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ER β Expression and Breast Cancer Risk Prediction for Women with Atypias

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Abstract

Estrogen receptor beta (ER β) is highly expressed in normal breast epithelium and a putative tumor suppressor. Atypical hyperplasia substantially increases breast cancer risk, but identification of biomarkers to further improve risk stratification is needed. We evaluated ER β expression in breast tissues from women with atypical hyperplasia and association with subsequent breast cancer risk. ER β expression was examined by immunohistochemistry in a well-characterized 171 women cohort with atypical hyperplasia diagnosed 1967–1991. Nuclear ER β percent and intensity was scored in the atypia and adjacent normal lobules. An ER β sum score (percent + intensity) was calculated and grouped as low, moderate or high. Competing risks regression was used to assess associations of ER β expression with breast cancer risk. After 15 years median follow-up, 36 women developed breast cancer. ER β expression was lower in atypia lobules than normal lobules, by percent staining and intensity (both $p < 0.001$). Higher ER β expression in the atypia or normal lobules, evaluated by percent staining, intensity or sum score, decreased the risk of subsequent breast cancer by 2 ($p = 0.04$) and 2.5-fold ($p = 0.006$). High normal lobule ER β expression conferred the strongest protective effect in pre-menopausal women: the 20-year cumulative incidence of breast cancer was 0% for women $< \text{age } 45$ with high versus 31% for low-moderate ER β expression ($p = 0.0008$). High ER β expression was associated with a significantly decreased risk of breast cancer in women with atypical hyperplasia. These data suggest ER β may be a useful biomarker for risk stratification and a novel therapeutic target for breast cancer risk reduction.

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Keywords

breast cancer risk prediction; estrogen receptor beta; atypical hyperplasia

Introduction

Estrogen receptor beta (ER β) is a member of the nuclear receptor superfamily of transcription factors that was identified in 1996 as the product of the *ESR2* gene on chromosome 14q22-24.[1,2] ER β is distinct from ER α (*ESR1* gene on chromosome 6), the form of the estrogen receptor that is assayed in routine clinical practice for all newly diagnosed breast cancers and used to determine treatment and prognosis.[3,4] ER α and ER β share 96% homology in their DNA-binding domains, but differ considerably at the hinge region, AF1 domain and ligand binding domain.[2,5] Unlike ER α , ER β is highly expressed in normal breast epithelium but declines in expression in pre-cancerous and cancerous breast lesions.[6–13]

Further, ER β (specifically the full-length form, termed ER β 1) is postulated to function as a tumor suppressor of breast cancer as well as other cancers.[14–17] Its expression is diminished or absent in invasive breast cancers compared to preinvasive or benign lesions.[8,9,12,18–20] In breast cancer models, ER β expression alone, or in combination with ER α , inhibits breast cancer cell proliferation and enhances sensitivity of ER α expressing breast cancer cells to the anti-proliferative effects of selective estrogen receptor modulators.[14,21–24] Tumoral expression of ER β is also associated with increased effectiveness of tamoxifen and aromatase inhibitor therapy.[14,25–30]

There is little data on the association of ER β expression in benign breast disease with subsequent breast cancer risk. Atypias of the breast increase the lifetime risk of breast cancer four-fold.[31] In particular, given its possible tumor suppressor role, ER β expression in breast tissues with atypia and a possible role for ER β in mitigating breast cancer risk in high-risk individuals are of interest. The identification of novel biomarkers for risk prediction within this high-risk group of patients is desirable to ascertain those individuals at the highest risk, to better guide women in their choice of pharmacologic or surgical risk reduction strategies, and to identify pharmacologic targets to lower risk.

To better understand the role of ER β in benign breast disease and its possible impact on future breast cancer risk, we undertook this study in women with atypical hyperplasia and known long-term outcome with regard to subsequent development of breast cancer. Our aim was to examine the relationship of ER β expression in the atypical epithelium and in the adjacent histologically normal lobules with the risk of future breast cancer.

Materials and Methods

Patient Cohort

With IRB approval, we studied breast tissues from women with atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH) from the Mayo Clinic Benign Breast Disease Cohort. The cohort and verification of atypical hyperplasia has been described previously.

[32] Among 334 women in the cohort diagnosed with atypia between 1967 and 1991, adequate tissue for ER β staining was available for the 171 women who form the basis of this study. Median follow-up of this atypia subcohort was 15 (range 1 to 36) years.

Histology and Immunohistochemistry

The atypia tissue samples were characterized for the presence of unifocal versus multifocal atypia defined as more than one terminal ductal lobular unit clearly containing atypical hyperplasia.[33] Additionally, the degree of involution of the breast tissue was categorized as none, partial or complete.[34] Immunostaining and assessment for ER α and Ki-67 was performed as previously described.[32,35] For this analysis of ER β expression the tissues studied included the epithelia of the atypical hyperplasia lesion(s), referred to as “the atypia”, and epithelia of histologically normal lobules from the same tissue section as the atypia, hereafter called normal or adjacent normal lobules.

The ER β subcohort was defined after review of all the tissue sections by two pathologists (JMC and DWV). Subsequently, ER β immunohistochemistry was performed on archival formalin-fixed paraffin-embedded tissue sections which were stained with an ER β specific monoclonal antibody (PPG5/10, Thermo Scientific) which is known to detect only the full length form of ER β (termed ER β 1) without cross reacting with any of its splice variant forms.[11,36] This antibody also has been shown to be the most sensitive and specific commercially available ER β antibody for use in immunohistochemical studies.[11,36,37] In brief, sections were first deparaffinized in xylene, washed in decreasing concentrations of ethanol and rehydrated in distilled water. Antigen retrieval was performed by treatment with a pre-heated, citrate-based solution (low-pH Target Retrieval Solution (Dako) in a steamer at 98° C for 40 minutes. Staining was performed in a Dako Autostainer Plus as previously described.[11]

Nuclear ER β staining in epithelia was scored semi-quantitatively by a single pathologist (JMC) blinded to patient identity as well as clinical characteristics and outcome. Both the atypical and adjacent histologically normal breast epithelia were scored for percentage of nuclei stained (scores 0–4 for <1%, 1–25%, 26–50%, 51–75%, and >75%, respectively) and staining intensity (scores 0–3 for negative, weak, intermediate, and strong, respectively). All epithelial atypical foci were scored for categorization, on average this was one focus per case. Similarly, all background adjacent normal lobules were reviewed and scored for categorization, an average of 3 adjacent normal lobules per case. An ER β expression sum score (percent plus intensity, range 0–7) was created and grouped as negative/low (score 0–2), moderate (score 3–5), and high (score 6–7) as previously described.[14]

Statistical Analysis

Descriptive statistics were reported with frequency and percentage for categorical variables and median (range) for continuous variables. Paired comparisons of ER β expression between the atypia and adjacent normal lobules were performed using Wilcoxon signed-rank tests. Independent sample comparisons between the two types of atypia (ADH versus ALH) used Wilcoxon rank-sum tests. Follow-up time was calculated from the date of the index benign biopsy until the diagnosis of breast cancer or until the earliest of the following:

prophylactic mastectomy, last follow-up, or death. The cumulative incidence estimator was used to estimate the incidence of breast cancer while taking the competing risk of death into account.[38] The association of ER β expression with breast cancer risk was assessed using Fine and Gray competing risks regression with the Firth penalized bias-reduction method and was summarized with hazard ratio (HR) and 95% profile-likelihood confidence intervals.[39] P-values < 0.05 were considered statistically significant. Correlation coefficients (ρ) were calculated using the Spearman's rank method. Analysis was performed using SAS (Version 9.3) with the %pshreg macro and R (<http://www.r-project.org>) including the cmrsk package.[40,41]

Results

The median patient age was 56 years (range 28–84 years). All women underwent excisional biopsy of the atypia lesion. Seventy-nine women had ADH and 92 had ALH. No women in this cohort received chemoprevention with endocrine therapy. One patient underwent bilateral prophylactic mastectomy 3 years after her atypia biopsy. During follow-up, 36 of the 171 women developed breast cancer at a median of 13 (range 1–29) years.

ER β expression, assessed by nuclear percent staining, intensity and sum score, was lower in the atypia than in adjacent normal lobules (all $p < 0.0001$) as shown in Table 1 and illustrated Figure 1 which shows low and high ER β expression, respectively, in the atypias (Panels A and B) and in the adjacent normal lobules (Panels C and D). ER β expression was slightly lower in ADH than ALH (supplemental Table 1S), particularly for nuclear percent staining ($p=0.0002$), sum score ($p=0.0004$) and sum score category ($p=0.001$). However, ER β expression in the adjacent normal lobules did not differ between ADH and ALH cases (data not shown).

Association of ER β Expression with Other Risk Factors

The association of ER β expression with age, lobular involution and the number of atypical foci is summarized in Figure 2. Generally, ER β expression in either the atypia or normal lobules was independent of these factors. The only significant association was for age category with normal lobule ER β expression, with significantly lower ER β expression in women ≤ 55 years as compared to women > 55 ($p = 0.03$). ER β expression in the atypia was not significantly associated with the three variables.

Correlation of ER β expression with other markers

Data were available on ER- α and Ki-67 for the atypia lesion only. ER α in the atypia was not correlated with ER β expression in the atypia for either percent staining ($\rho = 0.02$) or intensity ($\rho = 0.08$). ER β expression in the atypia lesion did show a trend ($p=0.09$) toward an inverse association with Ki-67 expression with 39% (12/31), 24% (20/86), and 14% (1/7) showing elevated Ki-67 expression ($\geq 2\%$ cells positive in the atypia) across the levels of low, moderate, and high ER- β sum score, respectively.

Association of ER β with Breast Cancer Risk

ER β in the atypia—By sum score, 44 (26%) of atypias had low ER β expression, 117 (68%) moderate expression, and 10 (6%) high expression. Higher nuclear ER β expression in the atypia was associated with decreased breast cancer risk for all methods of evaluation including nuclear percent staining ($p=0.03$), intensity of staining ($p=0.03$) and sum score ($p=0.01$). Low nuclear ER β expression (sum score 0–2) in the atypia was associated with a 2-fold increased risk of subsequent breast cancer (HR 2.0, 95% CI: 1.02–3.8, $p = 0.04$) compared to atypias with moderate-high expression (sum score 3–7) (Figure 3A).

ER β in the normal lobules—In the adjacent normal lobules ER β expression assessed by sum score was high in 96 (56%), moderate in 74 (43%) and low in 1 (0.6%). As the distribution was substantially different in normal lobules as compared to the atypia, low-moderate expression was compared to high expression in further analysis of the normal lobule scores. The normal lobule sum score showed a moderate correlation of $\rho = 0.39$ with atypia sum score within subject. Of those with low expression in the atypia, 28/44 (64%) showed low-moderate expression in the adjacent normal lobules, which was significantly more frequent ($p=0.002$) than for the moderate-high atypia expression group at 47/127 (37%). However, there were also a substantial number of subjects discordant between the atypia and the normal lobules in terms of favorable ER β expression ($\kappa = 0.22$), with a favorable score for the normal lobules but not the atypia in 16/171 (9%) and a favorable score for the atypia but not the normal lobules in 47/171 (27%).

High ER β expression in the normal lobules was also protective against future development of breast cancer, and as with the atypia lesion, nuclear percent staining ($p=0.002$), intensity of staining ($p=0.01$), and sum score ($p=0.001$) were each significantly associated with risk. A low-moderate sum score (score 0–5) conferred a HR of 2.5 (95% CI 1.3–5.1) for future breast cancer risk versus a high sum score (score 6–7), $p=0.006$ (Figure 3B).

Multivariate analysis—The potential for multivariate analysis was somewhat limited due to the small number of breast cancer events ($n = 36$). Yet, after adjustment for the key risk factors of age, degree of lobular involution, and number of foci of atypia, lower ER β expression remained significantly associated with increased breast cancer risk, in both the atypia (adjusted HR 2.3, 95% CI: 1.1–4.5, $p=0.007$) and in the normal lobules (adjusted HR 2.3, 95% CI: 1.1–4.8, $p = 0.006$). When both atypia and normal lobule ER β expression were included in the same model with the above mentioned covariates, their respective hazard ratios showed some attenuation (adjusted HRs 1.91 and 1.96, $p=0.07$ and $p=0.08$, respectively), yet each measure continued to show a trend.

We noted a slight shift in ER β scores over time with higher scores observed in more recent years (normal lobule median sum score 6 versus 5 and atypia median sum score 4 versus 3 for 1982–1991 versus 1967–1981, respectively, $p < 0.01$ for both). However, although these differences were statistically significant, the absolute differences were modest and hazard ratio estimates adjusting for year of BBD biopsy remained significant with only small attenuations relative to the unadjusted estimates [HR 1.9 (95% CI: 0.9–3.6) for low versus

high-moderate scores in the atypia, and HR 2.3 (95% CI: 1.2–4.9) for low-moderate versus high scores in normal lobules].

To further explore whether considering ER β expression from both the atypia and normal added value to either alone, three categories of ER β expression were compared, derived from the combination of the binary variables for the atypia lesion (low or moderate-high) and the normal lobules (low-moderate or high), (Figure 4). Those with low expression in the atypia and low-moderate expression in the normal lobules (poor/poor) had the highest risk of future breast cancer with an estimated cumulative incidence of 29.2% at 20 years and an unadjusted hazard ratio of 3.7 (95% CI: 1.6–8.8) or an adjusted HR of 3.8 (95% CI: 1.6–9.5, $p=0.001$) as compared to subjects with moderate-high expression in the atypia and high expression in the normal lobules (favorable/favorable) who had an estimated cumulative incidence of 10.4% at 20 years. Those with favorable expression levels for either the atypia or normal lobules but not both showed an intermediate degree of risk that was not significantly different from the most and least favorable categories (data not shown), although these analyses are limited by sample size.

Effect of age on ER β expression and breast cancer risk—To explore the effect of hormonal status on the protective effect of higher ER β expression, associations of ER β expression and breast cancer development were determined within age strata roughly corresponding to pre-, peri-, and post-menopausal states based on age at benign biopsy. The effect of low ER β expression in the atypia lesion was greatest for older patients. For women over age 55 the HR was 3.4 (95% CI 1.1–10.3), for those age 45 to 55 the HR was 1.8 (95% CI 0.7–4.6) and for those less than age 45 the HR was 1.1 (95% CI 0.2–4.8) as shown in Figure 5. In contrast, lower ER β expression in normal lobules showed a stronger effect in younger women. In women under age 45 at biopsy, low-moderate versus high ER β expression was strongly associated with future breast cancer risk with a HR of 15.2 (95% CI 1.8 to >100). Smaller effects of ER β expression in normal lobules were observed for individuals 45 to 55 (HR 1.5, 95% CI 0.6–3.8) and over age 55 (HR 2.3, 95% CI 0.8–7.4) at the time of benign biopsy. Strikingly, no patient (0 of 15) less than age 45 with high normal lobule ER β expression developed breast cancer during follow-up versus 40% (6 of 15) with low-moderate normal lobule ER β expression, $p=0.008$, as shown in Figure 5.

Discussion

Atypical hyperplasia is identified in 4 to 10% of benign breast biopsies.[42–44] Recent data suggest that the absolute risk of future breast cancer imparted after a biopsy demonstrating atypia is about 30% at 25 years, comparable to the risk associated with mantle radiation prior to age 31, a well-recognized indication for breast cancer risk reduction interventions. [33,45–47] While a prior benign breast biopsy showing atypia confers a substantial risk for the future development of breast cancer, currently utilized models to predict breast cancer risk are poorly applicable to these women, although a risk model incorporating benign breast biopsy histology recently has been published.[48] In current practice, the use of cumulative incidence data is recommended when counseling women with atypical hyperplasia on their future risk of breast cancer and the number of atypical foci is reported as the best further discriminant of risk for these high-risk women.[47] Despite these advances, further

individualization of risk prediction is needed, as the majority of these very high-risk women will not be diagnosed with breast cancer. To this end, we evaluated ER β expression using a sum score incorporating both the extent and intensity of staining and found that preserved nuclear ER β expression in the atypia and in the normal lobules of women with atypical hyperplasia was significantly associated with a decreased risk of subsequent breast cancer.

In our longitudinal cohort study we found that when ER β expression was diminished within the atypical epithelium, the risk of future breast cancer was doubled. Low ER β expression in the adjacent normal lobules also was associated with a 2.5 -fold increase in future breast cancer risk. Further, high ER β expression in the normal lobules was most protective against future breast cancer for women who were under 45 years of age (and likely premenopausal) at the time of their biopsy. To the best of our knowledge, this is the first study to evaluate ER β expression and breast cancer risk in a well-defined cohort of women with long-term follow-up.

Shaaban et al reported a case-control study in which they evaluated patients who were diagnosed with breast cancer at least 6 months after a prior benign breast biopsy and compared them to an age and year of biopsy-matched group of controls who had a benign breast biopsy between 1979 and 1999 and who did not develop cancer.[20] In this study, ER β expression was assessed in 54 cases and 71 controls in foci of usual type hyperplasia without atypia using the same antibody we utilized, specific for the full-length ER β 1 receptor. In the hyperplastic lesions of cases versus controls they found a non-statistically significant difference in mean nuclear percent staining for ER β : 68% in the cases versus 81% in the controls. In 116 normal lobules evaluated for ER β expression, 56 from patients who later developed cancer and 60 who did not, mean percentage nuclear staining was 89% in the cases versus 97% in the controls. These findings lend support to our hypothesis that high levels of ER β expression in both the atypical lesion and adjacent normal breast tissue are protective against future breast cancer. To the best of our knowledge, no other case-control studies evaluating ER β expression and subsequent breast cancer risk have been reported.

We confirmed that ER β expression was lower in the atypia than in adjacent normal lobules whether assessed by nuclear percent staining, staining intensity or sum score. Roger and colleagues evaluated 13 cases of atypia (12 ADH and one ALH) of which two were from patients with coincident cancer. They evaluated total ER β expression with a polyclonal ER β 503 antibody and described a statistically significant decrease in the ER β to ER α ratio in the lesional epithelia versus both adjacent normal epithelia and normal lobules from reduction mammoplasties.[8] Ellis et al reported similar findings on ER β expression in a study of ADH grouped with DCIS, and ALH grouped with LCIS from both patients with and without cancer combined.[10] This study used the 14C8 ER β antibody which also detects total ER β , including splice variants, and described similar levels of ER β expression in normal lobules from breast reduction specimens and cancer patients and a steady decline in expression from benign breast disease with usual hyperplasia to atypia combined with carcinoma in situ. Using a different ER β 1 specific antibody and categorization of staining similar to our methodology, Chantzi et al found that ER β 1 expression was slightly, but not significantly, higher in 14 samples of normal breast tissue versus 16 cases of atypical

hyperplasia.[12] Unlike our study and another [10] in which there was no association between age and ER β expression in the atypia or adjacent normal lobule, they further found that ER β 1 expression declined after menopause in normal breast lobules and benign breast disease including atypical hyperplasia as well as in DCIS.

Our finding of the dramatic protective effect on future breast cancer risk of high ER β expression in the normal adjacent breast lobules in premenopausal patients is intriguing and raises several interesting hypotheses for further investigation. Might early intervention to preserve or create a high ER β breast environment prior to menopause confer a risk-reducing benefit that extends for decades? Why is it that ER β is so effective when patients are younger (pre-menopausal) and why is the effect so long lasting even after menopause? Is this analogous to the tamoxifen effect of five years of treatment affecting 20-year event rate? Would therapeutic targeting of ER β represent a novel treatment for breast cancer prevention? These findings lend further support to the notion that ER β functions as a potent tumor suppressor in the breast and lay the foundation for future studies aimed at addressing these questions.

Strengths of our study include use of an established and well-characterized patient cohort with long-term follow-up information on breast cancer events, as well as careful central pathology review and verification of cases. Limitations include the relatively modest absolute number of events and use of a manual, semi-quantitative scoring system for assessing ER β expression. Along with development of digital quantitation algorithms to permit semi-automated reads of immunohistochemical ER β expression, confirmation of our findings in a validation cohort is needed prior to clinical application of these data.

Conclusions

Here we show for the first time that preserved ER β expression in atypical hyperplasia and within normal adjacent breast epithelium is protective against the future development of breast cancer. These data suggest that ER β may be both a biomarker of elevated breast cancer risk and a potential target for therapeutic intervention. Analysis in a validation cohort to confirm our findings, and further investigation in a larger group of women at lower risk for breast cancer, is indicated to confirm the role of ER β as a novel biomarker of breast cancer risk. Studies are warranted to further elucidate the mechanisms by which ER β functions in normal breast epithelial cells and/or alters the microenvironment surrounding atypical breast lesions, in order to elicit a breast cancer preventative effect. Finally, strategies aimed at enhancing ER β expression levels and activating the tumor suppressive effects of this receptor appear promising for the prevention of breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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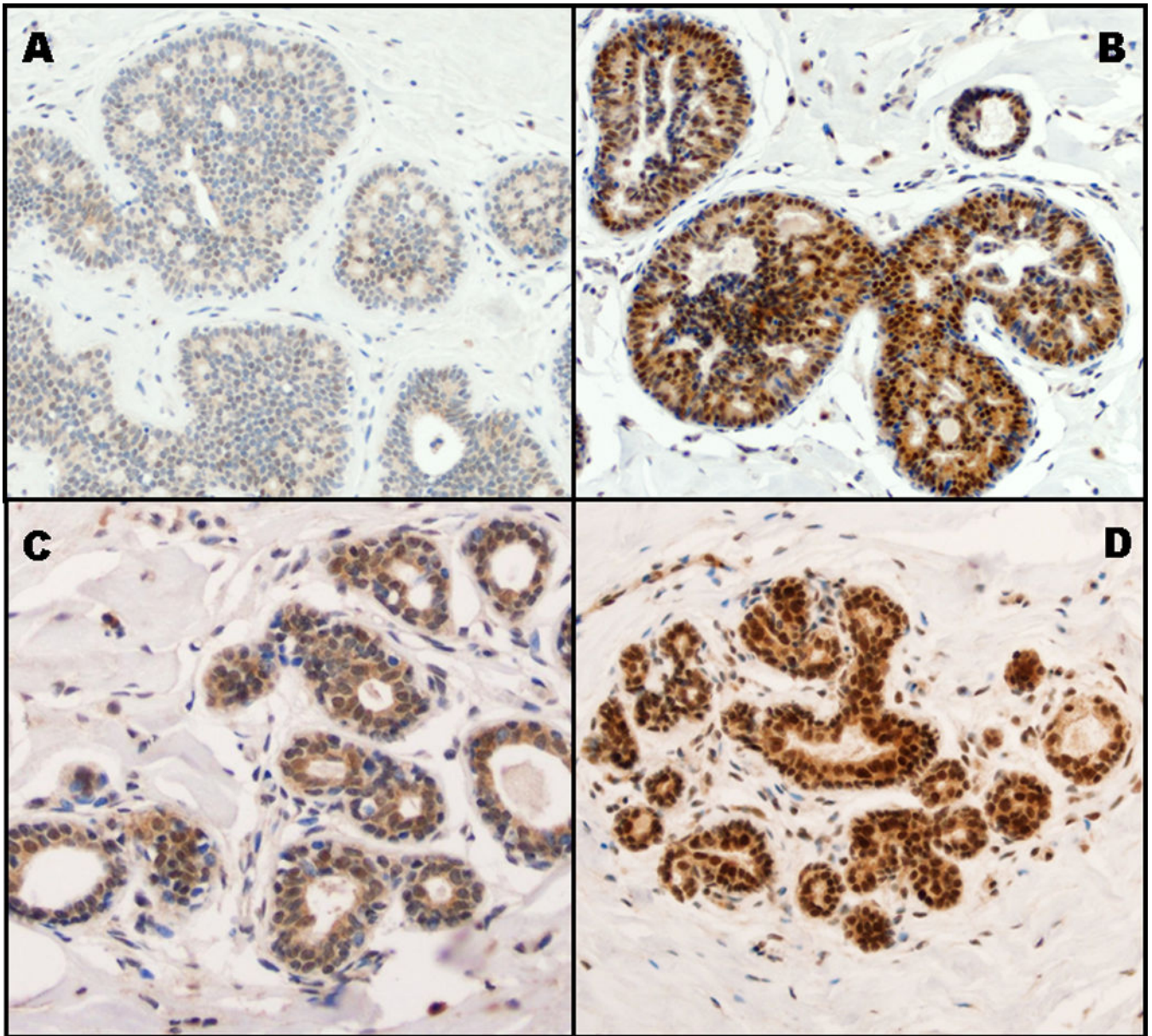


Figure 1.

Illustration of ER β nuclear immunostaining stratified by sum score incorporating extent and intensity of staining in both atypical and normal lobules. (A) Low ER β expression in atypia: nuclear percentage score of 1 and intensity score of 1, representing a sum score of 2. (B) High ER β expression in atypia: nuclear percentage score of 3 and intensity score of 3, representing a sum score of 6. (C) Low ER β expression in normal lobule: nuclear percentage score of 1 and intensity score of 1, representing a sum score of 2. (D) High ER β expression in normal lobule: nuclear percentage score of 4 and intensity score of 3, representing a sum score of 7.

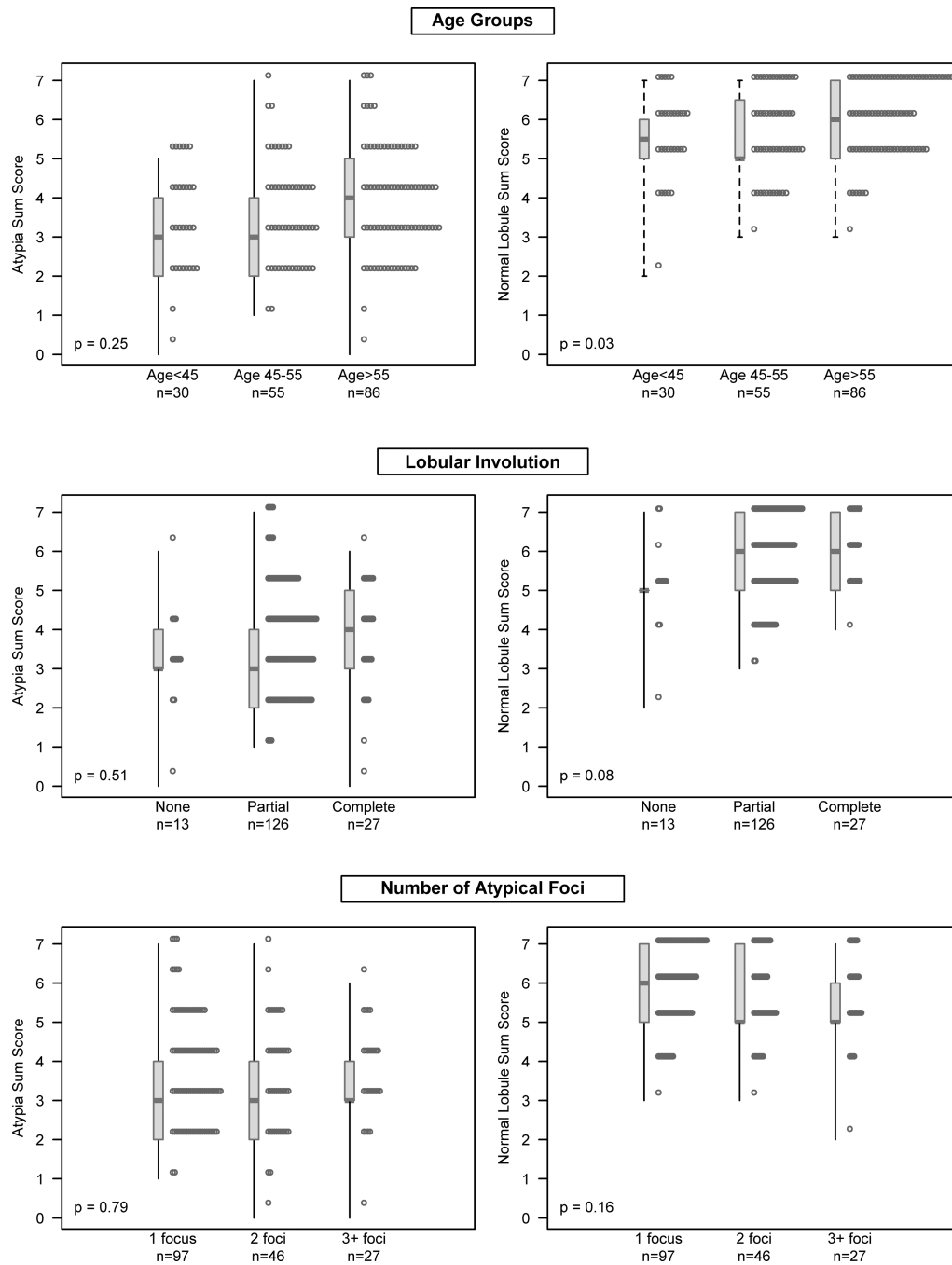


Figure 2. The relationship between ER β expression in the atypia (left panels) and in the normal lobules (right panels) with age, lobular involution and the number of atypical foci.

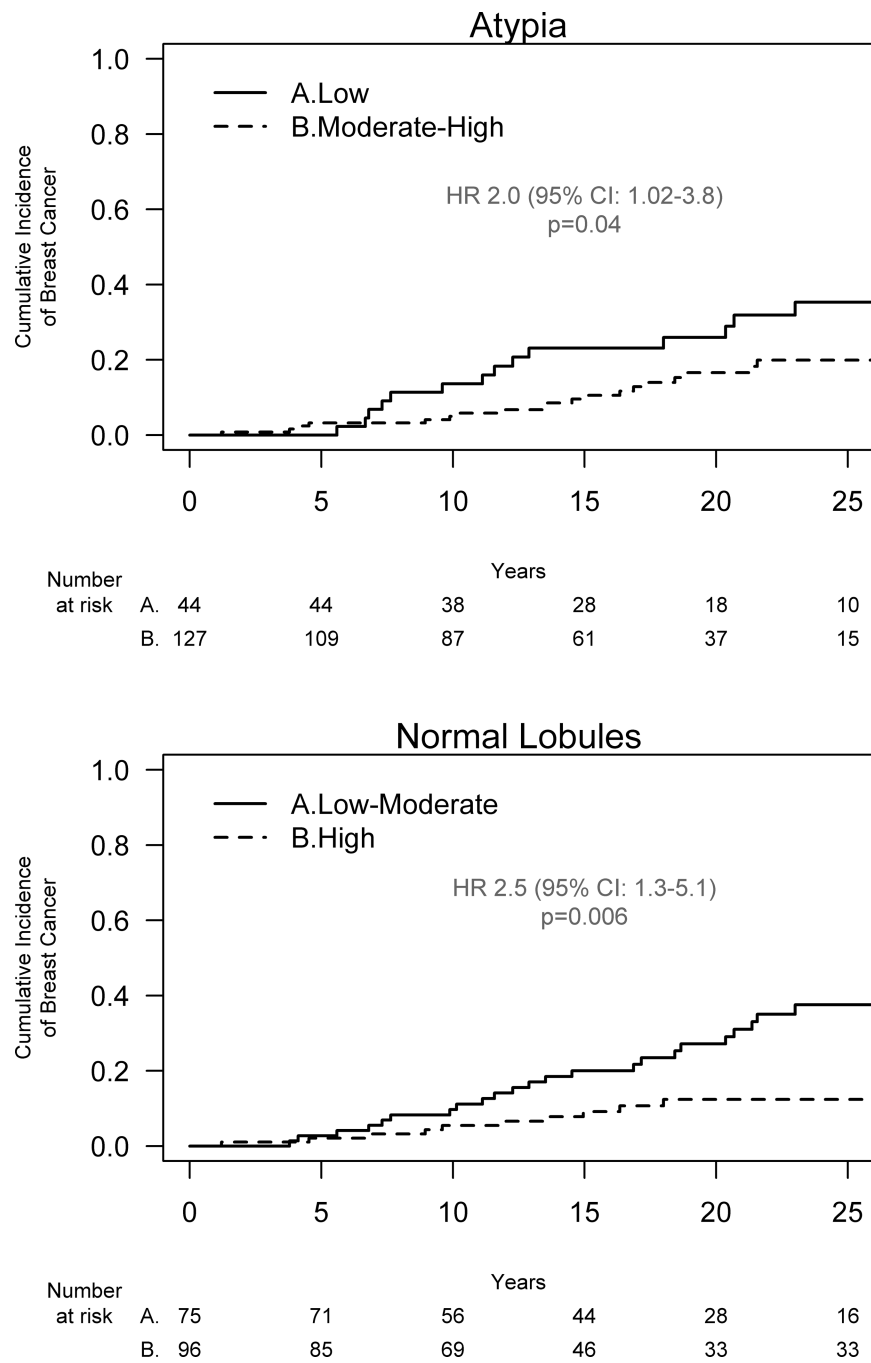


Figure 3. ER β expression in the atypia (top panel) and adjacent normal lobule (lower panel) and subsequent breast cancer risk.

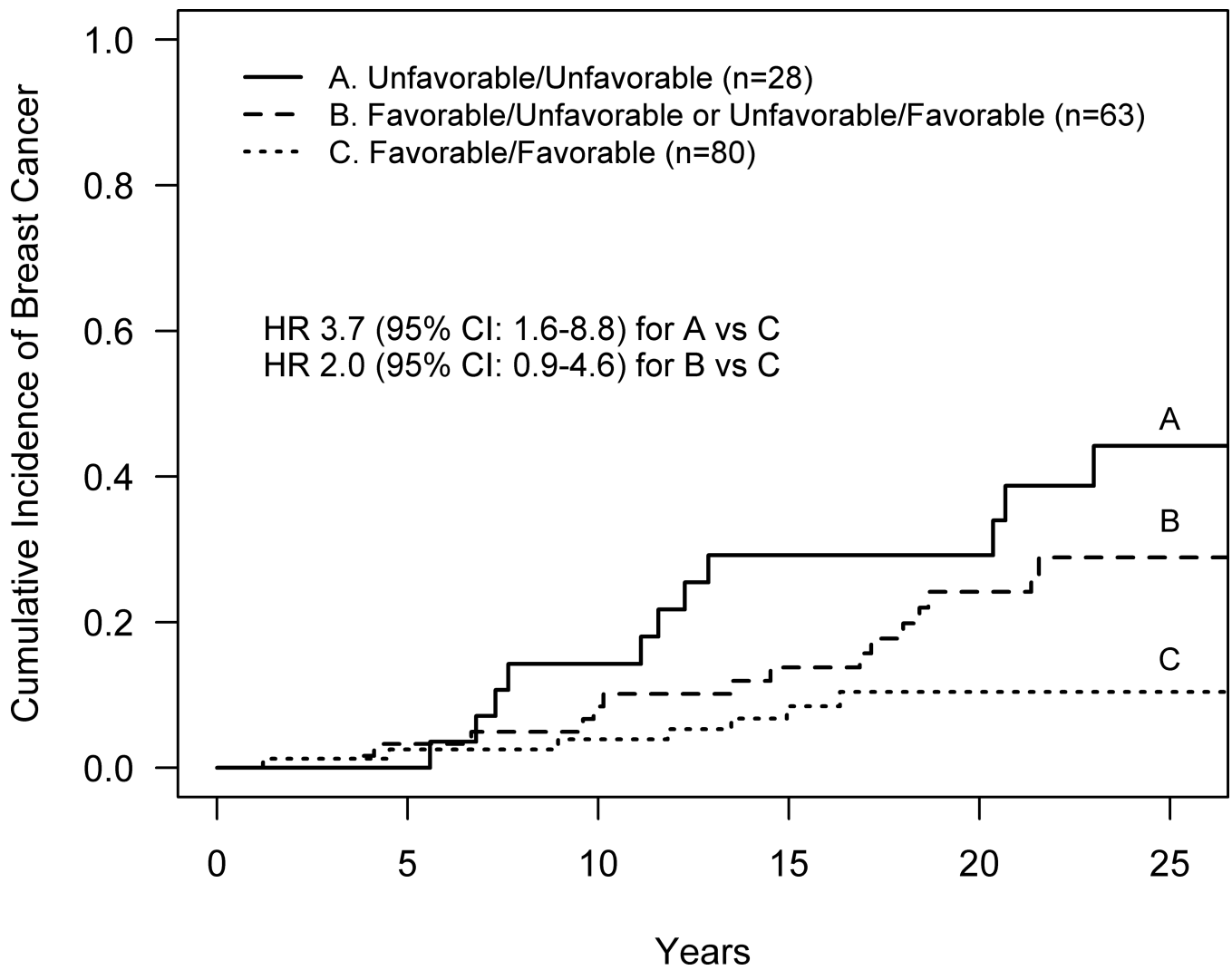


Figure 4. Breast cancer risk for combinations of ER β expression in the atypia and adjacent normal lobules. Unfavorable/Unfavorable refers to those women with low expression in the atypia and low-moderate expression in normal lobules; Favorable/Favorable refers to those with moderate-high expression in the atypia and high expression in the normal lobules; the intermediate category captures those with favorable expression in either the atypia or normal lobules but not both.

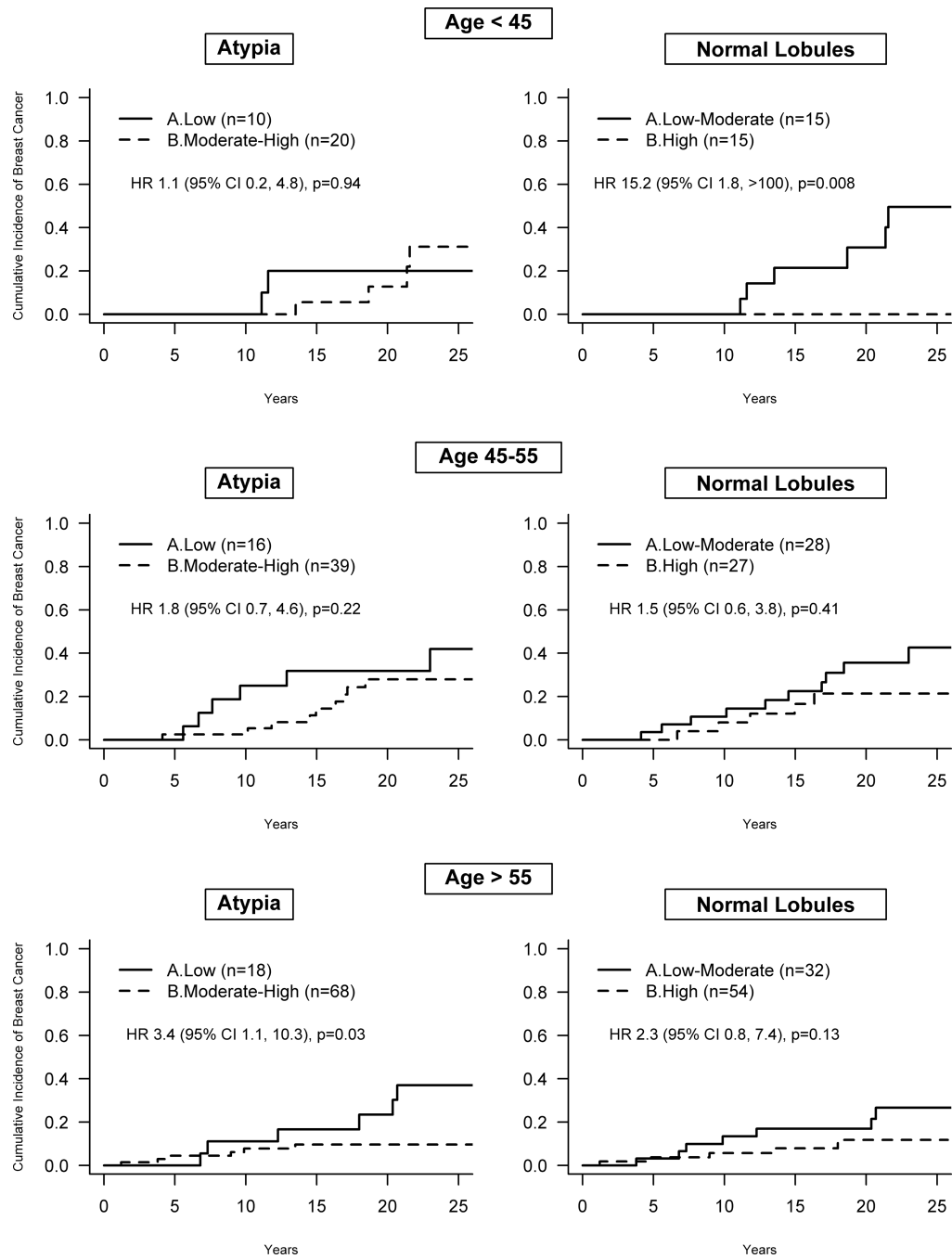


Figure 5. Association of ER β expression in the atypia and adjacent normal lobules with subsequent breast cancer risk stratified by age groups.

Table 1ER β staining in the atypia and adjacent normal lobules.

	Atypia N=171	Normal Lobule N=171	p-value
Nuclear % Staining			p<0.0001
<1%	8 (4.7%)	0	
1–25%	41 (24.0%)	1 (0.6%)	
26–50%	59 (34.5%)	12 (7.0%)	
51–75%	56 (32.7%)	81 (47.4%)	
>75%	7 (4.1%)	77 (45.0%)	
Nuclear Intensity			p<0.0001
Negative	2 (1.2%)	0	
Weak	109 (63.7%)	21 (12.3%)	
Intermediate	48 (28.1%)	73 (42.7%)	
Strong	12 (7.0%)	77 (45.0%)	
Sum Score, median (range)	3 (0–7)	6 (2–7)	p<0.0001
Sum Score Group			p<0.0001
Negative/Low	44 (25.7%)	1 (0.6%)	
Moderate	117 (68.4%)	74 (43.3%)	
High	10 (5.8%)	96 (56.1%)	