A High-Risk Lesion for Invasive Breast Cancer, Ductal Carcinoma in Situ, Exhibits Frequent Overexpression of Retinoid X Receptor

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Abstract
The development of prevention strategies for breast cancer will require a molecular map of carcinogenesis. We have investigated gene expression patterns in premalignant and early carcinomatous human breast lesions that confer to the patient varying risks for developing invasive breast cancer. The relative expression levels of one of the retinoid receptors, retinoid X receptor (RXR), was determined by in situ hybridization to 58 biopsy specimens; RXR mRNA grain density over each lesion was compared to that over the normal ductal/lobular units in each section. Overexpression of RXR mRNA was observed in 66% of noncomedo ductal carcinoma in situ (DCIS), which confer a >8-fold increase in breast cancer risk, and 88% of comedo DCIS lesions, which are associated with a yet higher risk. In contrast, only 8% of lesions that confer little or no increase in breast cancer risk showed RXR overexpression. The implications for retinoid chemoprevention are discussed.

Introduction
Although a vast number of oncogenes and suppressor genes have been identified in invasive human breast cancer, relatively little is known of the molecular events associated with the premalignant and early carcinomatous stages of this disease. We and others have examined gene sequence and/or expression patterns in cohorts of human breast lesions, including p53, c-erb-B-2 (Her-2/neu), cyclin D, aneuploidy, loss of heterozygosity, and estrogen receptor (1–17). Such information can be compared to epidemiological data, which associate a histological type of breast lesion with a patient’s risks for the development of invasive breast cancer. These risks vary from low to no risk (typical hyperplasia, adenosis, fibrocystic changes, and apocrine metaplasia) and rise to approximately 4-fold in atypical ductal hyperplasia and to greater than 8-fold in DCIS (18–21). Within DCIS, a more aggressive subtype is also identified, comedo DCIS, defined by central necrosis, high nuclear atypia, and frequent mitotic figures. These studies can therefore identify molecular events associated with an increased breast cancer risk, which can be tested subsequently in transfection model systems, transgenic mice, or other systems for a causal involvement in early breast neoplasia.

Many of the molecular events studied to date have shown the greatest change in expression coincident with the highest risk correlate, comedo DCIS. These markers include p53 overexpression or mutation, c-erb-B-2 overexpression, and loss of heterozygosity at several loci (reviewed in Ref. 6). We recently reported that cyclin D1 mRNA levels exhibited a different pattern, as a high proportion of DCIS lesions, comedo or noncomedo, exhibited cyclin D as compared to normal ductal/lobular units within the specimen or premalignant lesions associated with a lower risk for breast cancer development. Cyclin D1 has been widely reported as a target of gene amplification and overexpression (22–32) in infiltrating ductal breast carcinoma, and transgenic mouse and transfection studies have indicated a causal role in oncogenesis (33, 34). Our cohort data indicate that alterations in cyclin D1 expression occur early in human breast neoplastic progression and suggest this marker as a target for prevention efforts.

Of the 16 markers that we have studied for expression trends in premalignant and early carcinomatous breast lesion cohorts, only two have shown a correlation to epidemiological cancer risk estimates. We report herein the expression patterns

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The abbreviations used are: LOH, loss of heterozygosity; DCIS, ductal carcinoma in situ; RXR, retinoid X receptor; RAR, retinoic acid receptor; ATRA, all-trans-retinoic acid; 9CRA, 9-cis-retinoic acid; RXRE, retinoid X response element; T3R, thyroid hormone receptor.
of RXR, a member of the RAR family, which regulates differentiation, proliferation, and apoptosis in many cell types (35-41), but for which limited data are available in breast cancer. The RARs and RXRs are ligand-inducible transcriptional regulatory factors and are included within the steroid/thyroid receptor superfamily (reviewed in Ref. 42). RXR is an intranuclear heterodimerization partner that provides transactivational capacity with other receptors in the family, such as vitamin D3 receptor, T3R, peroxisome proliferator activator receptor, and the RARs (reviewed in Ref. 43). We report the increased RXR expression in those human breast lesions that confer increased risk levels common to all forms of DCIS. The potential implications of these data for breast neoplastic progression, including retinoic chemoprevention, are discussed.

Materials and Methods

In Situ Hybridization Probes. Riboprobes were generated by PCR with the oligonucleotides 5'-GGGTTACATGGGCTTAAGTGTCTAGAC-3' and 5'-CCACGTCTGGCTTGAGCTGTCAGGTACC-3' (includes polylinker) and human teratocarcinoma cDNA template. PCR was performed at 94°C, for 60 s, 55°C for 60 s, and 72°C for 5 min for 35 cycles. The amplified fragment was subcloned into the double-stranded KpnI site of pGEM3Z or pBluescriptKS vectors (Promega) and confirmed by double-stranded sequencing. In vitro transcription with the respective RNA polymerase (Promega) generated antisense and sense strands labeled with 35S-labeled UTP (Amersham Corp.) according to the manufacturer’s directions.

Cohort. Sections from formalin-fixed, paraffin-embedded blocks obtained from consultation practice without any attached clinical data were utilized. The diagnosis was based on standard criteria, which correlate with epidemiological data (18-21). The pathological diagnosis of each case was evaluated independently by two pathologists. For the in situ hybridization, the cohort included lesions of DCIS and benign breast lesions. The sections on which immunohistochemistry was performed overlapped the original cohort and, in addition, contained lesions of invasive breast cancer with an adjacent intraductal component.

In Situ Hybridization. Sections on sialized slides were deparaffinized twice in xylene for 10 min and 50% xylene in ethanol for 30 s, hydrated sequentially in graded ethanol, and warmed to 68°C in 2× SSC for 30 min. The slides were incubated in each of the following reagents and washed in between with 1× PBS: 200 ng/ml proteinase K, 27 mm glycine, 40 mg/ml paraformaldehyde, 1.36% (vol/vol) triethanolamine, and 0.27% (vol/vol) acetic anhydride; they were then washed in water. Slides were hybridized with 1.25 × 106 cpm 35S-labeled riboprobe in hybridization buffer containing 50% formamide, 5× SSC, 0.1 M NaHPO4 (pH 7.0), 1× Denhardt's solution, 5% (wt/vol) dextran sulfate, 0.1 M DTT, 0.1 mg/ml tRNA, and 0.1 ng/ml Escherichia coli DNA. Hybridization was overnight at 42°C. Slides were washed in 50% (vol/vol) formamide, 2× SSC, and 10 mm DTT 50% (vol/vol) and 50% formamide, 1× SSC, and 10 mm DTT each for 30 min at 37°C. This was followed by three washes at room temperature of 1× SSC and 10 mm DTT for 10 min each, and then the slides were left O/N in 1× SSC and 10 mm DTT. On the following day, slides were incubated in 5 pg/ml RNase A for 45 min at room temperature, followed by a final wash of 2× SSC for 1 hour. Slides were dehydrated in graded ethanol and air dried. Under light-free conditions, slides were coated in NTB2 emulsion (Kodak) and stored in a light-tight box for 4 weeks. After exposure, slides were developed in D-19 Developer/Fixer (Kodak) and stained with H&E.

In Situ Hybridization Cohort Analysis. Sections were hybridized in sets of 18, together with a section of a single lactating breast specimen used for normalization purposes. The pathological diagnosis and grain densities over the cells of normal lobular units were determined by microscopic inspection at ×40 magnification by consensus of a pathologist and oncolgist using an arbitrary 1+ to 5+ (most dense) system. The lactating breast control was set at 3+ . Grain densities and pathological diagnosis were determined independently by a second pathologist. In cases in which variability of grain densities were observed within a lesion, the range of densities was recorded. Only sections in which the grain densities in all areas of a lesion matched those of the normal lobular units were recorded as “normal = lesion.” For one set of in situ hybridizations, grains were quantitated. The number of grains over 10 eosin-stained nuclei from each type of lesion (i.e., normal, DCIS, and so on) within each slide were determined. The means ± SE for each 1-5 intensity evaluation given by the pathologist were as follows: 1, 0.48 ± 0.09 grains; 2, 1.26 ± 0.12 grains; 3, 3.95 ± 0.31 grains; 5, 16.05 ± 3.25 grains.

Immunohistochemistry. Paraffin-embedded sections on sialized slides were deparaffinized, washed in ethanol, soaked in 0.5% hydrogen peroxide in methanol, and rehydrated. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) in a microwave for 5-15 min past boiling. A Ventana machine was used for the application of immunostaining reagents for an avidin-biotin-peroxidase assay. Rabbit polyclonal antibodies for RXRα, -β, and -γ (Santa Cruz Biotechnology) were applied in a 1:20 dilution. Rabbit IgG (Jackson ImmunoResearch Laboratories) was used for a negative control.

Immunohistochemistry Cohort Analysis. Slides were evaluated independently by two pathologists. The percentages of positively stained nuclei in normal ductal/lobular elements and in lesions were scored. An intraslide comparison of the percentages of positively stained nuclei in lesion versus normal ducts was then performed.

Results

Differential Expression of RXR mRNA. In situ hybridization was used to determine the relative expression levels of RXR mRNA among biopsied human breast lesions that confer to the patient varying risks for the development of invasive breast carcinoma. These lesions include typical hyperplasia, sclerosing adenosis, fibrocystic changes and radial scar, which confer no or low risk; noncomedo DCIS, which is associated with an 8-10-fold increase in cancer risk; and comedo DCIS, which confers a yet higher cancer risk (18, 19). A riboprobe encoding RXRα was prepared; this probe is approximately 80% identical to RXRβ and RXRγ and therefore likely detects composite RXR expression (44-46). The fragment used encodes a portion of the ligand-binding region of the protein product. 35S-radiolabeled probes were hybridized to sections of 58 formalin-fixed, paraffin-embedded biopsy specimens. After autoradiography, grain intensity was determined independently by two pathologists and compared to that of normal ductal/lobular units located in the margin of each specimen.

Table 1 summarizes the results of this investigation. Benign lesions signifying no or only slight increased cancer risk were compared to matched normal ductal/lobular units on the same slide. RXR was overexpressed in only 8% of the specimens (1 of 12). In contrast, 42% (5 of 12) of these lesions were
Riboprobes encoding the E region of RXR were generated, and 35S-labeled antisense probes were hybridized in situ to sections of formalin-fixed, paraffin-embedded biopsy specimens. Grain densities over the normal ducts and breast lesions were determined by microscopic inspection at ×40 magnification by consensus of a pathologist and an oncologist using an arbitrary scoring system of 1+ to 5+ (most dense) system. A lactating breast specimen was included in each set of in situ hybridization for normalization purposes and was set at 3+. Grain densities were determined independently by a second pathologist. Quantitation of grain densities is detailed in "Materials and Methods."

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Percentage (fraction) of cases</th>
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<tr>
<td></td>
<td>Lesion &gt; normal</td>
</tr>
<tr>
<td>Benign&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3% (1/12)</td>
</tr>
<tr>
<td>DCIS (total)</td>
<td>66.9% (32/48)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Comedo</td>
<td>87.5% (7/8)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Noncomedo</td>
<td>65.8% (25/38)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cribriform</td>
<td>73.3% (11/15)</td>
</tr>
<tr>
<td>Solid</td>
<td>58.8% (10/17)</td>
</tr>
<tr>
<td>Papillary</td>
<td>66.7% (4/6)</td>
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<sup>a</sup> Includes lesions of typical ductal hyperplasia, fibrocystic changes, apocrine metaplasia, radial scar, and papilloma.
<sup>b</sup> Significantly different from benign (no or only slightly increased risk lesions); P = 0.0002, Fisher's exact test.
<sup>c</sup> Significantly different from benign (no or only slightly increased risk lesions); P = 0.0008, Fisher's exact test.
<sup>d</sup> Significantly different from benign (no or only slightly increased risk lesions); P = 0.0006, Fisher's exact test.

Exhibited fewer RXR mRNA grains of hybridization than the normal structures.

For any form of DCIS, a striking trend of RXR mRNA overexpression was noted, with 70% (32 of 46) of lesions exhibiting greater grain intensities than matched normal lobular units. Fig. 1 illustrates the RXR overexpression in an area of DCIS in comparison to normal ductal/lobular units within the same slide. This trend held for all DCIS subtypes examined, with the highest overexpression rates (88%) in the most aggressive comedo DCIS. These findings were compared to our previous cohort data on cyclin D1 overexpression (3). Of the present DCIS cohort, 65% of the specimens were also assayed for cyclin D1 mRNA levels and, of these, 50% exhibited an overexpression of both markers.

The interpretation of the in situ hybridization data was subject to several important controls. Neither the acellular stroma nor stromal cells exhibited significant grain densities of RXR mRNA overexpression. Overexpression rates of 13% (1 of 8) and 0% for RAR α and RAR β, respectively. Thus, limited data indicate that not all of the members of the retinoid receptor superfamily exhibit mRNA overexpression in carcinoma in situ lesions.

Fig. 1. Overexpression of RXR mRNA in DCIS in comparison to a matched normal breast duct. Sections were hybridized in situ to the antisense strand of an RXR riboprobe. Hybridization was detected by autoradiography, and photomicrographs were taken at ×40 magnification. Grain densities, indicative of RXR mRNA expression levels, were quantitatively lower in the normal breast ductal/lobular units (A) than in DCIS (B). Photomicrographs A and B are taken from the same slide.

The data indicate that a high proportion of DCIS lesions exhibited a quantitative increase in RXR mRNA, as compared to matched normal ductal/lobular units. This overexpression is associated with increased risk for the development of invasive breast cancer, given that lesions associated with low or no risk rarely exhibited RXR overexpression.

Expression of RXR Subtypes in Normal Breast Ductal/ Lobular Units and Noninvasive Lesions. The in situ hybridization data indicated an overexpression of RXR mRNA in lesions conferring a high risk for patient development of invasive carcinoma. Immunohistochemical studies were then conducted using antibodies specific for the α, β, and γ forms of the RXR family to (a) confirm the RXR mRNA overexpression trends in DCIS at the protein level and (b) identify which RXR family member(s) was responsible. Specificity of the antibodies was confirmed on Western blots, in which each recognized a Mr 48,000-56,000 band from lysates of breast carcinoma cell lines, without apparent cross-reactivity (data not shown). Fig. 2 shows representative immunohistochemical staining for RXRa in the normal ductal/lobular units and a multifocal DCIS from a single breast biopsy lesion. Immunostaining localized RXRa expression to breast myoepithelial and ductal epithelial cells and spared the stroma except for positively stained lymphocytes. The subcellular ductal epithelial staining pattern included intense nuclear staining consistent with its intranuclear func-
Overexpression of RXR in DCIS

Fig. 2. Increased intranuclear staining for RXRa in a DCIS lesion in comparison to the matched normal ductal/lobular units. Sections were immunostained for RXRa, and photomicrographs were taken at ×40 magnification. Arrowheads, nuclei of intraductal cells; arrows, nuclei of myoepithelial cells. A, normal breast ducts; B, DCIS. Photomicrographs A and B are taken from the same slide.

Table 2 Comparison of RXR protein expression in matched normal ducts and breast DCIS lesions

<table>
<thead>
<tr>
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<th>Lesion &gt; normal</th>
<th>Lesion = normal</th>
<th>Lesion &lt; normal</th>
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<tbody>
<tr>
<td>RXRa</td>
<td>64% (16/25)</td>
<td>32% (8/25)</td>
<td>4% (1/25)</td>
</tr>
<tr>
<td>RXRB</td>
<td>21.4% (3/14)</td>
<td>71.4% (10/14)</td>
<td>7.1% (1/14)</td>
</tr>
<tr>
<td>RXRγ</td>
<td>23.1% (3/13)</td>
<td>61.5% (8/13)</td>
<td>15.4% (2/13)</td>
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*Significantly different from cases stained for RXRB (P = 0.019, Fisher’s exact test) and significantly different from cases stained for RXRγ (P = 0.038, Fisher’s exact test).

Discussion

The development of prevention strategies for breast cancer in women identified as high risk will depend on an accurate molecular map of the carcinogenesis process. Of the many possible investigative approaches that can be pursued toward this goal, we have chosen an analysis of relative gene expression in human biopsy lesions that confer to the patient varying degrees of risk for the development of invasive breast cancer. Genes thus identified are relevant to human disease given that they were subject to regulation in human breast lesions and can be tested in more general transfection or transgenic mouse model systems for functional oncogenic activity.

Our findings demonstrate an overexpression of RXR mRNA in human DCIS breast lesions that confer an 8-fold or greater risk for the development of invasive disease. Among a variety of biopsy lesions associated with low or no risk for the development of invasive breast cancer, overexpression of RXR mRNA was infrequent. Immunohistochemistry studies confirmed the in situ hybridization trends at the protein level and identified the specific receptor to be predominately RXRa. The relative expression levels of many genes involved in breast cancer have been determined in similar cohort studies (reviewed in Ref. 6). Most markers have shown a change in expression levels only at the highest risk category comedo DCIS, including increased expression of p53, c-erb-B-2, and transforming growth factor β (1–5, 8–17, 47). Our data, coupled with our previous findings of cyclin D overexpression, signal molecular events associated with a significant but lower positive nuclei comparable to that of the normal ductal/lobular units.

Immunohistochemical evaluation of RXRB and -γ expression patterns are also summarized on Table 2. All specimens exhibited staining for RXRB and RXRγ. However, the percentage of positively staining nuclei in the DCIS lesions was equivalent to that of the matched normal ductal/lobular units in 71% (RXRB) and 62% (RXRγ) of specimens examined. Overexpression of RXRB and -γ was observed in 21% and 23% of lesions, respectively. These percentages were significantly below those obtained for RXRa, for RXRB (P = 0.019) and RXRγ (P = 0.038). However, the data suggest a potential role for the β and γ members of the RXR family in a subset of DCIS.

In summary, the immunohistochemical data confirm and extend the RXR mRNA overexpression data in DCIS, with an increase in the intracellular protein receptor level and identify RXRa as the major isoform involved in the overexpression.
level of risk (3). The data suggest the hypothesis that these events may be causally involved in neoplastic progression at an earlier stage or along a different pathway. The recent finding that cyclin D1 can independently activate the estrogen receptor DNA-binding domain (48) potentially ties both markers to the steroid/thyroid receptor superfamily. Comparison of our RXR and cyclin D in situ hybridization cohort data, however, indicates that only 50% of lesions show dual overexpression. These data suggest that the two molecular events, if functionally involved in neoplastic progression, may represent discrete mechanisms. Potential synergistic interactions would also be of interest in this case.

RXR is an integral member of the larger steroid/thyroid receptor superfamily which includes the estrogen receptor, progesterone receptor, glucocorticoid receptor, RARs (RARα, -β, and -γ), vitamin D$_3$ receptor, T3R, peroxisome proliferator-activated receptor, liver-enriched receptor, farnesoid X receptor, orphan receptors, and the three RXRs (RXRα, -β, -γ; Refs. 43, 49, and 50). The natural ligand for the RARs is ATRA, which can also activate the RXRs (45). A ligand with higher affinity for RXR is a natural metabolite of ATRA, 9CRA (51, 52). All of the receptors are ligand-activated transcription factors. RXR is unique to this group for its ability to interact with other receptors and form heterodimer complexes (50, 53–58). The heterodimer formation with RXR is necessary for the receptor:ligand complex to bind to a DNA hormone response element and activate transcription (59). An alternative pathway of activation for RXR is as a homodimer by binding to a unique response element (RXRE) (60). The downstream RXRE activation pathway is incompletely understood. Genes that have been identified as downstream of RXRE, CRBPIIb (61) and apolipoprotein A1 (62), each have a promoter sequence containing the RXRE, but data demonstrating induction of gene expression by retinoic acid have not been reported. Other genes that have been demonstrated to be activated by RXRE are 25-hydroxyvitamin D$_3$-24-hydroxylase (63) and growth hormone in rat (64). Thus, RXR serves as an accessory factor for multiple hormonal receptor pathways and, in addition, has its own pathway of activation.

The causes of increased RXR expression are not generally known. In rat hepatoma cell lines, RXR mRNA was induced by dexamethasone, but not 9CRA, ATRA, thyroid hormone, or estrogen (65). Induction of RXR by ATRA or 9CRA was observed in F9 embryonal carcinoma cell lines (66), but not in the MCF-7, T-47D, BT20, or HBT126 breast carcinoma cell lines (67, 68). We have noted a trend among the immunohistochemistry cohort suggesting that the normal ductal/lobular cells may differ in patients whose lesions overexpress or fail to overexpress RXR. Of the specimens in which DCIS lesions exhibited RXR overexpression, approximately two-thirds contained normal ductal/lobular units in which <60% of cells were RXR positive. In contrast, among cases in which RXR expression was comparable between DCIS and normal ductal/lobular units, only one-third of specimens contained normal ductal/lobular units with <60% RXR positive cells. These data, based on very limited numbers of specimens, suggest the hypothesis that lower RXR expression among normal breast epithelial cells may signal a sensitivity to those factors that increase RXR expression, which may be germane to the neoplastic process. This hypothesis will be further investigated in larger cohort studies.

Because of the complexity of the RXR functional pathways, several possible phenotypic effects may result from its overexpression. The increase in RXR expression may affect the breast phenotype through heterodimerization with other receptors of the steroid/thyroid receptor superfamily, resulting in transactivation. In reference to the clinical trials with retinoids for breast cancer prevention, this could potentially increase chemopreventative differentiation/growth inhibition/apoptotic effects of the retinoids via RARs. Alternatively, other heterodimerizations could potentiate these same preventive effects. For example, RXRβ has been reported to inhibit transactivation from the estrogen response element as a heterodimer with either T3Rα or an unidentified protein (69). A second possibility is that increased levels of RXR homodimers may form, with resultant differentiation/growth inhibition/apoptotic phenotypic effects. For instance, ligands that favored RXR homodimer formation blocked the enhancement of the estrogen-induced proliferative effects by thyroid hormone (70). RXR ligands have shown activity in preclinical models of breast cancer (71, 72), and some are currently under investigation in breast cancer.

In contrast to the role of retinoids in differentiation and chemoprevention, emerging data have also correlated RXR expression with the undifferentiated state. RXRα is highly expressed in an organ-specific pattern through mouse embryonic development (46). Increased RXRα mRNA was found in the more undifferentiated skin cancer lesions, as compared to normal skin and benign tumors, in a mouse in situ hybridization study (73). RXR homodimer formation was reported to be responsible for the transcriptional activation of the retinoic acid response element in undifferentiated melanoma cells (74). Thus, our data and those mentioned may support a role for RXR under some circumstances in the dedifferentiated state. Further information on the expression levels of steroid/thyroid receptor family members expressed in breast carcinomas and premalignant lesions, which could potentially interact with RXR, will be needed for these functional investigations. Optimally, transgenic mouse or transfection studies should approximate these expression patterns to make these studies relevant to the human condition.

Acknowledgments
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A high-risk lesion for invasive breast cancer, ductal carcinoma in situ, exhibits frequent overexpression of retinoid X receptor.

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