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Parity and expression of epithelial histopathologic markers in breast tissue

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Abstract

It is well established that pregnancies protect against breast cancer, but the mechanism is not fully understood. We explored the influence of parity on hormonal and proliferation markers in benign tissue from tumor blocks of breast cancer cases. Women with breast cancer were recruited from a case-control study nested within the Multiethnic Cohort study. Tissue microarrays of benign tissue cores were available for 159 participants. Immunostaining for estrogen receptor alpha (ERα) and beta (ERβ), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/neu), Ki-67, and Proliferating Cell Nuclear Antigen (PCNA) was evaluated in epithelial tissue by a pathologic expert. We applied logistic regression models to examine marker expression by parity (0, 1–2, and ≥3 live births with adjustment for age at diagnosis and body mass index. Of the 159 women, 24 were nulliparous, 63 had 1–2 live births, and 72 had ≥3 live births. Inverse associations were observed between parity and expression of ERα (p_{trend}=0.02) and PCNA (p_{trend}=0.04). Among nulliparous women, 45.5% were ERα positive in contrast to 18.0% and 18.9% of women with 1–2 and ≥3 live births. The respective values for PCNA were 56.5%, 44.3%, and 31.1%. No associations were detected for ERβ, PR, Her2/neu and Ki-67. The current findings suggest that pregnancies may protect against breast cancer by reducing susceptibility to estrogenic stimuli and proliferative activity as assessed by expression of ERα and PCNA in breast tissue.

Keywords

parity; breast tissue; marker expression; tissue microarray; estrogen receptor; PCNA

Introduction

Epidemiological studies across ethnic groups and regions suggest that parity is protective against breast cancer, specifically, estrogen receptor (ER) positive breast cancer (Britt *et al.,* 2007; Collaborative Group on Hormonal Factors in Breast Cancer, 2002); however, its mechanism is not fully understood. One or more pregnancies may induce physiological changes in the breast that, in turn, make the epithelial tissue less susceptible to malignant

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Conflicts of interest
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transformation in the future, especially if the pregnancies occur at early ages(Britt *et al.,* 2007; Russo *et al.,* 2005). Rodent studies indicate that pregnancy is profoundly protective against both the tumor-initiating effects of DNA-damaging carcinogens, such as dimethylbenzanthracene or ionizing radiation, and the tumor-promoting effects of endogenous or exogenous steroid hormones (Medina, 2005). During pregnancy, undifferentiated epithelial cells, which make up most of the breast lobules of nulliparous women, develop into mature epithelia (Russo *et al.,* 2005). Highly differentiated cells are thought to be more resistant to carcinogenesis. Such transformation may be observable in hormonal and proliferation markers of breast tissue of parous females. For example, gene expression analyses in the mammary gland of 28 female cynomolgus macaques in different life phases demonstrated distinct patterns during pregnancy and lactation including changes in ER and progesterone receptors (PR) (Stute *et al.,* 2012). As shown in previous reports of small numbers of premenopausal women (Battersby *et al.,* 1992; Taylor *et al.,* 2009), the expression of histopathologic markers, such as ER and PR, may be lower in parous than nulliparous women. The presence of these hormone receptors influences tumor growth in the breast in response to estrogenic stimuli; ERα promotes proliferative activity while ERβ appears be more anti-proliferative by stimulating cell differentiation (Barkhem *et al.,* 1998; Fox *et al.,* 2008; Britt *et al.,* 2007). The expression of PR, which has subtypes PRA and PRB with possible opposing effects, is regulated by ERα in the presence of estrogens and is a sensitive indicator of estrogenic effects in cells (Potter *et al.,* 1995; Nardulli *et al.,* 1988). ER and PR expression status also serve as prognostic factors and in the selection of the appropriate breast cancer treatment. Other immunohistochemical markers associated with breast cancer prognosis include those that detect specific nuclear antigens, such as human epidermal growth factor receptor 2 (HER2/neu), Ki-67, and proliferating cell nuclear antigen (PCNA). These proteins are involved in cell proliferation and their expression indicates cancer growth and progression (Schwartz *et al.,* 1993). In contrast to ER or PR, their relation with parity has not been investigated. To add to the limited knowledge how parity may protect against breast cancer risk by altering breast tissue, we explored the expression status of these markers in benign tissue from tumor blocks of breast cancer patients in the Hawaii component of the Multiethnic Cohort (MEC).

Materials and Methods

Study population

Breast cancer cases were recruited from a case-control study (Maskarinec *et al.,* 2005) investigating mammographic density nested in the MEC (Kolonel *et al.,* 2000). Benign tissue adjacent to malignant tumor tissue was available for 159 of 279 breast cancer cases in the case-control study. The project was approved by the Institutional Review Boards of the University of Hawaii and Wake Forest School of Medicine; all subjects provided informed consent in writing. Further details of the study were reported previously (Verheus *et al.,* 2009). At entry into the MEC, all women completed a questionnaire that inquired about demographics, reproductive history, anthropometric measures, and family history of breast cancer. As part of the case-control study, women completed a one-page breast health questionnaire that asked about previous breast surgery, menopausal status, mammography history, and hormone replacement therapy (HRT) use.

Tumor microarrays

Tissue microarrays (TMAs) were prepared according to standard procedures and separately immunostained for ERα (6G11; Novacastra Labs, Newcastle-upon-Type, UK) and ERβ (EMR02; Novacastra Labs, Newcastle-upon-Type, UK), PR (1A6; Novacastra Labs, Newcastle-upon-Type, UK), HER2/neu (rabbit polyclonal DAKO Corporation, Carpinteria, CA) Ki-67 (Clone SP6; Labvision NeoMarkers, Fremont, CA), and PCNA (PC10;

Novacastra Labs, Newcastle-upon-Type, UK) as described elsewhere (Verheus *et al.,* 2009). Among all stained specimens, 159 women were identified with at least one benign epithelial tissue sample. Missing data were recorded for PR and PCNA (N=1); for ERα and ERβ (N=2); for Her2/neu (N=3); and for Ki-67 (N=4). A pathologic expert quantified staining on individual TMA core sections at a magnification of 20×, using a Nikon Labophot 2 microscope, a 3 megapixel digital camera (Infinity 2–3, Lumenera Inc., Ottawa, Ontario), and color imaging software (Image Pro Plus, Media Cybernetics, Bethesda, MD). Stromal tissue was not evaluated for any of these markers.

Statistical analysis

The SAS statistical software package version 9.2 was used for all analyses (SAS Institute Inc., Cary, NC). For all histology markers, the mean percentage of stained cells of all available cores per sample was calculated. For all six markers, the distributions of samples were skewed with strong left tails (Verheus *et al.,* 2009). Therefore, samples were divided into two categories: negative staining (<10% of cells stained) and positive staining (≥ 10% of cells). Because the number of women with positive staining for PR and Ki-67 was very low (4 and 13 respectively), we dichotomized the results by no vs. any epithelial staining. Given the known estrogen dependency of PR expression, we also compared the expression status of ER and PR in benign tissue using chi-square tests. We applied logistic regression models to examine marker expression (positive vs. negative) by parity category (0, 1–2, and ≥3 live births as continuous variable). Associations were adjusted for age at diagnosis (continuous) and body mass index (BMI in kg/m^2). BMI was significantly associated with positive ERα expression (p_{trend}=0.02). Although age at first-live birth was related to ERα expression (p_{trend}<0.01), it was not included as a covariate due to its high correlation with parity: 125 of 135 women (93%) had at least one live birth before or at 30 years. Menopausal status was also positively associated with parity because of the older age at diagnosis of parous than nulliparous women (Table 1), but showed was not related to marker expression and, thus, not included as a covariate. We explored other potential breast cancer risk factors, i.e., HRT use, physical activity, years of education, family history of breast cancer, ethnicity, age at menarche, alcohol consumption, and smoking, but no association with marker expression was detected. In addition, we performed stratified analyses by menopausal status and by ethnicity for the largest ethnic groups, i.e., Caucasians and Japanese Americans. Subsequently, we evaluated the expression status of selected markers in malignant tissue that showed statistically-significant associations with parity in benign tissue.

Results

Of the 159 women, 24 (15%) reported no children, 63 (40%) had 1–2 children, and 72 (45%) had ≥3 children (Table 1). Japanese Americans constituted the largest proportion of participants, followed by Caucasians with smaller numbers of Native Hawaiians and other ethnic backgrounds. Across parity categories, significant differences were noted for age at diagnosis (p<0.001), HRT use (p<0.01), menopausal status (p<0.001), and age at first-live birth (p<0.0001). Women with children tended to be older at breast cancer diagnosis than nulliparous women (63.4±7.6 years for ≥3 children and 58.9±8.8 years for 1–2 children vs. 56.8±9.5 years for nulliparous women). HRT use was more common in women with ≥3 children (75%) than in women with no or 1–2 children (50% or 46%). Nearly all women (99%) with ≥3 children and 84% in the women with 1–2 children had their first child before age 30. No differences in BMI, menopausal status, years of education, family history of breast cancer, ethnicity, or age at menarche were apparent across parity categories. Expression of PR was not associated with ERα (49% in agreement; p=0.95) and weakly associated with ERβ (42% in agreement; p=0.06).

We observed inverse associations between parity and positive expressions of ERα (p_{trend}=0.07) and PCNA (p_{trend}=0.04). Among nulliparous women, 45.5% were identified as ERα positive in contrast to 18.0% and 18.9% of women with 1–2 and ≥3 children. The respective values for PCNA were 56.5%, 44.3%, and 31.1%. The inverse associations both became statistically significant (p_{trend} =0.02 for ERα and p_{trend}=0.04 for PCNA; Table 2) after adjustment for age at diagnosis and BMI. Interestingly, stratified analyses by ethnicity showed similar trends in the 49 Caucasian women (75%, 17%, and 9% ERα positive across 0, 1–2, and ≥3 children; p_{trend}<0.01; 75%, 41%, and 30% PCNA positive; p_{trend}=0.03) but no association in the 70 Japanese Americans (33%, 18%, and 25% ERα positive; p_{trend}=0.47; PCNA: 44%, 41%, and 38% PCNA positive; p_{trend}=0.78). However, the interaction terms between ethnicity and parity were not statistically significant ($p_{interaction}$=0.21 and 0.76). In stratified analyses by menopausal status, the inverse associations remained significant or borderline significant only for the 99 post-menopausal women (p_{trend} =0.01 for ERα and p_{trend} =0.08 for PCNA) and not for the 60 pre-menopausal women (p_{trend} =0.50 for ERα and p_{trend} =0.10 for PCNA), but again the interaction terms were not significant. In malignant tissue, neither the expression of ERα (p_{trend}=0.15) nor that of PCNA (p_{trend}=0.59) was associated with parity in both unadjusted and adjusted models.

No significant associations with parity were observed for ERβ, PR, Her2/neu and Ki-67 in unadjusted and adjusted models, even with different parity categories (0 vs. ≥1 or 0–2 vs. ≥3) or restricting the analyses to pre-menopausal or post-menopausal women only.

Discussion

In this exploratory study, parity was inversely associated with the expression of ERα and PCNA in benign tissue from a multiethnic population of pre- and post-menopausal breast cancer cases. The percentage of ERα staining was similar between the women with 1–2 and ≥3 children suggesting a threshold effect; the percentage of nulliparous women with ERα positive staining was more than twice as high as that of parous women. On the other hand, the percentage of PCNA staining showed a linear decline with increasing number of children from 56.5% to 31.1%. No associations were detected for ERβ, PR, Her2/neu and Ki-67. These findings support the idea that pregnancies modify the expression of hormonal and proliferation markers in the breast tissue in a manner that lowers breast cancer risk later in life.

The inverse association of parity with ERα expression in the present study was in agreement with a previous report (Taylor *et al.,* 2009) that evaluated the expression of ERα and PRA and PRB in the breast terminal duct lobular unit epithelium of 26 premenopausal women and 30 pregnant women. In this study, lower expression of ERα was observed in 10 parous than 16 nulliparous pre-menopausal women although the results were not statistically significant. The mean percentage of ERα positive cells was lowest in parous women, intermediate in pregnant women, and highest in nulliparous women indicating a transitional process during pregnancy. These observations support the hypothesis that a decline in hormone receptor positive cells occurs during breast maturation associated with pregnancy (Russo *et al.,* 2005). A recent study of non-human primates showed that ERα expression is diminished with parity and that ER-related markers including PR are markedly down-regulated during pregnancy and lactation (Stute *et al.,* 2012). On the other hand, no association between parity and ERα was seen in 158 normal breast samples from pre-menopausal women after adjustment for menstrual cycle phase and oral contraceptive use, which appear to influence ER expression (Battersby *et al.,* 1992; Khan *et al.,* 1998). The heterogeneity by menopausal status observed in our study may be due to lack of adjustment for menstrual cycle phase.

In contrast to our null findings even after stratification by menopausal status, trends of lower PR expression in parous vs. nulliparous women were observed elsewhere (Taylor *et al.,* 2009; Battersby *et al.,* 1992). In particular, PRA was significantly lower in parous than nulliparous pre-menopausal women (10.2% vs. 32.2% stained; p=0.01). The lack of such a pattern in the present study may be related to the unexpectedly weak associations of PR with ERα and ERβ that were observed.

To our knowledge, no previous reports have described the association between parity and PCNA expression. PCNA is a nuclear protein expressed during cell cycle and plays a role in DNA synthesis (Schwartz *et al.,* 1993; Leonardi *et al.,* 1992; Malkas *et al.,* 2006). In previous studies, an overexpression of PCNA in tumor cells was associated with shorter overall survival, as well as with shorter disease free survival (Stuart-Harris *et al.,* 2008), but little is known about its relation to known breast cancer risk factors. Because PCNA is thought to assess longer-term, cumulative proliferative activity in cells (Leonardi *et al.,* 1992), an inverse relation with parity might reflect the pregnancy-related reduction in cell growth in breast tissue.

The present study had several strengths including its multiethnic participants from a population-based cohort, the evaluation of benign tissue samples in tumor blocks of breast cancer cases, and the use of the TMA method. More than half of the study population consisted of non-Caucasian women distributed evenly across parity categories. The overall sample size was, although not ideal, fairly large for a pathologic study that required access to tumor blocks and sufficient amounts of tissue. The TMA approach made it possible to examine multiple markers in specimens stained under identical conditions.

This study also had a number of limitations. Given the lack of readily-available tissue samples and the invasive nature of breast biopsies, the study population did not include women without cancer to compare cases with controls. Because of field effects, benign tissues from unaffected women would have been preferable over benign tissue components from breast cancer patients. Concerns about statistical power did not allow more detailed stratified analyses by HRT use or breast cancer subtype. In addition, we did not adjust for age at first-live birth because of serious collinearity with parity. Among the breast cancer cases included in the present study, the number of nulliparous women was relatively small. Thus, the findings should be interpreted with caution and need to be further evaluated in larger studies.

The results of this study suggest that ERα and PCNA in breast tissue reflect the cancer protective effects of pregnancies on the breast that reduce its susceptibility to estrogenic stimuli and proliferative activity. Although the potential mechanisms of action are unclear, these pathologic markers may be related to physiological changes in the breast associated with parity.

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Table 1

Characteristics of breast cancer cases by parity[a]

Parity	All	0	1–2	3	*P*-value[b]
N	159	24 (15%)	63 (40%)	72 (45%)	
Age at diagnosis, *years*	60.6±8.7	56.8±9.2	58.9±8.8	63.4±7.6	**<0.001**
Body mass index, kg/m^2	24.4±4.3	24.1±6.5	24.3±3.7	24.7±3.9	0.69
Hormone replacement therapy use					**<0.01**
Never	64 (40%)	12 (50%)	34 (54%)	18 (25%)	
Current/past user	95 (60%)	12 (50%)	29 (46%)	54 (75%)	
Menopausal status					**<0.001**
Premenopausal	60 (38%)	14 (58%)	31 (49%)	15 (21%)	
Postmenopausal	99 (62%)	10 (42%)	32 (51%)	57 (79%)	
Education, *years*	14.6±2.4	14.7±2.5	14.9±2.2	14.4±2.5	0.34
Family history of breast cancer	17 (11%)	3 (13%)	5 (8%)	9 (13%)	0.66
Ethnicity					0.97
Japanese American	70 (44%)	9 (37%)	29 (46%)	32 (44%)	
Caucasian	49 (31%)	8 (33%)	18 (28%)	23 (32%)	
Native Hawaiian	21 (13%)	3 (13%)	8 (13%)	10 (14%)	
Other	19 (12%)	4 (17%)	8 (13%)	7 (10%)	
Age at first-live birth, *years*					**<0.0001**
No children	24 (15%)	24 (100%)	--	--	
< 21	21 (13%)	--	7 (11%)	14 (20%)	
21–30	103 (65%)	--	46 (73%)	57 (79%)	
≥ 31	11 (7%)	--	10 (16%)	1 (1%)	
Age at menarche, *years*					0.63
< 13	86 (54%)	12 (50%)	36 (57%)	38 (53%)	
13–14	58 (37%)	10 (42%)	19 (30%)	29 (40%)	
> 14	15 (9%)	2 (8%)	8 (13%)	5 (7%)	

[a]Data are presented as mean±SD or N (%). Of the 159 women, missing marker expression data were recorded for N=1 for PR and PCNA, N=2 for ERα and ERβ; N=3 for Her2/neu; and N=4 for Ki-67.

[b]*P*-values from χ^2 test (categorical variables) or Students t-test (continuous variable).

Table 2

Marker expression of benign tissue in breast cancer cases by parity

Immunohistochemical marker[a]		Parity				P_{trend}[b]
		All	0	1–2	≥ 3	
ERα	Total N	157	22	61	74	
	N, positive	35	10	11	14	**0.02**
	%, positive	22.3	45.5	18.0	18.9	
ERβ	Total N	157	22	62	73	
	N, positive	47	6	17	24	0.37
	%, positive	29.9	27.3	27.4	32.9	
PR	Total N	158	23	61	74	
	N, positive	81	12	32	37	0.75
	%, positive	51.3	52.2	52.5	50.0	
Her2/neu	Total N	156	22	61	73	
	N, positive	52	8	21	23	0.90
	%, positive	33.3	36.4	34.4	31.5	
Ki-67	Total N	155	22	60	73	
	N, positive	90	15	30	45	0.61
	%, positive	58.1	68.2	50.0	61.6	
PCNA	Total N	158	23	61	74	
	N, positive	63	13	27	23	**0.04**
	%, positive	39.9	56.5	44.3	31.1	

[a] ERα = estrogen receptor alpha; ERβ = estrogen receptor beta; PR = progesterone receptor; HER2/neu = human epidermal growth factor receptor 2; PCNA = proliferating cell nuclear antigen. Positive marker expression is defined as <10% vs. ≥10% epithelial staining for ERα, ERβ, Her2/neu, and PCNA and as no vs. any epithelial staining for PR and Ki-67.

[b] P_{trend} value from logistic regression analysis using parity categories (0, 1–2, and ≥3) as a continuous variable adjusted for age at diagnosis (continuous) and body mass index (continuous).