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PRINCIPAL INVESTIGATOR: Craig Rowell

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27710

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**4. TITLE AND SUBTITLE**

Stromal-Epithelial Interactions and Tamoxifen-Sensitivity: A Bench-to-Bedside Model of Chemoprevention

**6. AUTHOR(S)**

Craig Rowell

Email: craig.rowell@duke.edu

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**14. ABSTRACT**

The microenvironment of the breast likely plays a critical role in changes to cytology during the development of cancer. Understanding of changes to the genetic as well as broader biochemical constituents of individual cells or cell types may have greater influence in our ability to detect and or track cancer development. It is likely that these changes are dynamic and affected by external stimuli including therapeutic regimes. This work examined changes in the methylation profile of estrogen responsive genes (estrogen receptor and progesterone receptor) as well as establishing early protocols for examination of tissue-level steroids that may function through these important receptors. Finally, we initiated studies to evaluate the role of changing environment on tissue development by isolating, culturing and differentiating adipose derived pluripotent (stem) cells from the breast tissue. The results of these studies showed that estrogen receptor methylation status does not change with respect to tamoxifen treatment. Current focus on progesterone methylation will soon determine if this is treatment has effect on this gene or not. Finally, we have demonstrated through our pilot study that changes in breast tissue level steroids are likely related to body mass index and menopausal status.

**15. SUBJECT TERMS**

RPFNA, Estradiol, Progesteron Receptor, Estrogen Receptor, GC-MS
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Stromal-Epithelial Interactions and Tamoxifen-Sensitivity: A Bench-to-Bedside Model of Chemoprevention

Abstract:

The microenvironment of the breast likely plays a critical role in changes to cytology during the development of cancer. Understanding of changes to the genetic as well as broader biochemical constituents of individual cells or cell types may have greater influence in our ability to detect and or track cancer development. It is likely that these changes are dynamic and affected by external stimuli including therapeutic regimes. This work examined changes in the methylation profile of estrogen responsive genes (estrogen receptor and progesterone receptor) as well as establishing early protocols for examination of tissue-level steroids that may function through these important receptors. Finally, we initiated studies to evaluate the role of changing environment on tissue development by isolating, culturing and differentiating adipose derived pluripotent (stem) cells from the breast tissue. The results of these studies showed that estrogen receptor methylation status does not change with respect to tamoxifen treatment. Current focus on progesterone methylation will soon determine if this is treatment has effect on this gene or not. Finally, we have demonstrated through our pilot study that changes in breast tissue level steroids are likely related to body mass index and menopausal status.

Introduction:

The National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention Trial (BCPT) demonstrated that tamoxifen (Tam) reduced the incidence of estrogen receptor-"positive" (ER(+)) invasive cancers. In contrast, Tam did not reduce the incidence of estrogen receptor-"negative" (ER(-)) breast cancer. While the BCPT demonstrated a clinical benefit from Tam in reducing the incidence of ER(+) breast cancer, we still do not understand the mechanism of Tam action during early mammary carcinogenesis. Random Periareolar Fine Needle Aspiration (RPFNA) is a research technique prospectively validated in high-risk women to test for (1) short-term breast cancer risk and (2) response to chemoprevention. Approximately 250 RPFNA have been performed at Duke University in the initial 24 months of my mentor?s studies. Importantly, >90% of women who underwent initial RPFNA underwent subsequent RPFNA, thus allowing us to (1) prospectively monitor cytology in high-risk women and (2) assess individual response to chemoprevention. In preliminary data, we used RPFNA to test for cytological response to Tam chemoprevention in women who (1) are at high risk for breast cancer and (2) have mammary atypia on their initial RPFNA. After 6 months of Tam chemoprevention, (1) 15/30 women demonstrated a disappearance of atypia on RPFNA at 6 months, (2) 13/30 women exhibited persistent atypia, and (3) 2/10 exhibited an increase in Masood Cytology Score. Our preliminary data demonstrate the feasibility of using RPFNA to test for cytological response to Tam chemoprevention in women who (1) are at high risk for breast cancer and (2) have mammary atypia on their initial RPFNA. After 6 months of Tam chemoprevention, (1) 15/30 women demonstrated a disappearance of atypia on RPFNA at 6 months, (2) 13/30 women exhibited persistent atypia, and (3) 2/10 exhibited an increase in Masood Cytology Score. Our preliminary data demonstrate the feasibility of using RPFNA to test for cytological response to Tam chemoprevention. This proposal aims to test whether (1) low ER expression by immunohistochemistry (IHC) and (2) methylation of the ERAlpha promoter (ESR1) predicts resistance to Tam chemoprevention. In addition, we will isolate mammary epithelial and stromal RPFNA cells obtained from women who have persistent atypia after 12 months of Tam chemoprevention and test these cells for resistance to estrogen-mediated co-activator recruitment and activation of the estrogen response element (ERE).

Hypothesis: The proposed multidisciplinary training proposal will test the hypothesis that in premenopausal women with cytological atypia on initial RPFNA, (1) hypermethylation of the ERAlpha promoter (ESR1) predicts RPFNA-monitored cytological response to Tam
chemoprevention and (2) mammary epithelial cells isolated from women who have persistent atypia after Tam chemoprevention will fail to exhibit estrogen-mediated activation of ERE.

Relevance: One of the greatest challenges we face is to move research discoveries from the laboratory to the clinic where they can directly benefit women at risk for breast cancer. The area of investigation I propose is highly relevant to improving breast cancer prevention. Recent studies suggest that breast cancer incidence may be substantially reduced in high-risk women by tamoxifen chemoprevention. As a result of these studies, tamoxifen is routinely offered to high-risk premenopausal women to prevent breast cancer. However, tamoxifen can have significant side effects and many women will not respond. Currently, we give tamoxifen blindly without being able to track whether it is working in individual women. By defining the molecular markers that predict response to tamoxifen chemoprevention, we can rapidly identify women who will or will not have a clinical benefit from tamoxifen. Observations gained in these studies can be rapidly translated by our multidisciplinary prevention group to benefit high-risk women.

I. ESR1 Promoter Hypermethylation does not predict atypia in RPFNA nor persistent atypia after 12 months Tamoxifen chemoprevention (see Appendix A). Submitted for publication in Cancer Epidemiology, Biomarkers and Prevention.

II. Progesterone Receptor Methylation in Breast Cancer

Abstract

Normal and malignant tissues contain two progesterone receptor (PR) isoforms, PR-A and PR-B. The PRs are estrogen-regulated genes, and their synthesis in normal and cancer cells require estrogen and estrogen receptors (ER). Emerging evidence suggests that methylation of the 5’ CpG island of the PR gene predicts estrogen receptor (ER) status. We evaluated the frequency of PR-A and PR-B promoter methylation by methylation specific PCR in 1) five normal mammary epithelial cell strains, 11 cancerous mammary cell lines and 16 primary breast tumors. Primers were optimized to detect 5 methylated cells in 104 non-methylated controls cells. The PR-A promoter was methylated in 60% of the normal, 54% of breast cancer cell lines and 81% of the primary tumors. The PR-B promoter was methylated in 40% of the normal, 45% of breast cancer cell lines and 12% of the primary tumors. Most of the cancerous mammary cell lines that were ER negative were methylated for both PR-A and PR-B. In contrast, there was only 37% incidence of methylation of the ER promoter (ESR1 promoter) in the primary breast tumors. The status of ER and PR can change over the natural history of the disease or during treatment. Our initial results suggest that the PR-A promoter may be methylated at an earlier stage in mammary carcinogenesis than the PR-B or ER promoter.

Background

Progesterone receptors (PR) are members of the nuclear receptor superfamily and are required for normal mammary gland development. The two PR isoforms, PR-A and PR-B, are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters. Normal breast express equimolar ratios of PR-A:PR-B, however, this is altered by hormone replacement therapies, E and E+P, and use of tamoxifen in cancer treatments. PR-A excess has been associated with poor clinical outcome and with more rapid disease recurrence after tamoxifen treatment. The status of ER and PR can change over the natural history of the disease or during treatment (figure 1). PR levels decrease more
dramatically during tamoxifen therapy, with up to half of tumors completely losing PR expression when resistance develops. Emerging evidence suggests that the relation between gene methylation and protein expression for ER and PR’s is more complicated than that of other genes. The current hypothesis is that methylation of PR gene may be a better predictor of ER expression and that methylation of the ER gene may be a better predictor of PR expression. In this pilot study we attempt to determine the rate of PR methylation in commonly used mammary derived cell lines (both normal and cancerous) as well as clinically derived tumor samples.

Figure 1. Progression of cellular atypia from non-proliferative to cancer is classified using the Masood cytology index which follows a score from 9-19. Evaluation of methylation of genes (e.g., Estrogen receptor alpha) can be examined by PCR and matched to cytology index. This comparison allows for longitudinal comparisons of changes in methylation and cytology and potential evaluation of therapeutic interventions.

Methods

The frequency of PR-A and PR-B promoter methylation was tested by methylation specific PCR in 1) five normal mammary epithelial cell strains and 11 cancerous mammary cell lines and 2) 16 primary breast tumors. Mammary epithelial cell strains and primary breast tumor DNA underwent bisulfite treatment prior to PCR-based analysis of methylation status. Primers were optimized to detect 5 methylated cells in 104 non-methylated controls cells.
Results

The PR-A promoter was methylated in 60% of the normal mammary cell strains and in 54% of breast cancer cell lines. The PR-B promoter was methylated in 40% of the normal mammary epithelial strains and in 45% of breast cancer cell lines. Most of the cancerous mammary cell lines that were ER negative were methylated for both PR-A and PR-B (Table 1).

<table>
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<th>PRB-M</th>
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Table 1: Progesterone receptor methylation profiling of common and unique breast cell lines.

PR-A methylation was observed in 81% of the primary breast tumors. In contrast, there was only 12% incidence of PR-B promoter methylation and 37% incidence of methylation of the ER receptor promoter (ESR1 promoter) in the same primary breast tumors (Table 2).

<table>
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<th>Tumors</th>
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Table 2: Methylation profile of tumor samples

Conclusion

In estrogen receptor-positive (ER+) breast cancers when PR’s are shown to be present, tumors are highly likely to respond to estrogen suppression therapies, tamoxifen or aromatase inhibitors. The status of ER and PR can change over the natural history of the disease or during treatment. DNA methylation events occur early in carcinogenesis, making methylation a potentially important marker of risk. Our initial results suggest that the PR-A promoter may be
methylated at an earlier stage in mammary carcinogenesis than the PR-B or ER promoter. Future research will involve monitoring PR methylation status using RPFNA samples from a cohort of women at high-risk for developing breast cancer.

III. Evaluation of Breast-Tissue Estradiol and Triglycerides in a High-Risk Cohort *
*This material was presented in greater detail in the mid-term report for this grant

Abstract
Using the extracellular fluid from random pariareolar fine needle aspiration samples (RPFNA-EF) obtained from women at high-risk for developing breast cancer we evaluated estradiol (E2), triglyceride (TG), and compared them to patient-matched serum levels. Additionally, we compared these values to the menopausal status and BMI values for each patient.

The results demonstrated that for this pilot study there were no statistical differences in the raw estradiol levels based on menopausal status. However, when we used TG levels for correction there was a slight, but significant, negative correlation between BMI and RPFNA estradiol levels.

In general, the estradiol levels of the RPFNA-EF compartment were over 3X that observed in the serum.

Background
It is well established that steroid hormones play a role in the progression and maintenance of tumors as demonstrated by results of the 2002 Women's Health Initiative (WHI) and the effectiveness of selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) in chemoprevention trials. However, we still have a minimal understanding of the role of tissue-derived steroids in breast cancer. Random Periareolar Fine Needle Aspiration (RPFNA) is a research technique, prospectively validated in high-risk women to test for 1) short-term breast cancer risk and 2) response to chemoprevention. As a result of this procedure we are also able to collect extracellular fluid (RPFNA-EF) from the breast tissue. Evaluation of this compartment may provide important information with respect to the concentration of steroids in the microenvironment of the breast.

Methods
We evaluated RPFNA-EF and matched patient serum estradiol and triglyceride levels and compared them to patient BMI, menopausal status and Masood cytology index. Estradiol and triglyceride levels were evaluated using an ELISA kit (ADI, San Antonio, TX) and colorimetric assay (TR-0100, Sigma-Aldrich), respectively. All assays were done in duplicate. Body weights and heights were clinically measured on a platform scale with a fixed stadiometer. Estradiol measurements were performed on 81 samples, Triglyceride values were determined for 73 samples and BMI was established for 74 samples. Masood cytology was determined by a single, dedicated, pathologist.
Figure 1. Using the extracellular fluid from random pariareolar fine needle aspiration samples (RPFNA-EF) obtained from women at high-risk for developing breast cancer we evaluated directly evaluated estradiol (E2), and triglyceride (TG). These values were then compared to patient-matched serum levels for E2 and TG as well as the menopausal status and BMI values for each patient.

Results

Women with a BMI below 20.6 (bottom 25th percentile of our group) had significantly lower mean estradiol levels compared with those with a BMI greater than 20.6 (371 mg/ml and 458 mg/ml, respectively; \( p = 0.004 \)). Concentrations of triglyceride and estradiol had a weak but significant negative correlation (\( r = -0.26, \ p = 0.028 \)). Therefore, as triglyceride increases the estradiol concentrations tend to decrease, vice versa. There were no significant differences in mean estradiol concentrations between Pre- and Post- menopausal women (525 mg/ml and 542 mg/ml, respectively; \( p = 0.166 \)). However, there was a significant decrease in the ratio of estradiol/triglyceride in post-menopausal compared to Pre-menopausal women (576 mg/ml and 810 mg/ml, respectively \( p = 0.026 \)) (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>AVG RPFNA E2 (mg/ml)</th>
<th>TG (mg/ml)</th>
<th>AVG E2/TG</th>
<th>Serum E2 (mg/ml)</th>
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<td>525.76</td>
<td>0.809</td>
<td>810.17</td>
<td>190.39</td>
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<tr>
<td>Postmenopause</td>
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<td>1.012</td>
<td>576.36</td>
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<td>BMI &lt; 20.6</td>
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<td>0.879</td>
<td>722.35</td>
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<tr>
<td>BMI &gt; 20.6</td>
<td>458.31</td>
<td>0.926</td>
<td>673.31</td>
<td>185.87</td>
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</table>

Table 1. Women with BMI below 20.6 (25th percentile of our group) had a significantly lower mean estradiol level compared with BMI greater than 20.6 (\( p = 0.004 \)). There was no difference, however, between those classified as obese (BMI > 25) and non-obese (BMI < 25). While there was no significant difference in mean estradiol levels based on menopausal status (\( p = 0.166 \)), there was a significant decrease in the mean ratio (estradiol/TG) in postmenopausal women (\( p = 0.026 \)).
Figure 2. Comparisons of RPFNA derived E2 and Serum TG levels with masood index. RPFNA E2 shows an apparent plateau with masood scores over 13. This score, generally, represents an important shift toward proliferation.

Discussion
In general we found that breast tissue levels of estradiol were not significantly different based on menopausal status. The use of triglyceride values for correcting estradiol levels had a significant impact on the general results of this study. The effects of BMI were only at the bottom quartile, in general prior correlations between BMI and breast cancer risk have been associated with BMI’s greater than 26. As demonstrated in previous studies looking at nipple aspirate fluid the breast level steroid concentrations are significantly higher than those observed in the serum. Our demonstration that these levels did not change with menopausal status may indicate an important prolonged role for estradiol in the general maintenance of the breast tissue. Likewise though this may suggest that overall the role for estradiol in cancer initiation/progression may be overstated. However, interpretation is made difficult by the fact that our cohort consists of women who have already been designated as high risk. Because of this we also evaluated the effect of masood score that is why significant increase at 13 is important, masood 13 is generally considered the cutoff point for the start of cytological atypia.
IV. Isolation and Differentiation of Human Adipose-Derived Adult Stem Cells (hADAS) from the Mammary Gland

Background
The potential role of stem cells and/or progenitor cells in cancer development continues to gain interest. Recent evaluations have pointed to an important source of progenitor cells; Adipose-Derived Adult Stem cells (hADAS). The differentiation potential of these cells has been demonstrated by their conversion to phenotypes including adipocytes, chondrocytes, osteoblasts and others. The differentiation into an osteoblastic form is of particular interest given the metastatic potential of breast cancer to bone. In this pilot project we demonstrate that hADAS cells derived from the mammary gland can differentiate to osteoblast-like cells.

Methods
Mammary gland hADAS cells (MG) were isolated from tissue derived from a patient undergoing prophylactic mastectomy and abdominal fat was purchased from Zen-Bio (AB)(Research Triangle Park, NC). Isolated cells were cultured in either DMEM/F12 with 10% FBS or DMEM high glucose 10% FBA supplemented with ascorbate, B-glycerophosphate, dexamethasome and 1,25 vitamin D. Culture media was changed every third day for three weeks. Initial assessment of cell differentiation was evaluated with alizarin red staining, indicating the presence of calcium deposition by osteogenic cells.

Alizarin Red Staining
Plates of cells used for the differentiation assay were rinsed with 150 mM NaCl three times then fixed in ice cold 70% ethanol and placed for 1 hour at 4oC. 2% alizarin red solution in distilled water was prepared and adjusted to pH 4.1 to 4.3 with dilute NaOH. The ethanol was removed and each well was rinsed with water X 3. The well was then covered with 2% alizarin red solution. The stain was allowed to sit in the well for 10 minutes at room temperature. The wells were then rinsed 5 times with water with a final rinse (6th) time for 15 minutes in distilled water. The plates were then photographed immediately.

Cytokine Evaluation
Culture media was collected after the first week of culture and at the end of the three week treatment period for each cell type and culture condition. Media samples were frozen at -20 C till evaluated. Evaluations were conducted using the Bioplex human 27 plex cytokine assay (BioRad, Hercules, CA). Each sample was evaluated in duplicate. All evaluations are represented as a percent of the quantity found in the abdominal fat DMEM samples (AB).
hADAS from Abdominal fat (AB)  hADAS from Mammary Gland (MG)

Figure 1. Alizarin red identifies the presence of calcific deposits by cells of an osteogenic lineage.

Figure 2. Results of Bio-Plex assay for cytokine responses before and after treatment for differentiation to the osteogenic form.
Conclusion

Changes in cytokine expression are evident within the first week of treatment. Temporal changes occur over the course of treatment as the cells differentiate into and osteogenic form. Many of the cytokines exhibiting change, including IL-6, IL-8, RANTES and VEGF use the NF-kB signal transduction cascade.

Future Directions

Finalize analysis of cytokine profiling experiment. Conduct more stringent evaluations of osteogenic activity such as alkaline phosphatase activity assays. Evaluate changes in additional cell lines including our proprietary model of basal carcinoma as well as epithelial cell lines. Evaluate RPFNA samples from our high-risk cohort to look at changes in cytokines determined to be important in the differentiation of the stromal compartment.
Key Research Accomplishments:

- Preparation of **ESR1 Promoter Hypermethylation does not predict atypia in RPFNA nor persistent atypia after 12 months Tamoxifen chemoprevention** for publication.

- Establishment of protocol for evaluation of PR-A and PR-B methylation and progress toward manuscript preparation.

- Development of protocol for evaluating steroids in the RPFNA sample.

- First investigation of potential hADAS population in the breast

Reportable Outcomes:

- Preparation of **ESR1 Promoter Hypermethylation does not predict atypia in RPFNA nor persistent atypia after 12 months Tamoxifen chemoprevention** for publication.

- Presentation of poster at annual Breast Cancer and the Environment Research Centers (Fall 2007)
  - Evaluation of Breast-Tissue Estradiol and Triglycerides in a High-Risk Cohort
  - Progesterone Receptor Methylation in Breast Cancer

- Presentation of poster at the Era of Hope conference (Summer 2008)
  - Evaluation of Breast-Tissue Estradiol and Triglycerides in a High-Risk Cohort

Conclusion:

The dynamic changes evident in breast tissue require techniques that allow for longitudinal evaluation. Likewise, the establishment of biomarkers related to normal development likely influenced by cancer progression and/or therapeutic intervention will be paramount in the establishment of early intervention protocols and response monitoring. The use of techniques such as RPFNA combined with technologies that can adequately evaluate small sample sizes in a consistent and meaningful way will provide physicians with powerful tools in the treatment and detection of breast cancer. This work has demonstrated that RPFNA provides sufficient material in a minimally invasive manner to support numerous biomarker screening methodologies. Likewise, the willingness of the patient population to undergo repeated testing enhances the ability of this method to be used to develop biomarkers for detection and therapeutic response. We have demonstrated the ability to track changes in DNA methylation and evaluate these changes with cytology and therapy. We have also demonstrated that potential changes in steroid hormones exist within the breast in the absence of ovarian steroidogenesis. These changes will be important to understand particularly when anti-steroidal or steroid blocking therapies are prescribed. Furthermore, the myriad changes in cytokine levels within the breast may be an additional source of information related to the differentiation activities of the cells/tissues. Therefore, continued development of biomolecule analysis techniques from RPFNA will provide both the researcher and the physician with important tools for detecting following treatment of breast cancer.
References:

*See References in Appendix A


ESR1 Promoter Hypermethylation Does Not Predict Atypia in RPFNA nor Persistent Atypia after 12 Months Tamoxifen Chemoprevention

Joseph C. Baker Jr.,1 Julie H. Ostrander,1 Siya Lem,1 Gloria Broadwater,1 Gregory R. Bean,1 Nicholas C. D’Amato, Vanessa K. Goldenberg,1 Craig Rowell,1 Catherine Ibarra-Drendall,1 Tracey Grant,1 Patrick G. Pilie,1 Shauna N. Vasilatos,1 Michelle M. Troch,1 Victoria Scott,1 Lee G. Wilke,1 Carolyn Paisie,1 Sarah M. Rabiner,1 Alejandro Torres-Hernandez,1 Carola M. Zalles,2 and Victoria L. Seewaldt1

1Duke University Medical Center, Durham, North Carolina and 2Yale-New Haven Medical Center, New Haven, Connecticut

Abstract

Purpose: Currently, we lack biomarkers to predict whether high-risk women with mammary atypia will respond to tamoxifen chemoprevention.

Experimental Design: Thirty-four women with cytologic mammary atypia from the Duke University High-Risk clinic were offered tamoxifen chemoprevention. We tested whether ESR1 promoter hypermethylation and/or estrogen receptor (ER) protein expression by immunohistochemistry predicted persistent atypia in 18 women who were treated with tamoxifen for 12 months and in 16 untreated controls.

Results: We observed a statistically significant decrease in the Masood score of women on tamoxifen chemoprevention for 12 months compared with control women. This was a significant interaction effect of time (0, 6, and 12 months) and treatment group (tamoxifen versus control). However, neither ESR1 promoter hypermethylation nor low ER expression predicted persistent atypia in Random Periareolar Fine Needle Aspiration after 12 months tamoxifen prevention.

Conclusions: Results from this single institution pilot study provide evidence that, unlike for invasive breast cancer, ESR1 promoter hypermethylation and/or low ER expression is not a reliable marker of tamoxifen-resistant atypia.

Introduction

In cancer, epigenetic silencing through promoter hypermethylation of tumor suppressor and other genes has been discovered to occur at least as frequently as mutations or deletions (1). Numerous studies support the idea that promoter hypermethylation plays a causal role in the earliest stages of carcinogenesis (2–4). Evidence also suggests that hypermethylation of DNA repair genes may affect overall and disease-free survival in patients with malignancy (5).

The estrogen receptor-α (ESR1) promoter and first exon contain a CpG island of which aberrant methylation occurs in breast, endometrial, prostate, and lung cancer, as well as adult acute myeloid leukemia and hepatocellular carcinoma (6–11). ESR1 promoter hypermethylation is observed in both estrogen receptor ER(+) and ER(−) breast cancer specimens at widely varying rates—from 0% to 100%—although these studies often stratify specimens according to multiple characteristics such as hormone receptor status (12–18). Some reports have found that in breast cancer cell lines, ESR1 promoter hypermethylation exhibits a tight inverse relationship with ER expression (15). The suppression of estrogen receptor in these ER(−) cell lines is lifted after treatment with the demethylating agent 5-azacytidine (19), and previous findings suggest a correlation between ESR1 promoter hypermethylation and suppression of message and protein levels in breast cancer (15, 17). However, other reports find no or only a weak association, suggesting it may not be a clear inverse relationship (13, 14, 20). Recent studies show that ESR1 promoter hypermethylation outdid hormone receptor status as a predictor of clinical response to the selective estrogen receptor modulator tamoxifen in women with invasive breast cancer (20).

Random Periareolar Fine Needle Aspiration (RPFNA) is a research technique developed to repeatedly sample mammary cells from the whole breast of asymptomatic high-risk women to assess both breast cancer risk and response to chemoprevention (21–23). The presence of atypia in RPFNA has been prospectively validated to confer a 5.6-fold increase in breast cancer risk in high-risk women (23). The presence of persistent atypia in RPFNA has been recently used as a surrogate marker to track cytologic response to chemoprevention agents (21, 22). These studies underscore the utility of RPFNA as a translational research tool, and as in our previous work, RPFNA can be used to couple cytologic analysis, methylation studies, and chemoprevention (21, 24, 25).
Although ER expression is routinely used to predict tamoxifen sensitivity in invasive breast cancers and ductal carcinoma in situ, ER expression is not clinically used to predict the tamoxifen sensitivity of atypia. This is because normal breast tissue expresses low levels of ER (26-28). Over 90% of epithelial cells in normal breast tissue express low ER and only 5% to 10% cells express moderate levels of ER (26-28). Importantly though, unlike ER (−) estrogen-resistant invasive breast cancer, normal breast tissue is estrogen responsive. Furthermore, in normal breast tissue, only epithelial cells expressing low ER (and not ER+ epithelial cells) proliferate in response to estrogen (27, 28). It is hypothesized that this inverse relationship is disturbed during early mammary carcinogenesis (27).

In our single institution pilot study, we tested for the ability of ESR1 promoter hypermethylation and low ER expression to predict persistent atypia in RPFNA in 18 high-risk women who took tamoxifen chemoprevention for 12 months. Here, we provide evidence that although tamoxifen reduced the incidence of mammary atypia in high-risk women, neither ESR1 promoter hypermethylation nor low ER expression predicted persistent atypia after 12 months tamoxifen chemoprevention, suggesting they may not be suitable markers for tamoxifen-resistant atypia. Larger multi-institutional studies are needed to confirm these results.

Materials and Methods

Informed Consent. The study was approved by the Human Subjects Committee and Institutional Review Board at Duke University Medical Center, in accordance with assurances filed with and approved by the Department of Health and Human Services. Women were offered tamoxifen chemoprevention as part of standard-of-care for high-risk women.

RPFNA Eligibility. To be eligible for RPFNA, women were required to have at least one of the following major risk factors for breast cancer: (a) 5-y Gail risk calculation of ≥1.7%; (b) prior biopsy exhibiting atypical hyperplasia, lobular carcinoma in situ, and ductal carcinoma in situ; (c) known BRCA1/2 mutation carrier; or (d) contralateral breast cancer (23).

Tamoxifen Chemoprevention. To be eligible this study, women were required to have (a) a prior excisional biopsy exhibiting atypical hyperplasia, lobular carcinoma in situ, or contralateral ductal carcinoma in situ and (b) a minimum of one prior RPFNA demonstrating a Masood Cytology Index of 14 to 16 (atypia). Women with a history of clotting disorder, stroke, or abnormal uterine bleeding were not eligible to participate. Thirty-four women were offered tamoxifen chemoprevention; 18 women elected to take tamoxifen and 16 declined. This is consistent with our previously published studies demonstrating that approximately half of high-risk women in our cohort with atypia in RPFNA elect to take tamoxifen chemoprevention and half decline (29). Eighteen high-risk women with atypia (Masood 14-16) in a minimum of one prior RPFNA received 20 mg/d tamoxifen chemoprevention. Sixteen high-risk women with atypia in RPFNA (Masood 14-16) who declined tamoxifen chemoprevention served as controls. RPFNA was done at the time of starting tamoxifen chemoprevention (0 mo) and at 6 and 12 mo after initiation of tamoxifen. Persistent atypia was defined by the continued presence of aspirates with Masood Cytology Indices of 14 to 16 after 12 mo tamoxifen treatment.

RPFNA. RPFNA was done as previously published (25). A minimum of one epithelial cell cluster with at least 10 epithelial cells was required to sufficiently determine pathology; the most atypical cell cluster was examined and scored (22, 23). Cells were classified qualitatively as nonproliferative, hyperplasia, or hyperplasia with atypia (30). Cytology preparations were also given a semiquantitative index score through evaluation by the Masood Cytology Index (31). As previously described, cells were given a score of 1 to 4 points for each of six morphologic characteristics that include cell arrangement, pleomorphism, number of myoepithelial cells, anisonucleosis, nucleoli, and chromatin clumping; the sum of these points computed the Masood score: ≤10 nonproliferative (normal); 11 to 13 hyperplasia; 14 to 17 atypia; >17 suspicious cytology (23, 31).

Materials and Cell Culture Lines. Sodium bisulfite (Sigma; A.C.S.) and hydroquinone (Sigma; 99+%) were used under reduced lighting and stored in a dessicator. The T47D breast cancer cell line was obtained from the American Type Culture Collection and grown in supplemented αMEM (Life Technologies).

Methylation-Specific PCR. DNA was extracted from breast cancer cell lines and RPFNA as previously published; bisulfite treatment was as previously published (25). Previous work has elucidated appropriate methylation-specific PCR primers within exon 1 of the ESR1 promoter, from nt +376 to nt +494 relative to the transcription start site (18). The primer sequences used were as follows: Methylated (M) forward 5′-GTG TAT TTG GAT AGT AGT AAG TTC GTC-3′; M reverse 5′-GTT TGT-3′; Methylated (U) forward 5′-GTT GTA TTT GTA CCA TCT AAC CG-3′; Unmethylated (U) forward 5′-GTT GTA TTT GGA TAG TAG TAA GGT TGT-3′; U reverse 5′-CCA TAA AAA AAA CCA ATC TAA CAA-3′ (18). All PCR reactions consisted of 50 ng bisulfite-treated DNA, 1× PCR buffer, 250 μmol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 2.5 U of HotStar Taq polymerase (Qiagen) in 30 μL total volume. PCR buffers were individually optimized for the methylated and unmethylated programs. The 1× M buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 8.0), 4.0 mmol/L MgCl₂, and 100 mmol/L 2-pyridlidinone (Fluka; 99+%) and the 1× U buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 8.5), and 4.5 mmol/L MgCl₂. The methylated PCR program consisted of 95°C for 5 min followed by 40 amplification cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) and a final extension of 72°C for 4 min; the unmethylated PCR program was identical except a 52°C annealing temperature was used. A GeneAmp PCR System 9700 (Applied Biosystems) was used for all amplifications. PCR products were visualized on 1.5% ethidium bromide agarose gels using an Image Station 440 (Kodak). Optimization with methylated primers was achieved using minute amounts (~50 pg) of CpGenome Universal Methylated DNA (Chemicon) to model RPFNA samples. To estimate PCR sensitivity, titrated
ER Immunohistochemistry. **ER** protein expression was tested in RPFNA cytology specimens by immunohistochemistry (IHC) by a modification of published methods (32). Antigen retrieval was at 90°C with 0.2× nuclear decloaker (pH 9.5) for 2 min. Slides were then placed in glucose oxidase blocker at 37°C for 30 min. Cells were incubated with anti-ERα antibody (DAKO, 1D5). A minimum of 100 cells was assessed on the sample judged to be most abnormal by 2 readers. The proportion of cells stained at 5 intensity levels (0+ to 4+) were assessed to provide a weighted intensity score, calculated by multiplying each intensity level by the proportion of cells at that level and then summing over all levels. For example, if 80% of cells exhibited 0+ staining and 20% exhibited 1+ staining, the weighted intensity score would be 0.0 + 0.2 = 0.2. The slide was judged to be ER(+) if the intensity score was ≥0.2.

**Results**

**Study Demographics.** Eighteen high-risk women received 20 mg/day tamoxifen as part of standard-of-care for high-risk women. Sixteen high-risk women who declined tamoxifen chemoprevention served as controls. All 34 women had a minimum of one prior RPFNA that scored a Masood Cytology Index of 14 to 16 (atypia). Women underwent RPFNA aspiration at 0, 6, and 12 months. A Masood Cytology Index of 14 to 16 was confirmed on the 0 month RPFNA. The average age of women receiving tamoxifen prevention was 43.5 years (range, 36-50 years); average age for controls was 45.5 years (range, 39-55 years). The median RPFNA Masood score for women receiving tamoxifen prevention at 0 month was 15 and for controls subjects was 14 (Table 1). Seventeen of 18 high-risk women completed 12 months of tamoxifen prevention; 1 woman elected to undergo prophylactic surgery after 6 months of tamoxifen prevention and did not complete 12 months of tamoxifen.

The median RPFNA Masood score for women receiving tamoxifen prevention at 6 and 12 months was 14 and 13.5, respectively (Table 1). The median RPFNA Masood score for controls subjects at 6 and 12 months was 14 and 15, respectively (Table 1). There was a statistically significant difference in the average RPFNA Masood score at 12 months for women taking tamoxifen relative to control subjects (P = 0.028; Table 3) but not at 6 months (P = 0.32; Table 3). Furthermore, in a SAS mixed model analysis for repeated measures of Masood score, there is a significant interaction effect of time (0, 6, and 12 months) and treatment group (tamoxifen versus no tamoxifen; P = 0.0007; Table 3).

Atypia in RPFNA was defined by an initial (0 month) RPFNA with a Masood Cytology Index of 14 to 16. Persistent atypia was defined by the continued presence of RPFNA cells with a Masood score of 14 to 16 after 12 months of tamoxifen treatment. In the control group, 15 of 16 women had persistent atypia 12 months after their initial RPFNA. After 12 months of tamoxifen treatment, 8 of 17 (47%) women had persistent atypia, and 9 of 17 (53%) women had a loss of atypia. The median Masood score at 12 months for the group of}

**Table 1. Summary of Masood scores, ER methylation, and expression**

<table>
<thead>
<tr>
<th>Age in y (range)</th>
<th>T = 0</th>
<th>T = 6 mo</th>
<th>T = 12 mo</th>
<th>ESR1 Methylation</th>
<th>ER low (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>45.5 (39-55)</td>
<td>14</td>
<td>14</td>
<td>15</td>
<td>5/16 (31%)</td>
</tr>
<tr>
<td>Tamoxifen group</td>
<td>43.3 (36-50)</td>
<td>15</td>
<td>14</td>
<td>13</td>
<td>2/18 (11%)</td>
</tr>
</tbody>
</table>

**Statistical Methods.** The Wilcoxon Rank-Sums test was used to test for the association between ESR1 promoter hypermethylation and ER expression as well as the potential association between ESR1 promoter hypermethylation and/or ER expression and persistent atypia after 12 months of tamoxifen chemoprevention. A SAS Mixed model analysis for repeated measures was used to test for differences in Masood scores over time and between groups.
tamoxifen-treated women with persistent atypia was 14.75, whereas the median Masood score at 12 months for the group of tamoxifen-treated women with loss of atypia was 10.75 (Fig. 1). These medians are statistically significantly different ($P = 0.001$; Table 3).

**ESR1 Promoter Hypermethylation in Atypical RPFNA Samples Does Not Predict Low ER Expression.** Hypermethylation from nt +367 to nt +494 of the ESR1 promoter was tested using methylation-specific PCR in all RPFNA specimens obtained. Four representative samples are shown in Fig. 1B. Strong unmethylated bands were detected in all included samples, confirming both the presence of DNA and the promoter sequence itself (Fig. 1B). ER protein expression was tested by IHC using a modification of published methods. The frequency of ESR1 promoter hypermethylation and low ER in atypical RPFNA was 7 of 34 (21%) and 25 of 34 (74%), respectively (Table 1). ESR1 promoter hypermethylation was observed in 2 of 18 atypical RPFNA samples obtained from high-risk women who received tamoxifen prevention and in 5 of 16 high-risk control subjects who did not receive tamoxifen (Table 1). Low ER expression was observed in 13 of 18 (72%) atypical RPFNA samples obtained from women receiving tamoxifen prevention and in 12 of 16 (75%) control subjects (Table 1). No association was found between $ESR1$ promoter hypermethylation and ER expression by IHC in the combined group of subjects ($P = 0.15$; Table 3).

Neither $ESR1$ Promoter Hypermethylation nor Low ER Expression Predicts Persistent Atypia after 12 Months of Tamoxifen Prevention. Although there was no relationship between $ESR1$ promoter hypermethylation and ER protein levels in our cohort, we sought to test whether these variables may be potential biomarkers for predicting persistent atypia after tamoxifen chemoprevention. From the overall group of 17 women who received 12 months of tamoxifen prevention, 12 women had low ER expression (Fig. 2). Of these 12 women, 7 with low ER expression had persistent atypia, whereas the other 5 women with low ER expression had a loss of atypia at 12 months (Table 2). Only one woman who received tamoxifen chemoprevention had $ESR1$ promoter hypermethylation (Table 2). Of the 16 women without $ESR1$ promoter hypermethylation who received tamoxifen, 8 of 16 (50%) had persistent atypia. Neither $ESR1$ promoter hypermethylation nor low ER expression was statistically associated with the presence of persistent atypia in high-risk women who received tamoxifen prevention ($P = 0.32$ and $P = 0.5$, respectively; Table 3).

**Discussion**

The National Surgical Adjuvant Breast and Bowel Project Trial Breast Cancer Prevention Trial (P1) showed a 50% reduction in estrogen-sensitive breast cancer in premenopausal high-risk women who took tamoxifen chemoprevention (33). Importantly, tamoxifen decreased risk of invasive breast cancer in women with lobular carcinoma in situ and atypical hyperplasia by 56% and 86%, respectively (33). These findings provide evidence that women with lobular carcinoma in situ and atypia benefit most from tamoxifen (33). As a result, tamoxifen chemoprevention is an option for premenopausal women with mammary atypia. Although tamoxifen has been shown to have significant benefit in many women, not all women benefit from tamoxifen prevention and there are significant side effects associated with tamoxifen treatment. Progress in breast cancer prevention is currently limited by our lack of biological markers to identify which women will benefit from tamoxifen prevention therapies such as tamoxifen, as well as to identify women with tamoxifen-resistant atypia. Here, we tested whether $ESR1$ promoter hypermethylation predicted persistent atypia in high-risk women who were treated with tamoxifen chemoprevention. Recent reports show that $ESR1$ promoter hypermethylation outdid hormone receptor status as a predictor of clinical response to tamoxifen hormonal therapy in women with invasive breast cancer (33).

**Table 2. Summary of ER methylation and expression in control and tamoxifen groups divided into women with persistent atypia or loss of atypia**

<table>
<thead>
<tr>
<th></th>
<th>ESR1 promoter methylation</th>
<th>Low ER expression (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women on tamoxifen ($n = 17$)</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Persistent atypia ($n = 8$)</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Loss of atypia ($n = 9$)</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Control women ($n = 16$)</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Whole cohort ($n = 33$)</td>
<td>21%</td>
<td>74%</td>
</tr>
</tbody>
</table>

**NOTE:** Summary of ER methylation and expression in control and tamoxifen groups divided into women with persistent atypia or loss of atypia. This table provides information for $ESR1$ promoter methylation and ER expression on 17 women who completed 12 mo of tamoxifen chemoprevention and 16 women who served as controls. Both the tamoxifen and control groups were divided into either persistent atypia (Masood $\geq 14$) or loss of atypia (Masood $<14$) at 12 mo. The data for the two groups combined is also represented (whole cohort).
breast cancer (20). In contrast to these prior studies in invasive breast cancer, neither ESR1 promoter hypermethylation nor low ER expression predicted persistent atypia at 12 months in this study. These studies highlight potential differences between ER+ breast cancer and mammary atypia.

Although the presence of atypia has been shown to increase breast cancer risk, it is currently unknown if the presence of persistent atypia in RPFNAb can be used as a surrogate marker of breast cancer risk or as a marker of resistance to tamoxifen chemoprevention. We are unable to make conclusions relative to women who have a loss of atypia as this may be due to sampling error; further studies are necessary. However, in women with persistent atypia, it is clear that tamoxifen did not result in the elimination of atypia; yet the long-term implications of these observations must be interpreted with caution. It is possible that women who have persistent atypia on RPFNAb will still have a risk reduction benefit. Currently we are unable to identify whether an individual woman with either atypia on excisional biopsy or cytologic atypia on RPFNAb will progress to develop breast cancer.

Further longitudinal studies are ongoing to determine whether women with persistent atypia on RPFNAb are at increased short-term breast cancer risk relative to women who have had disappearance of atypia. It is important to recognize that this study is limited by being a small, single-institution study. Although these results are interesting, further validation in a larger multi-institutional study is required before our findings can be generalized to a larger, high-risk population.

Although the molecular mechanism of tamoxifen action in ER+ breast cancer is well-studied, there is little information on how tamoxifen may act in noncancerous human breast tissue that normally expresses low levels of ER (ER(-)‘poor’). Prevention models for tamoxifen action in ER-poor, noncancerous breast tissue have been primarily based on observations in ER+ breast cancer cells. Normal mammary tissue is composed of a heterogeneous population of cells. Greater than 90% of normal mammary epithelial cells express low ER, and only 5% to 10% of normal mammary epithelial cells express moderate ER (26). If normal breast tissue is evaluated solely by ER expression, it would be classified as ER(-). This classification system, however, may not be adequate for describing normal mammary tissue. Although normal mammary tissue exhibits low ER, it responds to estrogen and, therefore, is not equivalent to ER(-), estrogen-resistant invasive breast cancer.

There are a number of possible explanations why ER(poor) mammary atypia may respond to tamoxifen chemoprevention therapy. First, in vivo atypical mammary epithelial cells may be more sensitive to extragenomic effects of tamoxifen. In our in vitro cellular models of early mammary carcinogenesis, we observed that immediately after the acute loss of p53 function, primary human mammary epithelial cells exhibit a narrow window of tamoxifen-induced apoptosis sensitivity (34-37). It is possible that loss of p53 or other tumor suppressors early in mammary carcinogenesis (38, 39) sensitizes cells to tamoxifen chemoprevention, leading to elimination of these atypical cells in high-risk women.

Although inhibition of ER transcriptional activity and signaling is the predominate effect of tamoxifen in the breast, not all of the effects of tamoxifen can be directly attributed to competitive interactions with ER. Tamoxifen induces apoptosis in cholangiocarcinoma cells and inhibits angiogenesis in fibrosarcoma (40, 41). Tamoxifen also has a wide variety of other pharmacologic activities including stimulation of transforming growth factor-β, blockade of various chloride channels (42), inhibition of protein kinase C (43), and antagonism of calmodulin activity (44). Furthermore, tamoxifen-binding sites, independent of ER, have been identified. For example, Sutherland, et al. (45) reported a high-affinity antiestrogen binding site in human and rat uterine cells, as well as in other tissues. Tamoxifen also directly inhibits calmodulin in a calcium-dependent manner (44). Because therapeutic concentrations of tamoxifen are several orders of magnitude higher than required to saturate ER (46), these ‘extragenomic’ effects of tamoxifen may play an important role in ER-poor normal breast tissue.

The molecular mechanisms for tamoxifen resistance in ER(+) invasive breast cancer is an area of intense investigation. It is possible that similar mechanisms for tamoxifen resistance in invasive breast cancer may exist during early mammary carcinogenesis. For example, ER/progesterone receptor(+)-positive invasive breast cancer is more likely to respond to antiestrogen therapy than ER(+) /progesterone receptor(-) breast cancer. The present study did not examine progesterone receptor status. Growth factor signaling pathways downstream of ErbB family members, such as the Akt/mammalian target of rapamycin pathway, are often up-regulated in tamoxifen-resistant ER(+)- breast cancers. Therefore, if these pathways are up-regulated in high-risk women.
with mammary atypia, it is possible that these women would be resistant to tamoxifen chemoprevention. Furthermore, the ER coactivators AIB1/SRC-3 and MNA/THEL1 have both been shown to promote tamoxifen resistance in models of ER(+) breast cancer, both dependent and independent of ER signaling (47, 48). Finally, alterations in tamoxifen metabolism may also predict resistance. Production of tamoxifen metabolites occurs in the liver via cytochrome P450 CYP2D6 (49, 50). Several polymorphisms in CYP2D6 that result in a decrease in tamoxifen metabolism have been identified (51, 52). Although the effect of these polymorphisms on the efficacy of tamoxifen is still controversial (53, 54), it has been reported that specific mutations in CYP2D6 results in a higher risk of disease relapse but a decrease in the incidence of tamoxifen side effects (55). Although there is no clinical data associated with tamoxifen chemoprevention, it is reasonable to hypothesize that women with CYP2D6 polymorphisms may be resistant to tamoxifen chemoprevention.

In this study, we found that ESR1 hypermethylation or low ER expression was not able to predict persistent atypia in response to tamoxifen treatment in our high-risk cohort. Additionally, our results indicate that, similar to invasive breast cancer, not all mammary atypia is responsive to tamoxifen; 53% of women in this cohort showed a loss of atypia at 12 months. Although the implications of persistant atypia relative to breast cancer risk are still unclear, further analysis of RPFNA samples from atypia that is tamoxifen responsive versus tamoxifen resistant may be useful to determine biomarkers of tamoxifen sensitivity, as well as to increase our understanding of the early events underlying mammary carcinogenesis and the different tumor types that arise in the breast.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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