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EXPRESSION OF IGF1R IN NORMAL BREAST TISSUE AND SUBSEQUENT RISK OF BREAST CANCER

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Abstract

Introduction—The growth hormone and insulin-like growth factor (IGF) axis plays an essential role in the growth and development of the mammary gland. IGF1 and IGF1 receptor (IGF1R) may also play a role in the early transformation of mammary cells.

Methods—Using a nested case-control design, we examined the association between IGF1R expression in normal breast tissue from benign biopsies and subsequent risk of breast cancer within the Nurses' Health Study. We constructed tissue microarrays (TMAs) containing normal terminal ductal lobular units (TDLUs) from benign breast biopsies. Immunostains for IGF1R were performed on sections cut from the TMAs. A total of 312 women had evaluable IGF1R staining in normal TDLUs; 75 subsequently developed breast cancer (cases) and 237 did not (controls). The epithelial cells in the normal TDLUs were scored for both cytoplasmic and membrane staining for IGF1R.

Results—Cytoplasmic IGF1R expression was positively associated with subsequent risk of breast cancer (OR=2.47, 95% CI 1.41–4.33). Women whose TDLU epithelial cells showed little or no membrane expression of IGF1R but high levels of cytoplasmic IGF1R were at the highest breast cancer risk and were 15 times more likely to develop subsequent breast cancer when compared with women who had little or no membrane or cytoplasmic IGF1R expression in their TDLU epithelial cells (OR=15.9, 95% CI 3.6–69.8).

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Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

RMT was responsible for study design, data analyses, manuscript preparation and editing. GAC and BR made substantial contributions to the study design, analysis, and to the interpretation of data. YW, LCC, JLC, SJS were involved in the pathology review, staining, interpretation of slides, and contributed to manuscript editing. RH, HYI contributed substantially to manuscript editing. All authors read and approved the final manuscript.

Conclusion—In this study, IGF1R expression patterns in epithelial cells of normal TDLUs in benign breast biopsies were associated with an increased risk of subsequent breast cancer. Additional studies to confirm these findings are necessary.

Keywords

IGF1R; normal TDLU; benign breast disease; tissue microarrays

Introduction

The growth hormone and insulin-like growth factor (IGF) axis plays an essential role in the growth and development of the mammary gland (1). In addition, IGF1 has mitogenic effects on mammary cells (2). Rhesus monkeys treated with growth hormone or IGF1 show histologic evidence of mammary gland hyperplasia. Initial epidemiologic studies examining circulating levels of IGF1 found a positive association with the risk of premenopausal breast cancer, but not with postmenopausal breast cancer (3). However, more recently several large prospective studies have reported results that do not support the earlier findings (4–7). Reasons for inconsistencies between studies are not known but may involve differences in study populations assessed (e.g., by age or time period of follow-up) and variability in the IGF assay methodology.

The effects of IGF1 are mediated through its receptor, IGF1R, which is often overexpressed in breast cancers (8). IGF1R expression is required for mitogenesis and transformation *in vitro* (9, 10). More recently it has become evident that IGF1 and IGF1R may play a role in the early transformation of mammary cells (11–14). In one study, overexpression of IGF1R was sufficient to induce mammary epithelial hyperplasia in a transgenic mouse model (11). In addition, *in vitro* studies examining interference of IGF1R signaling have demonstrated inhibition of breast cancer cell growth (15–17).

In the current study, we hypothesized that elevated expression of IGF1R in normal breast tissue would be associated with an increase in the risk of subsequent breast cancer. Using a nested case-control design, we examined the association between IGF1R expression in normal breast tissue from benign breast biopsies and subsequent risk of breast cancer in women enrolled in the Nurses' Health Study.

Materials and Methods

Study population

Study Design and Population—The Nurses' Health Study (NHS) was initiated in 1976, when 121,700 U.S. registered nurses ages 30–55 returned an initial questionnaire. The Nurses' Health Study II (NHS II) is a separate cohort study consisting of 116,671 female registered nurses who were between ages 25 and 42 when the study began in 1989. These cohorts have been followed by mailed questionnaires biennially to update exposure information and ascertain non-fatal incident diseases. Information collected includes diagnosis of cancer, as well as benign breast disease (BBD), which is updated every two years through questionnaires. The methods developed to follow participants and confirm incident cancers and death in the Nurses' Health Study have been described previously in detail elsewhere (18) and have been applied to NHS II. In general, the questionnaire response rates among women who reported a previous diagnosis of BBD and among those who did not have been very similar (19).

Breast cancer nested case-control study

We conducted a case–control study nested within the subcohort of participants in the NHS and NHS II with a biopsy-confirmed BBD. Beginning with the initial NHS questionnaire in 1976, participants have been asked on every biennial questionnaire to report any diagnosis of fibrocystic disease or other BBD. Early questionnaires (1976, 1978, and 1980) asked whether the respondent had ever been diagnosed as having ‘fibrocystic disease’ or ‘other BBD’ and whether she had been hospitalized in relation to this diagnosis. Beginning in 1982, the NHS questionnaires sought specific details of a history of biopsy-confirmed BBD. The initial 1989 NHS II questionnaire and all subsequent biennial questionnaires also asked participants to report any diagnosis of BBD and to indicate whether it was confirmed by biopsy or aspiration.

Within the subcohort of women with a biopsy-confirmed BBD, eligible cases were women who reported a first diagnosis of breast cancer between 1976 and return of the 1996 questionnaire (NHS) or between 1989 and the return of the 1995 questionnaire (NHS II). Incident breast cancer cases in both cohorts were identified through the nurses’ own reports and were confirmed by review of medical records. Eligible controls were women who did not have a diagnosis of breast cancer at the time the matching case was diagnosed and also had a previous biopsy-confirmed BBD. Controls were matched to cases on year of birth and year of biopsy. Attempts were made to identify four matched controls for each case, although this was not always possible.

Benign breast biopsy confirmation

Hematoxylin and eosin (H&E) stained sections from the benign breast biopsies were independently reviewed by one of two pathologists (SJS, JLC) in a blinded fashion. Any slide identified as having either questionable atypia or atypia was jointly reviewed by the two pathologists. For each set of slides reviewed, a detailed work sheet was completed and the benign breast biopsy was classified according to the categories of Page *et al.* (20) as non-proliferative, proliferative without atypia or atypical hyperplasia (atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH)).

Benign breast biopsy block collection and tissue microarray (TMA) construction

After centralized review of H&E stained sections, we collected archived formalin-fixed paraffin-embedded benign breast biopsy blocks for participants. There were 463 cases and 1853 controls whose original slides had been reviewed and were eligible for the block collection. Of those, we successfully obtained benign breast disease blocks from 177 cases and 719 controls. There were 388 participants who were eligible for the TMA construction by having one or more of the following types of benign lesions: apocrine metaplasia, non-apocrine cysts, usual ductal hyperplasia, ADH or ALH. Upon further review, 15 women were excluded because there was either no breast tissue, or not enough tissue remaining on the block for coring. H&E sections of the corresponding paraffin-embedded tissue blocks were re-reviewed by a single pathologist (JLC) to identify areas of benign proliferative lesions and normal terminal duct lobular units (TDLUs), and to circle the areas from which the cores for the TMAs would be taken. We constructed 5 TMA blocks that contain normal TDLUs and benign lesions, representing 373 participants with one or more of the following types of benign lesions: apocrine metaplasia, non-apocrine cysts, usual ductal hyperplasia, ADH or ALH.

TMAs were constructed in the Dana Farber Harvard Cancer Center Tissue Microarray Core Facility, Boston, MA, by obtaining 0.6-mm cores from the targeted area in each donor block and inserting them into the recipient TMA blocks. For 96% of the targeted areas 3 cores

were obtained. Six cores were obtained in 23 cases and nine were obtained in one case. Normal TDLUs were targeted for 361 participants (21).

Tissue microarray analysis

For this study, one 5- μ m paraffin section was cut from each of the five TMA blocks and stained with antibodies for IGF1R(22–24). Immunostaining was performed in a single staining run on a Dako Autostainer (Dako Corporation, Carpinteria, CA). An anti-IGF1R antibody (Ab-1;clone 24–31; Lab Vision, Westinghouse Drive, Fremont, CA, USA) was used at a 1:100 dilution.

Immunostaining was performed on tissue sections following deparaffinization in two 5-minute changes of xylene and rehydration through graded alcohols to distilled water. After blocking endogenous peroxidase activity, sections were subjected to heat-induced epitope retrieval by heating in a vegetable steamer in EDTA (pH 8.0) for 20 minutes. Following heat-induced epitope retrieval, the primary monoclonal antibodies were applied to the sections for 30 minutes at room temperature. The slides were then incubated with HRP Labeled Polymer (Dako). The reactions were completed with the Envision detection system (Dako) using 3–3' diaminobenzidine as the chromogen. Appropriate positive and negative controls were included in all staining runs.

IGF1R staining in each core was scored on a 0–5 scale according to the proportion of normal TDLU epithelial cells with positive staining (0=0%, 1= $\leq$ 1%, 2=1–10%, 3=10–33%, 4=33–67%, 5= $\geq$ 67%+)(25). Because previous work by Happerfield et al demonstrated that there was a mixture of cytoplasmic and membranous IGF1R staining in normal and benign breast tissue(26), we scored both the cytoplasm and membranous staining separately. We considered a score of 2+ or higher (>1 % of cells staining) as positive. Nuclei of breast tissue cells exhibiting staining for ER or PR either at a low level (1 to 10% of cell nuclei staining) or high level ($>10\%$ cell nuclei staining) was considered to be positive for ER and PR respectively. ER and PR negative tissues were defined as those that exhibited complete absence of tissue cell staining. We did not score the intensity of staining in this study, because there is accumulating data indicating that staining intensity can be affected by both storage time and variability in processing(27). Because our samples from across the US over a large period of time, we did not feel that staining intensity would be a reliable measure. The current analysis is restricted to 312 participants with normal TDLUs and IGF1R cytoplasmic or membranous staining. We calculated an average score for each marker across available cores for each woman. We also examined patterns of IGF1R expression based on membranous and cytoplasmic staining (Figures 1–3). P-values for cross-tabulated frequencies were determined using Chi-square tests and Mantel-Haenszel Chi-square test for test for trend. Odds ratios (OR) and 95% confidence intervals (CI) for breast cancer were determined using multiple logistic regression. All analyses were adjusted for age (5 year age categories). Multivariate models were also adjusted for category of targeted benign breast lesion (nonproliferative, proliferative without atypia, proliferative with atypia). Data analysis was conducted with SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). All p-values presented are from two-sided tests of statistical significance. This study was approved by the Committee on the Use of Human Subjects in Research at Brigham and Women's Hospital. Completion of the self-administered questionnaire was considered to imply informed consent.

Results

In this study, there were a total of 312 women who had both normal TDLUs included in the TMAs and evaluable IGF1R staining; 75 subsequently developed breast cancer (cases) and 237 did not (controls). The average age at biopsy was 46.2 years (SD=8.8). As expected,

breast cancer cases were more likely to have had atypical hyperplasia relative to the controls (Table 1). Breast cancer cases and controls were equally likely to have normal TDLUs that were ER+ (92.3 versus 94.2%) and PR+ (91.2 versus 95.0%) on their benign biopsy.

We examined the association between both membrane and cytoplasmic expression of IGF1R in normal TDLU epithelial cells in relation to other biopsy characteristics among the controls (Table 2). Neither membranous nor cytoplasmic expression of IGF1R was associated with age at biopsy or type of benign breast disease (Table 2). However, normal breast epithelium with membranous IGF1R expression was more likely to be positive for ER ($P=0.02$) and PR ($P=0.007$). In contrast, there was no association between cytoplasmic IGF1R staining and ER status ($P=0.33$), although there was a positive association with PR status ($P=0.04$).

IGF1R membrane staining in normal TDLU epithelial cells was inversely associated with subsequent breast cancer risk independent of type of BBD, but this difference was not statistically significant (OR= 0.64; 95% CI 0.35–1.16; Table 3). Additional adjustment for cytoplasmic staining did not attenuate the association (OR=0.49; 95% CI 0.26–0.93). In contrast, cytoplasmic IGF1R expression was positively associated with subsequent risk of breast cancer (OR=2.47, 95% CI 1.41–4.33) (Table 3). Additional adjustment for membrane staining made the association slightly stronger (OR=2.88, 95% CI 1.60–5.19).

We also examined the association between patterns of cytoplasmic and membrane staining of IGF1R and breast cancer risk (Table 4). We found that women whose TDLUs were IGF1R-cytoplasm positive/IGF1R-membrane negative were more than 15-times more likely to develop subsequent breast cancer than women whose TDLUs were negative for both membranous and cytoplasmic IGF1R expression (OR=15.9, 95% CI 3.6–69.8). Additional adjustment for known breast cancer risk factors including age at menarche, age at first birth/parity, age at menopause, family history of breast cancer, postmenopausal hormone use, and weight gain since age 18 in a multivariate analysis did not alter the association (OR=14.0, 95% CI 2.6–75.4). We also examined the association between this high risk pattern (IGF1R-cytoplasm positive/IGF1R-membrane negative) and breast cancer risk stratified by category of BBD. Although the numbers were limited, the high risk pattern had a similar magnitude of association with breast cancer risk among women with nonproliferative (OR=13.6, 95% CI 0.8–240.9) and proliferative benign breast disease (OR=13.1, 95% CI 2.3–75.3) relative to women whose TDLUs were negative for both membranous and cytoplasmic IGF1R expression.

In an effort to better understand the mechanism by which the high risk IGF1R pattern influenced breast cancer, we also examined tumor characteristics of the subsequent tumor. Of the women with the high risk IGF1R expression pattern with available data 40% (2 out of 5) went on to develop ER- cancer, 100% had high grade tumors (3 out of 3), and 100% (6 out of 6) were invasive ductal tumors. However, because we do not have complete data for the breast cancer cases and are underpowered to draw conclusions, these descriptive data need to be interpreted with caution.

Discussion

We found that cytoplasmic expression of IGF1R in the epithelial cells of normal breast TDLUs in benign breast biopsies was positively associated with subsequent risk of breast cancer. Women whose TDLUs were IGF1R – cytoplasm positive/IGF1R-membrane negative were at a more than a 15- fold increased risk of developing breast cancer subsequent to their biopsy (median time=8 years later).

IGF1R is a tyrosine kinase growth factor receptor that when activated plays an important role in signal transduction pathways regulating cell proliferation, survival migration, and differentiation. Upon ligand binding, IGF1R stimulates downstream pathways responsible for these processes via phosphorylation of substrates including receptor substrate 1 (IRS1), Shc, and phosphatidylinositol-3 kinase (PI3K).

The differential staining patterns for IGF1R observed in our studies could result from various possible mechanisms; one intriguing possibility is the differential activation status of IGF1R. In several reports (28, 29) and in our own preliminary studies (unpublished data), cells cultured in the absence of serum or IGF ligands display a predominantly membranous staining pattern for IGF1R; this localized membrane staining pattern is less prominent following ligand stimulation.

Ligand stimulation induces internalization of IGF1R and other receptor tyrosine kinases like Met and EGFR (30, 31). In the case of IGF1R, the ligand stimulated receptor undergoes endocytosis via both caveolin and clathrin-mediated pathways (32, 33). Interestingly, recent studies have also reported nuclear localization of intact IGF1R that was dependent on both ligand stimulation and intact IGF1R kinase activity (28, 29). Furthermore, nuclear localization of IGF1R was also blocked by inhibition of endocytosis, suggesting that IGF1 receptors in the nucleus translocated from the cell membrane following ligand stimulation. Thus, the redistribution of IGF1R from the membrane to various intracellular locations could reflect changes in the activation status of IGF1R.

Although initially considered to be a mechanism by which receptor-mediated signaling was down-regulated, internalization of receptors into various intracellular compartments may actually be required for activation of specific signaling pathways (32, 34). For example, IGF1R internalization following IGF1 stimulation of oligodendrocyte precursor cells was required for phosphorylation and sustained activation of Akt (32). Studies of EGFR also suggest that clathrin-mediated receptor internalization is required for sustained activation of specific signaling molecules required for EGF-dependent biological processes including DNA replication and proliferation (35). The signaling capabilities of internalized receptor tyrosine kinases may also be responsible for the adverse outcomes reported for cytoplasmic Met localization in colon cancer (36).

In the current study, women whose benign breast biopsies showed little or no IGF1R expression on the membranes of normal TDLU epithelial cells but high levels internally within the cytoplasm were at the highest risk of subsequent breast cancer. These results raise the possibility that in a population of cells in this subset of women, the decrease in membrane staining reflects the early development of breast cancer which may provide new opportunities for breast cancer chemoprevention. Currently, there are anti-cancer agents in development targeting the IGF pathway either through receptor blockade or kinase inhibition (37).

The major limitation of the current study is the small sample size. Given the small sample size, these results should be interpreted cautiously, as chance may be a possible explanation for the observed results. However, the prospective nature of the study with benign tissue obtained several years prior to breast cancer diagnosis makes this study uniquely able to examine the association between IGF1R expression in normal breast epithelium and subsequent risk of breast cancer. In addition, all benign biopsies underwent centralized pathology review and IGF1R scoring with the same expert breast pathologists. The pathologists were blinded to the subsequent case-control status of the participants throughout the study; therefore it is unlikely that there is any differential misclassification of IGF1R scoring. We were unable to obtain tissue samples from all eligible participants. The

primary reason for this is due to the long time between BBD diagnosis and collection in our study. Many hospitals are not required to maintain formalin fixed paraffin embedded tissue samples beyond 5 to 10 years. Thus many of these samples had been destroyed by the time we attempted to collect them. There was no significant difference in our ability to obtain BBD tissue samples from cases versus controls. Interestingly, the 15-fold increase in breast cancer risk observed with the IGF1R – cytoplasm positive/IGF1R-membrane negative staining pattern was independent of the category of the benign breast disease category. Women with nonproliferative benign lesions, thought to be at low or no increased risk of breast cancer, were also at high risk of breast cancer if they had this high risk IGF1R staining pattern. It is important that these results be confirmed by other studies. Identification of tissue markers in normal breast tissue that are predictive of subsequent risk has important implication for risk stratification as well as preventive strategies.

Conclusion

In this study, IGF1R expression patterns in epithelial cells of normal TDLUs in benign breast biopsies were associated with an increased risk of subsequent breast cancer.

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Abbreviations

ADH	atypical ductal hyperplasia
ALH	atypical lobular hyperplasia
BBD	benign breast disease
BMI	body mass index
CI	confidence interval
ER	estrogen receptor
H&E	hematoxylin and eosin
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor receptor
IRS1	insulin receptor substrate 1
NHS	Nurses' Health Study
OR	Odds Ratio
PI3K	phosphatidyl inositol-3 kinase
PR	progesterone receptor
TDLUs	normal terminal ductal lobular units
TMA	tissue microarrays

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Figure 1. IGF1R immunostain in normal TDLUs at low (20x) and high (60x) powers: Negative staining.

Figure 2.
IGF1R immunostain in normal TDLUs at low (20x) and high (60x) powers: Membranous staining.

Figure 3.
IGF1R immunostain in normal TDLUs at low (20x) and high (60x) powers: Cytoplasmic staining.

Table 1

Descriptive characteristics of breast cancer cases and controls

	Cases	Controls
	N=75	N=237
Age at biopsy, years	N (%)	N (%)
<45	28 (37.3)	106 (44.7)
45–<50	24 (32.0)	46 (19.4)
50–<55	7 (9.3)	46 (19.4)
55+	16 (21.3)	39 (16.5)
Benign breast disease category		
Nonproliferative	16 (21.3)	56 (23.6)
Proliferative w/o atypia	30 (40.0)	124 (52.3)
Atypical Hyperplasia	29 (38.7)	57 (24.1)
Estrogen receptor expression in normal TDLU epithelial cells^I		
Positive	60 (92.3)	195 (94.2)
Negative	5 (7.7)	12 (5.8)
Progesterone receptor expression in normal TDLU epithelial cells^I		
Positive	62 (91.2)	189 (95.0)
Negative	6 (8.8)	10 (5.0)

^I Numbers do not add to total because of missing marker information.

Table 2

IGF1R membrane and cytoplasmic staining of normal TDLU epithelial cells according to biopsy characteristics among controls (n=237).

	IGF1 R Status			
	Membrane +	Membrane –	Cytoplasm +	Cytoplasm –
Age at biopsy, years	N (%)	N (%)	N (%)	N (%)
<45	83 (46.1)	23 (40.4)	29 (43.3)	77 (45.3)
45–<50	32 (17.8)	14 (24.6)	18 (26.9)	28 (16.5)
50–<55	34 (18.9)	12 (21.1)	13 (19.4)	33 (19.4)
55+	31 (17.2)	8 (14.0)	7 (10.5)	32 (18.8)
Benign breast disease category				
Nonproliferative	46 (25.6)	10 (17.5)	20 (29.9)	36 (21.2)
Proliferative w/o atypia	88 (48.9)	36 (63.2)	29 (43.3)	95 (55.9)
Atypical Hyperplasia	46 (25.6)	11 (19.3)	18 (26.9)	39 (22.9)
Estrogen receptor expression in normal TDLU epithelial cells^I				
Positive	153 (96.2)	42 (87.5)	58 (97.7)	137 (93.2)
Negative	6 (3.8)	6 (12.5)	2 (3.3)	10 (6.8)
Progesterone receptor expression in normal TDLU epithelial cells^I				
Positive	147 (97.4)	42 (87.5)	57 (100.0)	132 (93.0)
Negative	4 (2.7)	6 (12.5)	0	10 (7.0)

^I Numbers do not add to total because of missing marker information.

Odds ratio (95% Confidence interval) of developing subsequent breast cancer according to average proportion score of IGF1R membranous and cytoplasmic staining of normal TDLU epithelial cells.

Table 3

IGF1R status	Cases	Controls	OR (95%CI) ¹	OR (95%CI) ²	OR (95%CI) ³
Membrane -	24(32.0)	57 (24.1)	1.0 (REF)	1.0 (REF)	1.0 (REF)
Membrane +	51 (68.0)	180 (76.0)	0.71 (0.39–1.26)	0.64 (0.35–1.16)	0.49 (0.26–0.93)
Cytoplasm -	39 (52.0)	170 (71.7)	1.0 (REF)	1.0 (REF)	1.0 (REF)
Cytoplasm +	36 (48.0)	67 (28.3)	2.60 (1.50–4.53)	2.47(1.41–4.33)	2.88 (1.60–5.19)

¹ Adjusted for matching factors (age, year of biopsy, time since biopsy)

² Adjusted for matching factors and category of benign lesion

³ Adjusted for matching factors, category of benign lesion and IGF1R staining in other cellular compartment (e.g. cytoplasmic, or membrane)

Odds ratio (95% Confidence interval) of developing subsequent breast cancer according to IGF1R cytoplasmic and membrane staining of normal TDLU epithelial cells

Table 4

Membrane IGF1R	Cytoplasmic IGF1R	Cases	Controls	OR(95%CI) ¹	OR(95%CI) ²
-	-	14	54	1.0 (REF)	1.0 (REF)
+	+	26	64	1.8 (0.8-3.9)	1.6 (0.7-3.5)
-	+	10	3	13.8 (3.3-58.3)	15.9 (3.6-69.8)
+	-	25	116	0.9 (0.4-1.8)	0.8 (0.4-1.8)

¹ Adjusted for matching factors (age, year of biopsy, time since biopsy)

² Adjusted for matching factors and category of benign lesion