Identification of EZH2 as a Molecular Marker for a Precancerous State in Morphologically Normal Breast Tissues

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Abstract

The discovery of molecular markers to detect the precancerous state would have profound implications in the prevention of breast cancer. We report that the expression of the Polycomb group protein EZH2 increases in histologically normal breast epithelium with higher risk of developing cancer. We identify EZH2 as a potential marker for detecting preneoplastic lesions of the breast in vivo and as a possible target for preventative intervention. (Cancer Res 2006; 66(8): 4095-9)

Introduction

To date, the earliest recognizable precursor of invasive carcinoma of the breast is atypical ductal hyperplasia (ADH), the diagnosis of which is based on pathologic criteria. Although ADH is cytologically atypical and architecturally more complex than usual ductal hyperplasia, it still does not have the features necessary for the diagnosis of ductal carcinoma in situ (DCIS; ref. 1). Emerging data support the hypothesis that histologically normal breast epithelial cells from healthy women already contain genetic and epigenetic alterations which render them more susceptible to malignant transformation (2, 3). However, molecular markers identifying a precancerous state from otherwise morphologically normal breast epithelial cells remain largely unknown.

We previously reported that EZH2 is an independent predictor of breast cancer recurrence and death (4). The increased EZH2 expression is consistently associated with the aggressive behavior of breast cancer including invasiveness and metastatic potential. EZH2 interfered with the ability of breast cells to repair DNA double-strand breaks, which is a cause of genomic instability and a mechanism of cancer development (5). We propose here that elevated EZH2 protein expression detects a precancerous state in morphologically normal breast epithelium.

Materials and Methods

Breast sample collection. Breast tissue samples were obtained from the Surgical Pathology files at the University of Michigan with Institutional Review Board approval, and an arbitrary number was assigned to each sample for de-identification purposes. One hundred and nineteen breast tissue samples comprising a wide range of normal, ADH, and DCIS were used (Table 1). Among the 50 histologically normal breast samples, 25 were from healthy women who underwent reduction mammoplasty, 13 derived from women carrying a known BRCA1 heterozygous mutation who underwent a prophylactic mastectomy, and 12 were from women who underwent a biopsy which yielded benign breast parenchyma. BRCA1 mutation status and clinical information was obtained through the Breast and Ovarian Cancer Risk Evaluation Program at the University of Michigan Comprehensive Cancer Center (S.D. Merajver) and chart review, respectively.

Immunohistochemistry. Immunohistochemistry was done on tissue sections using standard biotin-avidin complex technique with a mouse monoclonal antibody against EZH2 (1:25, BD Biosciences, San Jose, CA) and a Ki-67 antibody (1:50; DAKO, Carpinteria, CA). DCIS samples were previously immunostained for estrogen receptor (ER) as part of the routine clinical evaluation using a monoclonal anti-ER antibody (clone 6F11, Ventana, Tucson, AZ). Once immunostained, the percentage of EZH2 and Ki-67-expressing epithelial cell nuclei was recorded independently by two observers (L. Ding and C.G. Kleer) in a blinded manner.

Statistical analyses. Statistical analyses were done by the epidemiologist in the study (C. Erdmann). The intensity of EZH2 staining in samples of normal peripheral tissue associated with ADH diagnosis, ADH diagnostic tissue, normal peripheral tissue associated with DCIS diagnosis, ADH peripheral tissue associated with DCIS diagnosis, and DCIS diagnostic tissue were compared with normal tissue obtained from normal individuals using the exact Wilcoxon test as the EZH2 distributions were not normally distributed, $P < 0.05$ was considered statistically significant. Among DCIS patients, the exact Wilcoxon test also was used to compare distributions of EZH2 staining intensities for nuclear grade, ER status, tumor size, and age. All statistical tests were two-sample and two-sided unless otherwise indicated. Spearman's rank correlation ($\rho$) was used to evaluate the correlation between EZH2, Ki-67, and DCIS grade. Spearman's $\rho$ statistic also was used to determine the intrarater reliability and the reproducibility of the scoring system.

Results and Discussion

To determine whether the expression level of EZH2 protein increases during the early phases of breast cancer development, we evaluated the expression of EZH2 protein in 119 breast tissue samples comprising a wide range of normal, ADH, and DCIS lesions (Table 1). When expressed, EZH2 was observed in the epithelial cells, mainly in the nucleus as described previously (refs. 4, 6, 7; Fig. 1). As compared with normal breast tissues from reduction mammoplasties that had little or no EZH2 expression (median, 0%), the EZH2 expression was elevated in ADH (median, 10%; Wilcoxon $P < 0.0001$; Table 2). The highest percentage of cells expressing EZH2 was observed in high nuclear grade (grade 3) comedo type DCIS (median, 80%; Wilcoxon $P = 0.0005$), which is consistent with the clinical evolution of DCIS, as high nuclear grade DCIS is associated with higher rate of recurrence and shorter disease-free survival interval than low (grade 1) and intermediate (grade 2) nuclear grade lesions (ref. 1; Table 3). These findings suggest that the expression of EZH2 protein increases as breast cancer develops and progresses.
Histologically normal lobules adjacent to ADH and DCIS had a ~3-fold and ~15-fold increase in the percentage of cells expressing EZH2 (median, 3% and 15%, respectively), when compared with normal lobules from women without ADH or DCIS (median, 0%; Wilcoxon $P = 0.1005$ and $P < 0.0001$, respectively). Furthermore, EZH2 expression was higher in ADH associated with DCIS (median, 40%) when compared with ADH alone (median, 10%; Wilcoxon paired $P = 0.065$; Fig. 1; Table 2). It is possible that the observed up-regulation of EZH2 in normal epithelium in the vicinity of ADH and DCIS is due to a “field effect” resulting from the activation of signaling pathways triggered by adjacent atypical cells, as has been suggested previously (8, 9). Alternatively, in light of our previous study (4) and present data showing that increased EZH2 expression is associated with progression in both early and late phases of breast cancer development, it is tempting to speculate that the expression of EZH2 in histologically normal epithelium may represent a molecular marker of a precancerous state that is associated with higher risk for the development of invasive breast cancer.

To test this hypothesis, we examined EZH2 expression in normal breast tissues from two populations of patients: BRCA1 mutation carriers and patients with benign lesions. It has been well documented that patients from these two groups have higher risk for breast cancer. First, we determined EZH2 expression in 38 histologically normal breast tissues derived from (a) 13 women carrying a known BRCA1 heterozygous mutation who underwent a prophylactic mastectomy, and (b) 25 patients who underwent a breast reduction and had no personal or family history of breast cancer (Supplementary Table S1). Women who inherit germ line mutations in the BRCA1 gene have up to an 85% lifetime risk of developing breast cancer by the age of 70 (10). Using immunohistochemistry, we found that BRCA1 mutation carriers had a marked increase in the percentage of epithelial cells expressing EZH2 when compared with controls (median, 25% versus 0%; Wilcoxon $P < 0.0001$; Fig. 2A). Our data suggest that increased EZH2 expression may be associated with higher risk for breast cancer. The elucidation of the possible relationship between EZH2 and BRCA1 genes warrants further investigation.

Prospective and retrospective studies have shown that healthy women who undergo a breast biopsy for benign breast disease have a relative risk of breast cancer of 1.5 to 1.6 compared with women in the general population, and that this risk persists for at least 25 years after the initial biopsy (11–13). Despite advances in the understanding of the molecular mechanisms leading to breast cancer, the only risk factors identified to date for breast cancer after the diagnosis of benign breast disease include the histologic classification of a benign breast lesion and a positive family history of breast cancer (11). We next determined EZH2 expression levels in 12 benign breast

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**Table 1. Clinical and pathologic characteristics of patients in this study ($n=119$)**

| Characteristics                  |  
|---------------------------------|---
| Diagnosis                        |  
| Normal/fibrocystic changes, no. | 25  
| Reductions                      | 12  
| Biopsies                        | 13  
| Prophylactic mastectomies (BRCA1) |  
| ADH, no.                        | 21  
| DCIS, no.                       | 48  
| Median age, years (range)       | 59 (36-90)  
| Median size, cm (range)         | 1.1 (0.1-5.0)  
| Nuclear grade, no. (%)          |  
| 1                               | 12 (25.00)  
| 2                               | 14 (29.17)  
| 3                               | 22 (45.83)  
| ER status                       |  
| Negative, no. (%)               | 13 (27.08)  
| Positive, no. (%)               | 30 (62.50)  
| Unknown, no. (%)                | 5 (10.42)  

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**Figure 1.** EZH2 expression is elevated in histologically normal breast epithelium and in ADH associated with DCIS (original magnification, ×200).
tissues samples from women with no family history or personal history of breast cancer (Supplementary Table S1). Seven women developed cancer within the following 12 years, and five did not. Consistently, we found that the histologically normal breast tissues from patients who developed cancer had significant up-regulation of EZH2 (median, 20%) when compared with patients who did not develop cancer (median, 5%; Wilcoxon P = 0.0088; Fig. 2B). These results further support the hypothesis that increased EZH2 expression in normal tissues is correlated with higher risk of breast cancer. These data may have profound clinical implications as the increasing use of mammography has resulted in an increased number of benign breast biopsies. Furthermore, the discovery of markers of breast cancer risk is necessary for the development of strategies to prevent breast cancer progression. Our data support the theory that precursors to breast cancer exist in histologically normal breast tissues and offer a way to identify such precursors.

EZH2 expression has been linked with cellular proliferation in breast and other neoplasms (6, 7, 14–16). Consistent with this, we found that EZH2 was expressed in epithelial cells undergoing mitosis (data not shown). Whereas EZH2 and the proliferative marker Ki-67 were nearly undetectable in normal breast samples from reductions, both were up-regulated in DCIS, and their levels increased with advancing histologic atypia (Spearman's $\rho = 0.77$; Fig. 3). Despite this positive correlation, the percentage of DCIS cells expressing EZH2 was higher than the percentage of Ki-67 positive cells suggesting that although EZH2 may be associated with the proliferative capacity of breast epithelial cells, it may have other nonproliferative functions in carcinogenesis. These data are in agreement with a previous study in bronchial carcinogenesis (16). Similar results were observed in breast tissues from patients who underwent a benign biopsy (Fig. 2B). In this group of patients, even though EZH2 and Ki-67 (17) were elevated in women who later developed carcinoma compared with those who did not, EZH2 expression was higher than Ki-67. Notably, whereas EZH2 expression was higher in normal breast tissues from BRCA1 mutation carriers, Ki-67 remained low (Fig. 2A), further suggesting that EZH2 has nonproliferative functions during neoplastic transformation. Our data show that EZH2 is a more sensitive marker than Ki-67 to predict breast cancer risk.

### Table 2. Comparison of EZH2 expression according to the pathologic diagnosis

<table>
<thead>
<tr>
<th>Pathologic diagnosis</th>
<th>n</th>
<th>EZH2 median</th>
<th>EZH2 range</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/fibrocystic changes</td>
<td>25</td>
<td>0.0</td>
<td>0-5</td>
<td></td>
</tr>
<tr>
<td>Normal/fibrocystic changes associated with ADH</td>
<td>11</td>
<td>3.0</td>
<td>0-20</td>
<td>0.1005</td>
</tr>
<tr>
<td>ADH</td>
<td>21</td>
<td>10.0</td>
<td>0-50</td>
<td>0.0009</td>
</tr>
<tr>
<td>Normal/fibrocystic changes associated with DCIS</td>
<td>15</td>
<td>15.5</td>
<td>0-80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ADH associated with DCIS</td>
<td>11</td>
<td>40.0</td>
<td>10-80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DCIS</td>
<td>48</td>
<td>45.0</td>
<td>0-100</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Compared with normal/FCC group, Wilcoxon exact test.

### Table 3. Associations between EZH2 protein expression and clinical pathologic characteristics of patients with DCIS (n = 48)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
<th>EZH2 (median)</th>
<th>EZH2 (range)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12 (25.00)</td>
<td>20.0</td>
<td>0-90</td>
<td>0.5138</td>
</tr>
<tr>
<td>2</td>
<td>14 (29.17)</td>
<td>30.0</td>
<td>0-100</td>
<td>0.0005</td>
</tr>
<tr>
<td>3</td>
<td>22 (45.83)</td>
<td>80.0</td>
<td>10-100</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>30 (62.50)</td>
<td>40.0</td>
<td>0-100</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13 (27.08)</td>
<td>80.0</td>
<td>0-100</td>
<td>0.3657</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (10.42)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>35 (72.92)</td>
<td>40.0</td>
<td>0-100</td>
<td>0.3455</td>
</tr>
<tr>
<td>≥2</td>
<td>13 (27.08)</td>
<td>80.0</td>
<td>10-100</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>12 (25.00)</td>
<td>60.0</td>
<td>20-100</td>
<td>0.0615</td>
</tr>
<tr>
<td>≥50</td>
<td>32 (66.67)</td>
<td>30.0</td>
<td>0-100</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (8.33)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*Wilcoxon exact test.

1P values between tumor grades 1 and 3 is 0.0005, between tumor grades 2 and 3 is 0.0011.
The advantage of using immunohistochemistry is that it is straightforward, widely available, and it allows for the evaluation of protein expression in situ, with direct visualization of the specific EZH2-expressing cells. We were able to achieve high interobserver reliability, as well as high reproducibility using our scoring method ($q = 0.96$, $P < 0.001$; and $q = 0.89$, $P < 0.0001$, respectively).

In conclusion, EZH2 detection in tissues by immunohistochemistry could potentially constitute the basis for a diagnostic test that distinguishes histologically normal breast tissues at higher risk for neoplastic progression. Finally, targeting EZH2 may provide a novel way to prevent breast cancer in women with increased risk at an earlier stage.

Acknowledgments

Received 12/1/2005; revised 2/3/2006; accepted 2/17/2006.

Grant support: National Cancer Institute grants K08CA090876 (C.G. Kleer) and R01CA107469 (C.G. Kleer); and the Histology and Immunohistochemistry Core at the University of Michigan Comprehensive Cancer Center through the University of Michigan’s Cancer Center support grant (5 P30 CA46592).

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We thank Dr. Yuan Zhu, Ph.D. (University of Michigan Department of Internal Medicine) for review of the manuscript and helpful critiques; Yanhong Zhang and Michael Zeidler for helpful suggestions during the execution of this project; and Kara Milliron, genetic counselor at the University of Michigan Comprehensive Cancer Center for providing information on the BRCA1 mutation status of the patients.

References

Figure 3. Expression of EZH2 and Ki-67 in DCIS. A, H&E staining of DCIS of nuclear grades 1, 2, and 3, and corresponding EZH2 and Ki-67 protein expression. EZH2 and Ki-67 proteins increase with advancing nuclear grade of the DCIS (original magnification, ×200). B, box plots illustrating that EZH2 and Ki-67 proteins are significantly higher in high nuclear grade DCIS (grade 3) when compared with low and intermediate grades (1 and 2). Note that the magnitude of association between EZH2 and DCIS grade is stronger than that for Ki-67 and DCIS grade.

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