MECs ($P = 0.14$) and ER-LB-negative tumors ($P = 0.98$), whereas ERα was more abundant in the ER-LB-positive ($P < 0.0001$) and PgR-LB-positive groups ($P < 0.0001$). Most importantly, ERRα levels were significantly greater than ERα levels in PgR-LB-negative tumors ($P = 0.030$). ERRα was present at greater levels than ERα in 13% (5 of 38), at approximately equivalent levels in 11% (4 of 38), and at lower levels in 76% (29 of 38) of the tumors. Therefore, ERRα may have played a prominent role in ERE-dependent transcription in almost one-fourth of the breast tumors, whereas ERα may have played a greater physiological role in the remaining tumors.

The Potential Role of ERRα in Breast Cancer. A primary conclusion from the above data is that ERRα showed a strong inverse relationship with ERα functionality in the tumors. Why might this be so? We hypothesize that ERRα functions in normal MECs as a modulator of the response to estrogen, competing with ERα for binding to EREs to achieve fine-tuned regulation of transcription. In support of this hypothesis, we have shown that ERα and ERRα directly compete for binding EREs, and that changes in the amount of ERRα modulates ERα-mediated ERE-dependent transcription (36). Misregulation can occur in tumors by several mechanisms. One common mechanism likely involves the overexpression of ERα, often accompanied by underexpression of ERRα relative to normal MECs, such that ERα outcompetes ERRα for binding to EREs. In this case, the modulatory effects of ERRα are largely lost. Alternatively, in ER-negative tumors or ones with high ERRα levels, ERRα becomes a major regulator of ERE-containing genes, acting constitutively because it functions independently of estrogen (31, 42).

Interestingly, ERRα has been shown to function actively as either a repressor (36) or activator (36, 44, 46, 48) of transcription in mammary carcinoma cell lines in a cell type-dependent manner. The factors that determine ERRα's transcriptional activity have yet to be identified, but likely involve, in part, the ErbB2 signal transduction pathway. Here, we found ERRα mRNA abundance strongly correlated with ErbB2 abundance in both the breast tumors and normal MECs (Table 2), suggesting a functional relationship between these factors. Consistent with this correlation, ERRα has been shown to function as a transcriptional activator in SK-BR-3 mammary cells, cells in which the *erb*B2 locus has been amplified such that ErbB2 mRNA levels are 128-fold higher than in MCF-7 cells (57), whereas it functions as a transcriptional repressor in MCF-7 cells (36). ERRα has also been demonstrated to exist as a phosphoprotein in COS-7 cells, another cell line in which ERRα activates transcription (58). Moreover, we have recently found that ERRα can serve as a substrate for activated MAPK *in vitro*.[4] Thus, ERRα and ErbB2 likely share a functional relationship through ErbB2-mediated modulation of ERRα's phosphorylation status. Combining these observations, we propose the following hypothesis: in cells containing low ErbB2 levels, ERRα down-modulates ERα-regulated ERE-dependent transcription; in cells containing high ErbB2 levels, ERRα constitutively activates transcription independent of ERα. A major prediction of this hypothesis is that tumors containing high levels of both ErbB2 and ERRα will not likely respond to antiestrogen therapy. This hypothesis also provides one of multiple mechanisms to explain ErbB2's relationship with tamoxifen resistance (18–20) and suggests that ERRα's phosphorylation status may have predictive value in assessing the effectiveness of therapeutic agents, such as Herceptin, that are directed against ErbB2. It also implicates ERRα itself as another likely efficacious target for therapy.

[4] E. A. Ariazi, unpublished data.

ERRβ mRNA Levels. ERRβ mRNA was increased in 8% (3 of 38) of tumors (Fig. 3*B*) and decreased in 3% (1 of 38) of tumors. Aberrant ERRβ expression was not associated with any of the clinical biomarkers, although too few tumors contained aberrant ERRβ amounts for strong statistical testing. Indicative of roles with other genes, ERRβ levels correlated with ERRα levels in normal MECs ($\rho_s = 0.77$, $P = 0.016$; Table 2), and with ERβ in the tumors ($\rho_s = 0.58$, $P = 0.0002$; Table 2). The potential role of ERRβ in breast cancer may lie in its correlation with ERβ, which has been associated with indicators of high tumor aggressiveness (7–9). Curiously, ERRβ levels inversely correlated with S-phase fraction ($\rho_s = -0.37$, $P = 0.026$; Table 2), perhaps suggesting that greater ERRβ levels inhibit cellular proliferation or, possibly, promote cellular differentiation. The importance for ERRβ in differentiation has been demonstrated by genetic ablation of this locus in mice, producing a severe defect in placental development that leads to embryonic lethality (59). However, the predictive value of ERRβ status remains unclear. It should be noted that ERRβ mRNA levels were quite low (Fig. 3*B*), indicating that the prognostic potential of ERRβ is not promising. However, ERβ mRNA levels were also quite low compared with ERα (Fig. 1), yet allowed accumulation of ERβ protein to levels clearly detectable by immunohistochemistry and participation in biologically significant roles in breast cancer (6, 8).

ERRγ mRNA Levels. The median ERRγ mRNA level was significantly elevated 3.9-fold in breast tumors relative to normal MECs (Kruskal-Wallis ANOVA, $P = 0.001$; Fig. 3*C*). Moreover, ERRγ mRNA was overexpressed in approximately 3/4 of the tumors, with high levels in 59% (22 of 37) and very high levels in an additional 16% (6 of 37; Fig. 3*C*). These findings may indicate that ERRγ could be involved in the development of breast cancer. The median ERRγ mRNA level was not significantly different among the ER-LB or PgR-LB tumor subgroups. Nonetheless, tumors that overexpressed ERRγ were associated with ER-LB-positive and PgR-LB-positive status (Fisher's exact test, $P = 0.054$ and $P = 0.045$, respectively; Table 1). Thus, tumors that overexpressed ERRγ were also frequently steroid receptor-positive, similar to tumors overexpressing ErbB3 or ErbB4. Hence, increased ERRγ levels may reflect hormonal sensitivity. ERRγ levels correlated with ErbB4 levels in both the tumors ($\rho_s = 0.32$, $P = 0.052$; Table 2) and normal MECs ($\rho_s = 0.76$, $P = 0.028$; Table 2), as well as with ErbB3 levels in normal MECs ($\rho_s = 0.81$, $P = 0.015$; Table 2). As discussed above, ErbB4 overexpression likely indicates a preferable clinical outcome; likewise, ERRγ overexpression may also indicate a more positive outcome. Interestingly, the median ERRγ level was 2.0-fold higher in the less aggressive-in-nature diploid tumors (157 copies/ng cDNA) compared with the aneuploid tumors (79 copies/ng cDNA; Kruskal-Wallis ANOVA, $P = 0.033$; data not shown), and the tumors that overexpressed ERRγ associated with diploid status (Fisher's exact test, $P = 0.042$; Table 1). Collectively, these findings indicate that ERRγ may serve as a marker of favorable clinical course. Furthermore, in light of the studies that demonstrated ERRγ binds 4-hydroxytamoxifen as an antagonist (40, 43, 45), ERRγ-overexpressing tumors may help identify a subset of patients that would benefit from this treatment.

In conclusion, the study described here represents an initial investigation into the potential utility of ERRs as biomarkers in human breast cancer, with the intent of generating hypotheses to test further. Given the large number of comparisons made with a modest sample size, the possibility that false-positive relationships were identified needs to be kept in mind. Nevertheless, several findings of likely significance were observed. Foremost was the finding that ERRα mRNA is a major species (Fig. 3), being expressed at levels greater than or similar to that ERα in 24% of the tumors (Fig. 4), with tumors containing the highest levels of ERRα being associated with a steroid receptor-negative status (Table 1; Fig. 3*A*) and, therefore, hormonal insensitivity. ERRα levels also directly correlated with levels of ErbB2 (Table 2), a marker of aggressive tumor behavior (13). Thus, ERRα may be an important unfavorable marker in a significant proportion of breast cancer patients. Additionally, ERRα status may indicate the effectiveness of ErbB2-based therapeutics, with ERRα itself being a candidate therapeutic target, especially for tumors lacking functional ERα. ERRγ was overexpressed in 75% of the tumors (Fig. 3*C*), indicating a role for this transcription factor in the pathogenesis of breast cancer. However, unlike ERRα, ERRγ overexpression associated with the presence of functional ERα (Table 1) and, hence, hormonal sensitivity. Furthermore, ERRγ levels correlated with levels of ErbB4 (Table 2), a likely positive indicator of clinical outcome (14, 17, 29, 30), as well as with less aggressive diploid tumors (Table 1). Therefore, ERRγ shows potential as a favorable marker of clinical course. Moreover, because 4-hydroxytamoxifen has been found to antagonize ERRγ (40, 43, 45), selection of patients for treatment with this SERM may be improved by knowledge of ERRγ status. In summary, the results presented here warrant additional investigations to evaluate whether the status of ERRα and ERRγ indicate clinical outcomes and sensitivity to hormonal therapy.

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