Transforming Growth Factor-β and Breast Cancer Risk in Women With Mammary Epithelial Hyperplasia


Background: Transforming growth factors-β (TGF-βs) regulate mammary epithelial cell division. Loss of expression of TGF-β receptor II (TGF-β-RII) is related to cell proliferation and tumor progression. Breast epithelial hyperplastic lesions lacking atypia (EHLA) are associated with a mild elevation in breast cancer risk. We investigated the expression of TGF-β-RII in EHLA and the risk of subsequent invasive breast cancer. Methods: We conducted a nested case–control study of women with biopsy-confirmed EHLA who did not have a history of breast cancer or atypical hyperplasia of the breast. Case patients (n = 54) who subsequently developed invasive breast cancer were matched with control patients (n = 115) who did not. Formalin-fixed, paraffin-embedded sections of breast biopsy specimens of all 169 patients (n = 115) who did not. Formalin-cancer were matched with control patients or atypical hyperplasia of the women with biopsy-confirmed EHLA conducted a nested case–control study of EHLA and the risk of subsequent invasive breast cancer. [J Natl Cancer Inst 1999;91:2096–101]

Conclusion: This study indicates that loss of TGF-β-RII expression in epithelial cells of EHLA is associated with increased risk of invasive breast cancer. 

Histologic and epidemiologic studies of proliferative breast diseases have identified lesions that are associated with a subsequent risk of breast carcinoma. Proliferative disease is subclassified as either atypical hyperplasia or proliferative disease without atypia. Atypical hyperplasia refers to rare lesions that meet some but not all of the criteria for carcinoma in situ. Women with these lesions have a fourfold elevation in relative risk of breast cancer (1–3). Women having proliferative disease without atypia have a twofold elevation in their risk for subsequent invasive breast cancer compared with women without proliferative disease (1–3). Despite the epidemiologic significance of proliferative disease without atypia, little is known about the underlying molecular alterations that are associated with these histopathologic changes. Knowledge of molecular markers of breast neoplastic progression is needed to identify women at high risk. Transforming growth factors-β (TGF-βs) are potent growth suppressors of mammary epithelial and breast carcinoma cells and act as negative autocrine growth inhibitors (4–6). In this study, we investigated the relationship between the content of the TGF-β type II receptor (TGF-β-RII) in breast epithelial hyperplastic lesions and the subsequent risk of invasive breast carcinoma.

Proliferative disease without atypia consists of a group of histologically defined lesions that are associated with a mild elevation in breast cancer risk (1,7). The most common of these lesions is moderate or florid hyperplasia of the usual type (1,7). This study focuses on the differences in TGF-β-RII expression among these latter lesions, which are referred to as epithelial hyperplasia lacking atypia (EHLA).

TGF-βs mediate their activity by high-affinity binding to a hetero-oligomeric complex composed of type I and type II receptors (8). Loss of response to growth inhibitory action of TGF-β is a common phenomenon in tumor progression. Previous studies (5,9–13) have shown that, in tumor systems, TGF-β responsiveness is positively associated with TGF-β-RII levels. We studied levels of TGF-β-RII in EHLA and risk of subsequent invasive breast carcinoma.

Subjects and Methods

Nashville Breast Study Cohort

We conducted a nested case–control study of women from the Nashville Breast Study Cohort. This cohort consists of women who underwent biopsy revealing benign breast tissue at three Nashville hospitals: Vanderbilt University Medical Center between 1952 and 1985, St. Thomas Hospital between 1952 and 1981, or Baptist Hospital between 1952 and 1968. Details on this cohort have been published elsewhere (1,14,15). In brief, a patient’s entry biopsy was her first biopsy that revealed benign breast parenchyma at a study hospital during the recruitment period. Patients were eligible for follow-up if, at the time of their entry biopsy, they were residents of Tennessee or Kentucky and did not have breast cancer diagnosed before or within 6 months of their entry biopsy. Patients were excluded if their entry biopsy slides or charts were lost. Follow-up was begun 6 months after the entry biopsy was performed. In this report, “follow-up” always refers to follow-up in the Nashville Breast Study Cohort.

This study was approved by the Institutional Review Board of Vanderbilt University Medical School. We obtained informed consent for participation and follow-up in the study from 9701 study subjects or their next of kin. This number represents 87% of those women eligible for follow-up. Follow-up was terminated when the first of the following events occurred: The subject had her exit interview.
Histologic Definitions and Analyses
The definitions that we use to classify benign breast disease have been described elsewhere (1,7). Proliferative disease connotes either atypical hyperplasia or proliferative disease without atypia (7). Proliferative disease without atypia refers to a group of lesions that are associated with a mild elevation in breast cancer risk (1–1.7). One of the most prevalent of these lesions is EHLA, which has also been described as moderate or florid hyperplasia of usual type (1,7). Complex fibroadenomas contain either cysts, sclerosed lesions, epithelial calcifications, or papillary apocrine change (15). We made these diagnoses on the entry biopsies of the entire cohort. The hematoxylin–eosin-stained slides of the diagnostic biopsies of case patients and control patients were assessed for the different components of proliferative disease without atypia, including EHLA without knowledge of the subsequent breast cancer outcome.

Immunohistochemical Evaluation
We performed immunostaining on a Ventana ES-automated immunostainer (Ventana, Tucson, AZ) by use of an affinity-purified rabbit polyclonal antibody (C-16; Santa Cruz Biotechnology, Santa Cruz, CA) raised against human TGF-β-RII. This antibody recognizes sequences corresponding to amino acids 550–565 located at the C-terminal region of the TGF-β-RII protein. To confirm antibody specificity, we incubated the TGF-β-RII antibody with a 10-fold excess of the TGF-β-RII synthetic peptide available from the manufacturer. Control sections were then stained, and no immunostaining was detected. Other investigators (18–20) have obtained similar results demonstrating the specificity of this antibody.

A pretest was done to establish the best staining conditions. Three antibody dilutions (1, 2, and 4 μg/mL) were tested by use of three conditions: no pretreatment, heat-induced epitope retrieval, and enzyme pretreatment. A concentration of 2 μg/mL by use of Protease 1 (Ventana) pretreatment gave the best results, and this method was applied to all specimens. We used normal breast tissue obtained from women with neither proliferative disease nor complex fibroadenoma. The definitions that we use to classify benign breast disease have been described elsewhere (7). The intensity of positive staining was classified as less than 25%, 25%–75%, and greater than 75%. We classified the pattern as homogeneous if more than 95% of positively stained cells had a similar staining intensity; otherwise, it was classified as heterogeneous. The intensity of positive staining was grouped into weak, moderate, and strong categories by making a comparison with positive epithelial cells of normal ducts and lobular units within the same biopsy specimen.

Statistical Analysis
We used the Kaplan–Meier method (21) to draw the cumulative morbidity curves in Fig. 1. Follow-up in this figure was censored after 30 years because of the small number of women with longer follow-up times. We estimated the risk of invasive breast cancer in patients with EHLA relative to women with neither proliferative disease nor complex fibroadenoma. This risk is given both for women in their first 12 years after EHLA diagnosis and for women in subsequent years. We estimated these risks by time-dependent hazard regression analysis (21). The covariates in this analysis were indicator step functions that denoted a diagnosis of EHLA and a time since diagnosis that was either less than or equal to 12 years or greater than 12 years, respectively. These risks were adjusted for age at diagnosis by including this age as a covariate in the model. We chose the 12-year cut point by maximizing the model deviance compared with models that used other cut points (22). The adequacy of our final model was assessed by comparison with more complex time-dependent covariate models. There is compelling evidence that the relative hazard of breast cancer morbidity associated with proliferative breast disease decreases with increasing time since the patient’s diagnostic biopsy (14). It is for this reason that we used a time-dependent covariate model when estimating relative hazard for such lesions. The advantage of using indicator step functions as covariates is that it permits the derivation of relative risks associated with different time intervals following biopsy. Although the true relative hazard is undoubtedly a continuous function of time, our model fitting indicated that a step function with a single step at 12 years provided a good fit to the data and that there was little to be gained by adding additional steps to the model.

We used both conditional and unconditional logistic regression analysis to assess the influence of TGF-β-RII on breast cancer risk in women in our nested case–control study (23). Our analyses are adjusted for both age at biopsy and year of biopsy by the matching criteria used to select control patients and by including these variables in the regression models. We included interaction terms in all models that investigated the combined effects of multiple covariates. In the unconditional analyses, the four control subjects who later became case patients were excluded from the control group. In analyses of patients with EHLA, we excluded case patients and control subjects who lacked EHLA in their diagnostic biopsy specimens. The results of our conditional (matched) and unconditional (independent) analyses were very similar. However, 20
matched triplets were excluded from the conditional analysis because either the case patient or both matched control patients lacked EHLA. This reduced the power of the matched analysis. For this reason, we give the results of the unconditional analyses in this report. We also analyzed the data adjusting for menopausal status, age of menarche, a first-degree family history of breast cancer, parity, and age at first birth. The parameters associated with these potential confounders were not statistically significantly different from zero, and these adjustments had little effect on our odds ratio estimates.

For these reasons, and to conserve statistical power, the odds ratios given in this report are adjusted only for age at biopsy and year of biopsy. The Clayton–Hills score test for trend (24) was used to assess whether breast cancer risk increased with decreasing TGF-β-RII expression or intensity. We used Wald statistics to derive all confidence intervals (CIs) given in this report (25). Analyses were performed by use of the SAS (26) and Stata (27) statistical software packages.

Breast cancer standard morbidity ratios (28) for women with different histologic diagnoses were adjusted for age at biopsy and year of biopsy in comparison to women from the Connecticut Tumor Registry (29). (These ratios equal the observed number of breast cancers divided by the expected number of breast cancers given the follow-up experience of the cohort.) All P values in this report are derived with respect to two-sided alternative hypotheses.

**RESULTS**

We obtained follow-up on 9701 women in the study cohort (87% of those eligible for follow-up). The average follow-up was 19 years. In their entry biopsies, 2185 had EHLA, 6036 had low-risk histology, and 1480 had other lesions. We will refer to patients with neither proliferative disease (1) nor complex fibroadenoma (15) as having low-risk histology. This latter group has an absolute breast cancer risk that is similar to that of women from the general population. (Their standard morbidity ratio compared with women from the Connecticut Tumor Registry is 1.04). Fig. 1 shows the cumulative morbidity from invasive breast cancer for cohort members with EHLA and with low-risk histology. Women with EHLA had 1.83 times the breast cancer risk of those with low-risk histology during their first 12 years of follow-up (95% CI = 1.3–2.5). Subsequently, this relative risk dropped to 1.07 in EHLA women who remained free of breast cancer for at least 12 years (95% CI = 0.73–1.6). This change in risk is reflected in Fig. 1. Note that the two cumulative morbidity curves diverge until 12 years after biopsy. Thereafter, they are almost parallel, suggesting little difference in risk for women who remain free of breast cancer for at least 12 years after their entry biopsy.

In the nested case–control study, the expression of TGF-β-RII was detectable in the epithelial cells of normal breast ducts and lobular units, exhibiting a cytoplasmic distribution (Fig. 2, A). The myoepithelial cells of terminal and large ducts stained homogeneously for TGF-β-RII; however, in the lobular units, these cells were rarely positive (Fig. 2, A). The cytoplasm of stromal cells and of endothelial cells, known TGF-β targets, also stained for TGF-β-RII (Fig. 2, A). There was a variation in the staining pattern and staining intensity for TGF-β-RII expression in epithelial cells of hyperplastic lesions among the specimens (Fig. 2, B–D).

Some of the cells were often negative alongside cells that were lightly or strongly positive. Fig. 2 indicates the range of positivity for individual cells. More than 75% of the epithelial cells of normal lobular units were positive for TGF-β-RII in 136 of 138 control patients and in 69 of 71 case patients. The intensity of staining of normal lobular units was similar between case patients and control patients.

**TGF-β-RII Expression Within EHLA Lesions and Breast Cancer Risk**

The relationship between TGF-β-RII expression within EHLA lesions and breast cancer risk is summarized in Table 1. This table shows the effects of extent of expression, pattern of expression, and intensity of expression on breast cancer risk. Breast cancer risk increased as the proportion of cells expressing TGF-β receptors decreased (test for trend P = .008). Women with less than 25% TGF-β-RII-positive cells in their EHLA lesions had 3.41 times the risk of subsequent invasive breast cancer than did women with histologically identical lesions but with more than 75% positive cells (P = .02).

**Interaction of TGF-β Receptors in EHLA Lesions and Normal Lobular Units**

The combined effects on breast cancer risk of TGF-β-RII expression in normal lobular units and epithelial hyperplasia lacking atypia are shown in Table 2. The results suggest an interaction between loss of TGF-β-RII expression in EHLA lesions and a heterogeneous pattern of TGF-β-RII expression in adjacent lobular units with normal morphology. Among women with normal breast lobular units having a heterogeneous staining pattern and EHLA, the odds ratios for breast cancer were 0.742 (95% CI = 0.3–1.8), 2.85 (95% CI = 1.1–7.1), or 3.55 (95% CI = 1.0–10.0) in women whose EHLA had greater than 75%, 25%–75% and less than 25% TGF-β-RII-positive epithelial cells, respectively (test for trend P = .003). Among all study subjects, regardless of whether they had EHLA or how TGF-β-RII was expressed in their EHLA, women who showed a heterogeneous pattern of TGF-β-RII expression in their normal lobular units had 1.66 times the breast cancer risk of patients whose normal lobular units had a homogeneous pattern of expression (95% CI = 0.91–3.0).

**DISCUSSION**

Estrogen receptors (30) and overexpression of p53 (31,32) and neu (32,33) protein are important prognostic factors in patients with well-established breast cancer. In contrast, the search for molecular
risk factors for breast cancer in patients with benign breast lesions has been less fruitful. Rohan et al. (34) reported that p53 expression in benign tissue from breast biopsy specimens was associated with a 2.5-fold elevation in breast cancer risk. The median interval between benign biopsy and subsequent cancer in their study was only 2.1 years (35), and their statistical power was modest. Millikan et al. (36) also observed evidence that p53 alterations in benign breast tissue are related to later breast cancer development. We have observed mutations of this gene and overexpression of the protein in some women with comedo (high-grade)-type ductal carcinoma in situ (DCIS) (37). Other molecular indicators of cancer progression, particularly cyclin D (38) and retinoid X receptor (39), were also found almost exclusively in DCIS. Rohan et al. (34) and Millikan et al. (36) did not show a significant effect of overexpression of neu on breast cancer risk.

To date, the most well-established breast cancer risk factors associated with benign breast lesions are histologic. Moderate or mild elevations of breast cancer risk are associated with proliferative disease with or without atypia, respectively (1–3,17,40). In this study, the TGF-β-RII findings are restricted to women who have proliferative disease lacking atypia. Thus, by examining TGF-β-RII expression, we are able to identify threefold difference in breast cancer risk among comparable women with histologically identical lesions.

Our knowledge of TGF-β biology enhances the plausibility of the epidemiologic findings of this report. TGF-β is a potent cell regulatory polypeptide that affects cell growth, differentiation, matrix production, and immune function (41) and acts as a negative autocrine growth inhibitor (4,41). Malignant progression is

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### Table 1. Relationship between expression of type II receptor for transforming growth factor-β (TGF-β-RII) and breast cancer risk in women with epithelial hyperplasia lacking atypia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of case patients (n = 54)</th>
<th>No. of control patients (n = 115)</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of TGF-β-RII-positive cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;75%</td>
<td>26</td>
<td>78</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>25%–75%</td>
<td>19</td>
<td>29</td>
<td>1.98</td>
<td>0.95–4.1</td>
<td>.008</td>
</tr>
<tr>
<td>&lt;25%</td>
<td>9</td>
<td>8</td>
<td>3.41</td>
<td>1.2–10.0</td>
<td></td>
</tr>
<tr>
<td>Pattern of TGF-β-RII expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogeneous</td>
<td>17</td>
<td>49</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>37</td>
<td>66</td>
<td>1.61</td>
<td>0.80–3.2</td>
<td>.18</td>
</tr>
<tr>
<td>Intensity of TGF-β-RII expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>12</td>
<td>41</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>31</td>
<td>58</td>
<td>1.83</td>
<td>0.80–4.0</td>
<td>.07</td>
</tr>
<tr>
<td>Weak</td>
<td>11</td>
<td>16</td>
<td>2.35</td>
<td>0.85–6.5</td>
<td></td>
</tr>
</tbody>
</table>

*P values next to braces test for an increasing trend in odds ratios. Other P values test the null hypothesis that the associated odds ratio equals 1. All P values are two-sided.

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Fig. 2. Immunohistochemical staining of type II receptor for transforming growth factor-β (TGF-β-RII). In panel A, the cytoplasm of epithelial cells of normal lobular units is positive for TGF-β-RII. The myoepithelial cells (arrows) are negative. In panel B, more than 75% of cells of epithelial hyperplasia lacking atypia (EHLA) stained for TGF-β-RII. In panel C, hyperplastic cells of EHLA show 25%–75% positive cells. The staining pattern is heterogeneous. Note the homogeneous expression in the adjacent normal lobular unit (arrows). In panel D, less than 25% of epithelial cells of this EHLA are positive for TGF-β-RII (avidin–biotin–peroxidase; original magnification: A = ×200; B = ×100; C = ×200; and D = ×200).
growth inhibition by TGF-β may be critical events in breast tumorigenesis (4,6,11,50).

In this study, we found variable expression of TGF-β-RII in EHLA by use of a semiquantitative grading scheme. Although interpretation of these data is subject to the quantitative limitations of immunohistochemical methods, our double-blind evaluation protects our findings from diagnostic bias. Our results demonstrate that TGF-β-RII is expressed by normal epithelium of ducts and lobular units and in mammary EHLA. Women with EHLA with reduced TGF-β-RII expression have an elevated risk of subsequent breast cancer risk factors for sporadic breast cancer are associated with modest levels of elevated cancer risk. In this report, we present evidence that differences in expression of the TGF-β-RII in ordinary hyperplastic breast lesions affect subsequent breast cancer risk. These results in combination with known histologic and epidemiologic breast cancer risk factors should help to refine the clinical management of proliferative breast disease.

REFERENCES


Table 2. Effects on breast cancer risk of expression of type II receptor for transforming growth factor-β (TGF-β-RII) in normal lobular units and in epithelial hyperplasia lacking atypia (EHLA)

<table>
<thead>
<tr>
<th>Pattern of TGF-β-RII expression in normal lobular units</th>
<th>Proportion of TGF-β-RII-positive cells in EHLA</th>
<th>No. of case patients (n = 71)</th>
<th>No. of control patients (n = 138)</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p*</th>
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<tbody>
<tr>
<td>Homogeneous &gt;75%</td>
<td>15</td>
<td>39</td>
<td>1.0</td>
<td>Referent</td>
<td>0.08–2.0</td>
<td>.53</td>
</tr>
<tr>
<td>Homogeneous 25%–75%</td>
<td>2</td>
<td>13</td>
<td>0.394</td>
<td>Referent</td>
<td>0.11–16</td>
<td>.53</td>
</tr>
<tr>
<td>Homogeneous &lt;25%</td>
<td>2</td>
<td>2</td>
<td>1.33</td>
<td>Referent</td>
<td>0.11–16</td>
<td>.53</td>
</tr>
<tr>
<td>Heterogeneous &gt;75%</td>
<td>11</td>
<td>39</td>
<td>0.742</td>
<td>Referent</td>
<td>0.30–1.8</td>
<td>.003</td>
</tr>
<tr>
<td>Heterogeneous 25%–75%</td>
<td>17</td>
<td>16</td>
<td>2.85</td>
<td>Referent</td>
<td>1.1–7.1</td>
<td>.003</td>
</tr>
<tr>
<td>Heterogeneous &lt;25%</td>
<td>8</td>
<td>6</td>
<td>3.55</td>
<td>Referent</td>
<td>1.0–10</td>
<td>.003</td>
</tr>
<tr>
<td>Homogeneous ‡</td>
<td>25</td>
<td>65</td>
<td>1.0</td>
<td>Referent</td>
<td>0.91–3.0</td>
<td>.10</td>
</tr>
<tr>
<td>Heterogeneous ‡</td>
<td>46</td>
<td>73</td>
<td>1.66</td>
<td>Referent</td>
<td>0.91–3.0</td>
<td>.10</td>
</tr>
</tbody>
</table>

*P values next to braces test for an increasing trend in odds ratios. Other P values test the null hypothesis that the associated odds ratio equals 1. All P values are two-sided.

†Includes seven case patients and 11 control patients with homogeneous normal lobular unit expression but no EHLA.
‡Includes 10 case patients and 12 control patients with heterogeneous normal lobular unit expression but no EHLA.

associated with the loss of sensitivity to growth inhibition by TGF-β (5,6,42,43).

Loss of TGF-β response has been shown to be temporally associated with tumor development and progression in cultured breast (13,44), colon (12,42), and other malignant (9,45,46) cell lines. Transgenic mice that overexpress TGF-β1 are resistant to induced mammary tumor formation (47). Loss of expression of TGF-β-RII has been demonstrated in human cancers (10,48,49), and loss of receptors or the ability to activate secreted latent TGF-β may be critical events in breast tumorigenesis (4,6,11,50).

In this study, we found variable expression of TGF-β-RII in EHLA by use of a semiquantitative grading scheme. Although interpretation of these data is subject to the quantitative limitations of immunohistochemical methods, our double-blind evaluation protects our findings from diagnostic bias. Our results demonstrate that TGF-β-RII is expressed by normal epithelium of ducts and lobular units and in mammary EHLA. Women with EHLA with reduced TGF-β-RII expression have an elevated risk of subsequent invasive breast cancer. Thus, reduction in TGF-β-RII expression appears to be an early event in the progression from hyperplasia to malignancy. This does not necessarily imply that EHLA with reduced TGF-β-RII expression is a deterministic precursor of breast cancer.

Breast cancer is the second largest cause of cancer mortality among North American women today; it has the highest incidence of potentially lethal cancers and is certainly the most feared. It is thus of great importance to be able to reassure women who are at low risk of breast cancer and to initiate preventive measures in women who are at high risk of this disease. Unfortunately, most of the known risk factors for sporadic breast cancer are associated with modest levels of elevated cancer risk. In this report, we present evidence that differences in expression of the TGF-β-RII in ordinary hyperplastic breast lesions affect subsequent breast cancer risk. These results in combination with known histologic and epidemiologic breast cancer risk factors should help to refine the clinical management of proliferative breast disease.
Gold LI, Sung JJ, Siebert JW, Longaker MT. Type I (RI) and type II (RII) receptors for transforming growth factor-beta isoforms are expressed subsequent to transforming growth factor-beta ligands during excisional wound repair. Am J Pathol 1997;150:209–22.


**NOTES**

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