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Estrogen receptor-β expression in invasive breast cancer in relation to molecular phenotype: results from the Nurses' Health Study

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The expression of estrogen receptor-α (ER-α) and related genes has emerged as one of the major determinants of molecular classification of invasive breast cancers. Expression of a second ER, estrogen receptor-β (ER-β), has not been previously evaluated in a large population-based study. Therefore, we examined ER-β expression in a large population of women with breast cancer to assess its relationship to molecular categories of invasive breast cancer. We constructed tissue microarrays from paraffin blocks of 3093 breast cancers that developed in women enrolled in the Nurses' Health Study. Tissue microarray sections were immunostained for ER-α, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6, epidermal growth factor receptor (EGFR) and with a monoclonal antibody to ER-β. Cancers were categorized as luminal A (ER-α+ and/or PR+ and HER2–); luminal B (ER-α+ and/or PR+ and HER2+); HER2 (ER-α− and PR− and HER2+); and basal-like (ER-α−, PR−, HER2− and EGFR or cytokeratin 5/6+). The relationship between expression of ER-β and molecular class of invasive breast cancer was analyzed. Overall, 68% of breast carcinomas were ER-β+. Expression of ER-β was significantly associated with expression of ER-α ($P<0.0001$) and PR ($P<0.0001$), and was inversely related to expression of HER2 ($P=0.004$), CK5/6 ($P=0.02$) and EGFR ($P=0.006$). Among 2170 invasive cancers with complete immunophenotypic data, 73% were luminal A, 5% luminal B, 6 % HER2 and 11% basal-like. ER-β expression was significantly related to molecular category ($P<0.0001$) and was more common in luminal A (72% of cases) and B (68% of cases) than in HER2 or basal-like types. However, despite their being defined by the absence of ER-α expression, 55% of HER2-type and 60% of basal-like cancers showed expression of ER-β. The role of ER-β in the development and progression of breast cancers defined by lack of expression of ER-α merits further investigation.
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Recent studies using microarray technology and unsupervised cluster analysis have provided new insights into the classification of invasive breast cancers.[1–4] These studies have resulted in the identification of several breast cancer subgroups that vary in their gene expression signatures and clinical course. The molecularly distinct breast cancer subgroups that have been the most reproducibly identified to date include luminal subtypes A and B (both of which are hormone receptor positive), the human epidermal growth factor receptor 2 (HER2) subtype and a group known as basal-like cancers.[1–4] Using this approach, the expression of estrogen receptor-α (ER-α) and related genes has emerged as one of the major determinants in defining molecular category, and

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ER-α is the primary target determining whether patients should receive hormonal therapy.

A second estrogen receptor, estrogen receptor-β (ER-β), was discovered in 1996.[5] Since then there has been increasing interest in its role in human breast cancers. Although its precise biologic role remains unclear, in part due to the fact that several isoforms exist, recent studies examining ER-β have suggested that its expression may be a prognostic factor and predictive factor in patients with breast cancer.[6–13] In addition, there is mounting evidence to support a role for ER-β in breast cancer onset and progression.[6,14,15]

Estrogen receptor-β is highly expressed in normal breast epithelium and its expression has been reported to decrease with tumor progression from *in situ* through to invasive carcinomas.[16,17] Nevertheless, up to 75% of invasive breast cancers have been shown to express ER-β, depending on the method used for its detection.[11,12,14,18–21] Of note, a subset of tumors that are ER-α− are ER-β+, and this may have implications for new therapeutic options for these patients.[10,22–24]

The expression of ER-β among the molecularly defined categories of invasive breast cancer has only been evaluated in smaller studies.[10,22–24] Therefore, using a large, well-characterized population of women with breast cancer, the objective of this study was to examine the expression of ER-β in relation to molecular phenotype.

Materials and methods

Study Population

Study design and population

The Nurses' Health Study was initiated in 1976, when 121 700 US registered nurses aged 30–55 returned an initial questionnaire. The cohort has been followed by mailed questionnaires biennially to update exposure information and ascertain nonfatal incident diseases. Information on body mass index, reproductive history, age at menopause and postmenopausal hormone use as well as diagnosis of cancer and other diseases are updated every 2 years through questionnaires. The follow-up rate among this cohort was over 90% through 1996.

Breast Cancer Case Confirmation

All women reporting incident diagnoses of cancer were asked for permission to review their medical records to confirm the diagnosis and to classify cancers as *in situ* or invasive, by histologic type, size and the presence or absence of metastases. To identify cases of cancer in nonrespondents who died, we obtained death certificates for all deceased participants and medical records for the incident cancers. Following medical record review, 99% of self-reported breast cancers were confirmed.

Breast Cancer Tissue Block Collection

In 1993, we began collecting archived formalin-fixed paraffin-embedded breast cancer blocks for participants with primary incident breast cancers over 20 years of follow-up (1976–1996). Cases who reported a prevalent cancer including breast cancer at baseline were excluded from collection. Of the 5610 breast cancers that were eligible for block collection, we were unable to obtain any pathology material for 1858 cases. The primary reason was because they had been destroyed by the hospital (45%). Because the majority of hospitals archive tissue blocks for only 5–10 years, we were more successful in obtaining more recent blocks. Because year of diagnosis and age at diagnosis are highly correlated (Spearman's correlation = 0.49; $P < 0.0001$), the temporal effect on our collections is evident not only in the differences in age at diagnosis, but also in the frequency of premenopausal breast cancers when comparing the women from whom we obtained specimens with those for whom we did not. However, these two groups of women were very similar regarding a number of other breast cancer risk factors and tumor characteristics (data published previously[25]). After taking into account age and year of diagnosis, the participants whose tumors were included in the tissue microarrays were very similar to those for whom we were unable to obtain tissue blocks.

We obtained pathology material for 3752 participants. Of these, 390 specimens were hematoxylin-and-eosin-stained slides only and 45 tissue blocks had to be returned to the lending hospital before construction of the tissue microarrays and thus could not be included. Hematoxylin and eosin sections of the corresponding 3317 paraffin-embedded tissue blocks were reviewed by a single pathologist to confirm the cancer diagnosis, classify the cancer according to histologic type and grade (Nottingham), and circle the area from which the cores for the tissue microarrays would be taken. Pathology review identified 420 tumor blocks as unusable for tissue microarray construction. The majority of exclusions were because the block did not contain residual tumor (60%) or there was insufficient tumor for the tissue microarray (26%). Tissue microarrays were constructed in the Dana-Farber Harvard Cancer Center Tissue Microarray Core Facility, Boston, MA. Three 0.6-mm cores were obtained from each breast cancer and were inserted into the recipient tissue microarray blocks. In total, 23 tissue microarray blocks were constructed from 3093 cancers and positive lymph nodes from 2897 participants. We excluded from the current analysis participants with positive lymph nodes only ($n = 25$), lobular or ductal carcinoma *in situ* ($n = 401$), and additional rare tumor types including malignant phyllodes tumors, neuroendocrine carcinoma and angiosarcoma ($n = 10$).

Immunohistochemical Analysis

We performed immunohistochemical staining for ER-α, progesterone receptor (PR), HER2, cytokeratin 5/6 and epidermal growth factor receptor (EGFR) on 5 μm paraffin sections cut from the tissue microarray blocks. Immunostains for each marker were performed in a single staining run on a Dako Autostainer (Dako Corporation, Carpinteria, CA, USA). These particular biomarkers were selected for analysis because they have been commonly used as a surrogate to classify invasive breast cancers according to their molecular phenotypes.[4,26–29] Sources and dilutions of the primary antibodies used in this study are listed in Table 1. The immunostaining protocols for ER-α, PR, HER2, cytokeratin 5/6 and EGFR have been previously described in detail.[25] Immunostaining for ER-β was performed on tissue sections following deparaffinization in two 5-min changes of xylene and rehydration through graded alcohols to distilled water. After blocking endogenous peroxidase activity, sections were subjected to heat-induced epitope retrieval in a vegetable steamer in citrate buffer (pH 6.1) for 30 min. Following heat-induced epitope retrieval, the primary monoclonal antibody ER-β1 (clone PPG5/10, Serotec) was applied to the sections at a dilution of 1:50 and the slides were incubated over night at 4°C followed by incubation with the biotinylated universal secondary antibody and the avidin–biotin complex. Visualization was performed with liquid 3,3′-diaminobenzidine as the chromogen substrate. Appropriate positive and negative controls were included in all staining runs.

Immunostained tissue microarray slides were evaluated for ER-α and PR expression, HER2 protein overexpression and expression of cytokeratin 5/6 and EGFR in each core. Tumor cells that showed nuclear staining for ER-α or PR were considered ER+ or PR+, whereas all ER− or PR− cases showed complete absence of tumor cell staining. Of note, low positive ER or PR (1–10% of tumor cell nuclei staining) and positive ER or PR (>10% of tumor cell nuclei staining) were collapsed into a single ER or PR 'positive' category for the purposes of this analysis. Tumor cells were considered positive for HER2 protein overexpression when more than 10% of the cells showed moderate or strong membrane staining (2+ and 3+). The results of analyses in which HER2 positivity was defined as 3+ were very similar to those presented with a definition of 2+ and 3+.[25] Cases were considered basal cytokeratin positive or EGFR+ if any cytoplasmic and/or membranous staining was detected in the tumor cells, even if focal. These latter criteria are similar to those previously used for scoring these markers in invasive basal-like cancers.[4,26,27] Tumor cells that showed distinct nuclear staining (regardless of the presence of cytoplasmic staining) were scored as ER-β+ (Figure 1).

Classification of Molecular Phenotype

Immunostained tissue microarray sections were reviewed under a microscope and visually scored for each individual tissue core as described earlier. We classified a case as positive if there was staining in any of the three cores from that case and negative if there was no immunostaining present. Cases that were ER-α+ and/or PR+ and HER2− were classified as luminal A cancers, cases that were ER-α+ and/or PR+ and HER2+ as luminal B cancers,

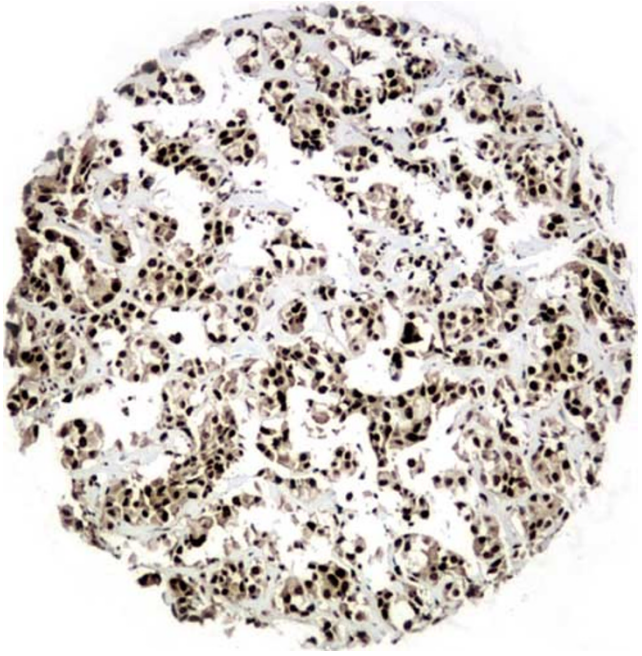

Figure 1 Tissue microarray core with positive ER-β nuclear immunostaining.

Table 1 Sources and dilutions of primary antibodies used in this study

Antibody	*Clone*	*Manufacturer*	*Dilution*
ER-α	1D5	Dako	1:200
ER-β1	PPG5/10	Serotec	1:50
PR	PgR 636	Dako	1:50
HER2	A0485 (rabbit polyclonal)	Dako	1:400
Cytokeratin 5/6	D5/16B4	Dako	1:50
EGFR	2-18C9	Dako	Prediluted (pharmDX kit)

cases that were ER-α−, PR− and HER2+ as HER2 type and cases that were negative for ER-α, PR and HER2 and positive for cytokeratin 5/6 and/or EGFR were categorized as basal-like. Cases that lacked expression of all five markers were considered 'unclassified'.

Statistical Analysis

χ^2-Tests were used to evaluate the independence of selected variables under the null hypothesis. All statistical tests were two sided and *P*-values <0.05 were considered statistically significant. Breast-cancer-specific survival was calculated from the date of diagnosis to the date of death from breast cancer or the follow-up cutoff. For the estimation of breast-cancer-specific survival, deaths from any other causes were censored. An additional 57 women were excluded due to missing data. The Kaplan–Meier product limit method was used to estimate survival according to ER-α/ER-β status and was compared across groups using the log-rank statistic.

Results

The population for this analysis consisted of invasive breast cancers that developed in women in the Nurses' Health Study after the baseline questionnaire (1976) through the 1996 follow-up cycle that could be classified into one of the four molecular phenotypes and that had evaluable ER-β-stained tissue microarray cores ($n=2170$). Based on immunostaining data from five of the markers used (ER-α, PR, HER2, EGFR and cytokeratin 5/6), 1585 invasive tumors were classified as luminal A (73%); 115 were luminal B (5%); 125 were HER2 type (6%) and 240 were basal-like (11%). There were also 105 invasive tumors that were considered unclassifiable (ER-α−/PR−/HER2−/EGFR−/cytokeratin 5/6−) for which ER-β staining results were available. An additional 234 invasive cases could not be classified because of non-evaluable staining, or lack of tumor tissue in the core.

Overall, 68% of invasive cancers were ER-β+. Expression of ER-β was significantly associated with expression of ER-α ($P<0.0001$) and PR ($P<0.0001$), and was inversely related to overexpression of HER2 ($P=0.004$), expression of cytokeratin 5/6 ($P=0.02$) and EGFR ($P=0.006$). ER-β expression was significantly related to molecular category ($P<0.0001$) and was more common in luminal A (72%) and luminal B (68%) subtypes than in HER2 or basal-like types (Table 2). However, despite their being defined by the absence of ER-α expression, 55% of HER2 and 60% of basal-like invasive cancers showed expression of ER-β. A similar percentage (48%) of ER-β positivity was seen in unclassified tumors.

The frequency of ER-β among the various histologic types of invasive cancer is shown in Table 3.

Table 2 Distribution of molecular phenotypes and frequency of ER-β expression according to molecular phenotype

Molecular category	*Frequency*	*ER*-β+	*ER*-β−
Luminal A	1585/2170 (73%)	1140/1585 (72%)	445/1585 (28%)
Luminal B	115/2170 (5%)	78/115 (68%)	37/115 (32%)
HER2	125/2170 (6%)	69/125 (55%)	56/125 (45%)
Basal-like	240/2170 (11%)	144/240 (60%)	96/240 (40%)
Unclassified	105/2170 (5%)	50/105 (48%)	55/105 (52%)

Luminal A, ER-α+ and/or PR+ and HER2−.
Luminal B, ER-α + and/or PR+ and HER2+.
HER2, ER-α− and PR− and HER2+.
Basal-like, ER-α−, PR− and HER2− and cytokeratin 5/6+ and/or EGFR+.
Unclassified, negative for ER, PR, HER2, cytokeratin 5/6 and EGFR.

Table 3 ER-β status according to histologic type

Histologic type	*ER*-β+	*ER*-β−
Infiltrating ductal, NOS	952/1500 (63%)	548/1500 (37%)
Infiltrating lobular	200/230 (87%)	30/230 (13%)
Infiltrating ductal with lobular features	254/333 (76%)	79/333 (24%)
Tubular	7/7 (100%)	0/7 (0%)
Invasive cribriform	5/10 (50%)	5/10 (50%)
Mucinous	30/36 (83%)	6/36 (17%)
Metaplastic	3/3 (100%)	0/3 (0%)
Other	30/51 (59%)	21/51 (41%)

ER-β expression was seen in all types, including 63% (952/1500), of invasive ductal carcinomas, 87% (200/230) of invasive lobular carcinomas, 83% (30/36) of mucinous carcinomas and 100% (7/7) of tubular carcinomas. Overall, ER-β− tumors at presentation were larger in size ($P=0.0008$), had a greater extent of lymph node involvement ($P=0.06$) and were higher grade at the time of diagnosis ($P<0.0001$) (Table 4). For 2145 cancers with available information on tumor grade, 85% of grade I, 71% of grade II and 49% of grade III cancers showed positive ER-β staining.

Breast cancer survival cross-classified by ER-α/ER-β status is shown in Figure 2. As expected, patients with ER-α+ tumors had greater survival than patients with ER-α− tumors ($P<0.0001$). However, overall ER-β status was not associated with improved breast cancer survival ($P=0.54$). Furthermore, no beneficial effect of ER-β positivity on survival was seen among ER-α+ tumors ($P=0.37$), ER-α− tumors ($P=0.10$), or ER-α+ tumors treated with tamoxifen ($P=0.55$).

Discussion

In this large population-based study, we have shown that 68% of invasive breast cancers express ER-β as

Table 4 ER-β status according to tumor grade, tumor size, lymph node status and stage at diagnosis

Characteristic	*ER*-β+	*ER*-β−	P-*value*
Tumor grade[a]			<0.0001
Grade 1	361/425 (85 %)	64/425 (15%)	
Grade 2	847/1190 (71%)	343/1190 (29%)	
Grade 3	260/530 (49%)	270/530 (51%)	
Tumor size (cm)[b]			0.0008
0.1–1.0	348/462 (75%)	114/462 (25%)	
1.1–2.0	536/788 (68%)	252/788 (32%)	
2.1–4.0	374/576 (65%)	202/576 (35%)	
4.1+	139/222 (63%)	83/222 (37%)	
Lymph node status[b]			0.06
0 nodes	861/1242 (69%)	381/1242 (31%)	
1–3 nodes	282/423 (67%)	141/423 (33%)	
4–9 nodes	120/183 (66%)	63/183 (34%)	
10+ nodes	67/116 (58%)	49/116 (42%)	
Stage at diagnosis[b]			0.10
I/II	1145/1660 (69%)	515/1660 (31%)	
III/IV	259/400 (65%)	141/400 (35%)	

[a]Data from centralized pathology review.
[b]Data abstracted from pathology report.

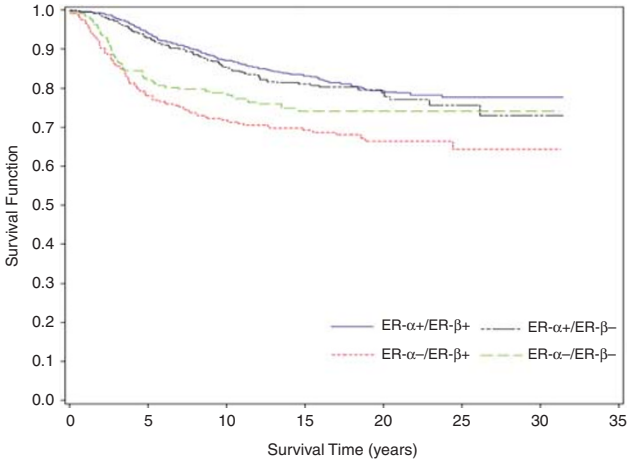

Figure 2 Breast cancer survival of women with nonmetastatic breast cancer by ER-α and ER-β status.

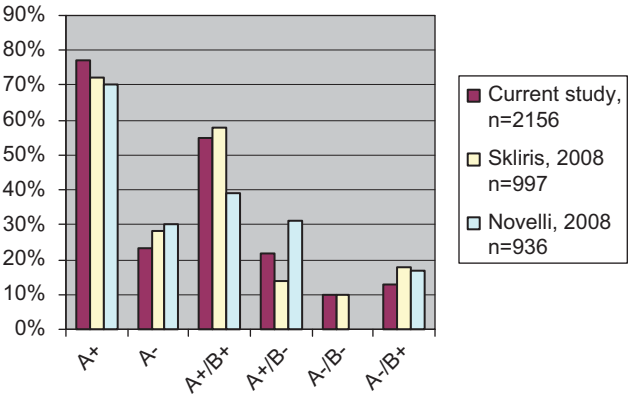

Figure 3 Frequency of estrogen receptor status as detected by ER-α (A) and ER-β (B) expression by immunohistochemistry.

detected by a monoclonal antibody to ER-β1. The frequency of ER-β expression among breast cancers in this series is similar to that reported in earlier studies that also used immunohistochemical methods for its detection.[11,12,14,18,21] In addition, we confirmed that there is a subset of ER-α− cancers that express ER-β. Among the ER-α− tumors in this analysis, 56% (279/501) were ER-β+. This finding is virtually identical to that of Skliris *et al*,[23] who reported that 58% of ER-α− tumors were ER-β1+. We found that 55% of cancers coexpress ER-α and ER-β, 22% of cancers expressed ER-α, but were ER-β−, and 13% of cancers were ER-α− and ER-β+. These results are also similar to summary data presented by Skliris *et al*[24] and to the recent findings of Novelli *et al*[10] (Figure 3).

Our understanding of the biology of ER-β and its role in invasive breast cancer remains elusive and results of earlier studies have been somewhat contradictory. ER-β has at least five isoforms (ER-β1-5)[30] of which three, ER-β1, ERβ-2 (also referred to as ER-βcx) and ER-β-5, have been identified in breast cancers. Much of the conflicting data in the literature are likely because of the fact that there are various ER-β isoforms and to the complexity of their interactions with ER-α, as well as to the use of a variety of detection methods. Recent gene expression profiling studies have shown that ER-β probably regulates a distinct subset of genes involved in cellular proliferation and apoptosis.[15] It is currently believed that ER-α promotes proliferation whereas ER-β is antiproliferative. Accordingly, declining levels of ER-β have been reported to be associated with an increased risk of progression to invasive cancer.[16,17,31] We found that ER-β− tumors were of higher grade, larger size, more likely to have lymph node involvement and of higher stage at time of diagnosis, all of which support a potential antiproliferative role for ER-β.

The prognostic and predictive importance of ER-β expression has been somewhat controversial, again in large part because of the issues mentioned above. Recently, however, there has been renewed interest in ER-β and its potential clinical relevance.[6,7,10,13,24] A recent study by Honma *et al*[13] examined ER-β expression in invasive breast cancers from 442 Japanese women, all of whom were treated with adjuvant tamoxifen. In that study positive immunohistochemical staining for ER-β1, using the PPG5/10 clone, was associated with a significantly increased disease-free survival and overall survival in tamoxifen-treated women. Of particular interest, this response was also seen in ER-α− tumors; a finding in agreement with earlier studies.[6,32] For example, Gruvberger-Saal *et al*[32] also noted better distant disease-free survival in women treated with tamoxifen who had ER-β+/ER-α− cancers. Two recent studies have provided additional insight into the potential prognostic significance of ER-β. In the first,

Novelli *et al*[10] reported that ER-β positivity was associated with a more aggressive clinical course among node-positive breast cancer patients. In contrast, these authors found that ER-β1 positivity predicted favorable response to endocrine therapy among lymph node-negative patients. It should be noted that the patients in the Novelli study were treated with a variety of adjuvant chemotherapy regimens so differences in outcome must be interpreted with caution. Nonetheless, these results suggest that ER-β may potentially be a useful prognostic and/or predictive factor among women with lymph-node-negative breast cancer. In the second study, Shaaban *et al*[7] reported that ER-β2 was a powerful prognostic indicator in women with breast cancer. Furthermore, these authors found that nuclear and cytoplasmic expression of ER-β2 differentially affected outcome.[7] Again though the possibility that differences in systemic therapies may have affected outcome is not addressed in this paper.

In the present study, ER-β positivity did not appear to have a beneficial effect on survival regardless of ER-α status. This lack of association with ER-β and improved survival seen in earlier studies may also be the result of considerable treatment variability; an inherent limitation in this large population of women treated over a long period of time. However, even when we examined survival in women with ER-α+ tumors treated with tamoxifen, ER-β positivity was still not associated with better outcome. Therefore, we were unable to further clarify the predictive and prognostic value of ER-β1.

Estrogen receptor-β expression was observed in each of the molecularly defined phenotypes of invasive breast cancer, even among those defined by the absence of ER-α expression, ie, the HER2 and basal-like subtypes. The association between ER-β and HER2 overexpression is unclear. Some studies have shown a positive association with HER2 overexpression[11,33,34] whereas others have shown an inverse relationship.[23,32] Cytokeratin 5/6, a marker of the basal-like subtype of breast cancer, was only weakly correlated with ER-β in one study.[23] In such ER-α−/ER-β+ tumors, it has been proposed that one or more of the ER-β isoforms are actually promoting proliferation, and this is supported by the frequent coexpression of ER-β with the proliferation markers Ki-67[14,23,33] and topoisomerase IIα.[11] In this study, ER-β was inversely related to HER2 expression as well as to the expression of the basal markers cytokeratin 5/6 and EGFR, in keeping with the findings of previous studies.[23,32]

Our study has several potential limitations. First, we were unable to obtain tissue blocks from all breast cancers arising in this cohort. Our success in doing so was highly correlated with time between diagnosis and initiation of our tissue block collection. After taking into account the effect of age and year of diagnosis, the women for whom we were able to obtain tumor specimens were very similar to those for whom we were unable to obtain specimens.[25] In addition, the frequency of ER-α and ER-β positivity among invasive tumors was very similar to other populations suggesting that samples included in this study are representative of the overall US population. Second, we used immunohistochemical markers as a surrogate to classify breast cancers into the molecular phenotypes defined by expression profiles. Although the antibody panel we used in this study has been shown to be a reliable proxy for classification of invasive breast cancers categorized by gene expression,[4,26–29] the correlation is not perfect and there will be some misclassification of these phenotypes. The categories as defined by the immunohistochemical markers have been shown to be associated with prognostic markers and survival consistent with what has been seen with classification based on RNA expression assays, suggesting that both methods are capturing distinct subgroups.[26,35] Misclassification of phenotypes may underestimate true differences between the subtypes. Finally, there is considerable variability in the specificity of commercially available ER-β antibodies[36] and caution should be used when directly comparing results among studies. In addition to there being several ER-β isoforms as discussed above, technical aspects such as fixation protocols and antigen retrieval methods could explain varying results. We used the Serotec PPG5/10 clone, an antibody directed against ER-β1, and a standard protocol as described above. The PPG5/10 clone has previously been validated by western blot analysis[17,20,31] and when compared with other commercially available ER-β antibodies, the PPG5/10 clone showed consistent strong nuclear staining and has been well validated in a number of studies at this point.[7,13,36] Moreover, ER-β1 is the only fully functional isoform.[37] As a result, it has been considered a favored antibody for ER-β detection.[38]

In summary, in this large population-based study, ER-β expression was commonly seen in luminal A and B types of invasive breast cancer. Furthermore, expression of ER-β was also seen in a subset of HER2 and basal-like cancers, which are considered to be hormone-receptor-negative breast cancers. The potential role of ER-β in the development and progression of invasive breast cancers and as a prognostic and predictive factor in breast cancers defined by lack of expression of ER-α expression merits further investigation.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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