Promoter Hypermethylation in Benign Breast Epithelium in Relation to Predicted Breast Cancer Risk

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ABSTRACT

Introduction: The tumor suppressor genes RASSF1A, APC, H-cadherin, RARβ2, and cyclin D2 are methylated more frequently in breast cancer than in adjacent benign tissue. However, it is unclear whether promoter methylation of tumor suppressor genes in benign breast tissue is associated with an increased risk for breast cancer.

Methods: Promoter hypermethylation was measured in benign and malignant breast samples obtained by fine needle aspiration biopsy from 27 breast cancer patients and 55 unaffected women whose risk of breast cancer had been defined using the Gail, Claus, and BRCAPRO models.

Results: Cyclin D2 methylation occurred in 57% of tumor samples but not in corresponding benign breast samples and in only one sample from an unaffected patient (P < 0.0001). RARβ2 methylation occurred in 32% of benign breast samples from cancer patients but only 9% of similar samples from unaffected women (P = 0.002). Promoter methylation of RASSF1A and APC occurred more frequently (70% and 56%, respectively) in unaffected women at high-risk for breast cancer as defined by the Gail model than in low/intermediate risk women (29% and 20%, P = 0.04 and P = 0.03). Of the Gail model risk factors, only number of prior breast biopsies was highly correlated with APC and RASSF1A methylation (P = 0.0001 and 0.02, respectively).

Conclusions: Since cyclin D2 promoter methylation occurs almost exclusively in tumors, it may be possible to exploit it for the early detection of breast cancer. Promoter methylation of APC, RARβ2, and RASSF1A in benign breast epithelium is associated with epidemiologic markers of increased breast cancer risk.

INTRODUCTION

Methylation of cytosines in promoter region CpG dinucleotides is a common mechanism for silencing tumor suppressor genes (TSG) and occurs frequently in breast cancer as well as in many other cancers (1, 2). Although there is a large body of literature reporting TSG methylation frequency in solid cancers, similar data for normal and preneoplastic tissue are lacking and it is not clear whether methylation in this setting is a marker of increased cancer risk. One study describing TSG methylation in patients with intestinal metaplasia or cancer of the esophagus found that CDKN2A, ESR1, or MYOD1 were only methylated in intestinal metaplasia from patients with dysplasia or cancer in other regions of the esophagus but not in patients with no evidence of progression beyond metaplasia (3). Furthermore, CALCA, MGMT, and TIMPS were methylated more frequently in normal stomach, normal esophageal mucosa and intestinal metaplasia from patients with distant dysplasia or esophageal cancer than patients without dysplasia or cancer. These data suggest that methylation of certain TSGs may occur as a field change and may be associated with increased risk for malignant progression.

Epidemiologic risk factors such as age, cancer family history, history of breast biopsies, and reproductive history have been combined in mathematical models that are useful for categorizing women as low, intermediate, or high risk for breast cancer. The Claus model (4) uses a patient’s family history of breast cancer to determine her risk, whereas the BRCAPRO model (5) uses family history of both breast and ovarian cancer to determine a patient’s risk of carrying a BRCA1 or BRCA2 mutation as well as the probability of developing breast cancer in the future. The Gail model is the most widely used and thoroughly validated model for calculating breast cancer risk (6–8). This model considers the patient’s ethnicity, age, age at menarche, age of first live birth, family history of breast cancer, and number of breast biopsies to determine the risk for developing breast cancer in the future. In addition, women diagnosed with one breast cancer develop contralateral breast cancer at the rate of 0.5% to 2% per year depending on the age at initial diagnosis and the family history of breast cancer (9, 10). An elevated Gail risk or personal history of breast cancer are validated epidemiologic markers of increased breast cancer risk suitable as surrogate end points for identifying putative biological markers of risk.

In this study, we analyzed the methylation status of five TSGs (APC, RASSF1A, H-cadherin, RARβ2, and cyclin D2) in benign and malignant breast cells obtained by fine needle aspiration (FNA) biopsies from 27 invasive breast cancer patients and 55 unaffected women whose risk of breast cancer had been defined using mathematical models. The purposes of this study were (1) to measure the frequency of TSG methylation in tumor tissue, benign breast tissue from cancer patients, and benign breast tissue from unaffected patients and (2) to determine whether TSG methylation frequency in benign breast tissue is associated with epidemiologic markers of breast cancer risk (personal history of breast cancer or model probabilities). Our analysis shows that
promoter methylation of cyclin D2 occurs almost exclusively in malignant tumor samples, whereas promoter methylation of RASSF1A and APC occurs more frequently in benign samples from high risk women than in samples from low or intermediate risk women as determined by the Gail model.

MATERIALS AND METHODS

Patients. This research was approved by the Institutional Review Board at the University of Texas Southwestern Medical Center, and written informed consent was documented for each subject. Between August 10, 2000 and March 18, 2003, 101 women were enrolled in this study. These included 63 unaffected women recruited from a breast cancer risk assessment clinic and 38 women with incident breast cancer (Table 1). Eight unaffected women were excluded from analysis due to inadequate DNA recovery (n = 5), a prior diagnosis of ductal carcinoma in situ (n = 1), or the absence of epithelial cells in the sample (n = 2). Eleven cancer patients were excluded due to chemotherapy treatment given prior to sampling (n = 3), matching cytology slides were not made (n = 2), inadequate DNA yield (n = 5), or improperly labeled samples (n = 1). The average age of the 55 evaluable unaffected women was 43 with a range of 20 to 63 years, and the average age of the 27 evaluable cancer patients was 50 with a range of 36 to 68 years (P < 0.001). Eighteen of the 55 unaffected women (33%) had prior biopsies and the histologies of these included lobular carcinoma in situ (1), atypical ductal or lobular hyperplasia (4), proliferative fibrocystic changes (4), nonproliferative fibrocystic changes (5), and normal breast tissue only (4). Three breast cancer patients were stage 0, nine stage I, eight stage IIA, and seven stage IIB.

Mathematical Risk Assessment. Comprehensive breast cancer risk assessment was done using the interactive software we developed. The software collects and archives information related to reproductive history, benign breast disease, family history, hormonal medication usage, and physical activity and automatically calculates age-specific breast cancer probabilities using the models of Gail (6), Claus (4), BRCAPRO (5), and Bodian (11). Certain components of this software have been made generally available in a package called CancerGene (12). Age- and race-matched general population risk is automatically calculated for each patient to provide a basis for comparison (13). Dividing the counseland’s risk by the age- and race-matched population risk produces a risk index that can be used to group women into low/intermediate and high-risk strata independent of age and race. We define high risk as a cumulative breast cancer probability that is greater than or equal to double the age- and race-matched general population risk. This is a better way to classify women as high risk than simply dichotomizing the absolute 5-year Gail risk as ≥1.7% or <1.7% as is commonly done. The arbitrary 1.7% threshold was the 5-year breast cancer risk for the average 60-year-old woman based on the 1985 to 1987 Surveillance, Epidemiology, and End Results data. Breast cancer incidence has increased over the last 20 years and the 5-year risk for the average 60-year-old Caucasian woman is now >2.0% and for the average 60-year-old African American woman <1.7%. Calculation of a risk index relative to age- and race-matched populations identifies women who are at increased risk compared with their peers. The distribution of the 55 evaluable risk-assessed patients by risk index is depicted in Fig. 1. The study sample represents a broad range of risk levels with a preponderance of women at low/intermediate risk: 44 of 55 (75%) by the Gail model, 47 of 55 (85%) by BRCAPRO, and 46 of 55 (84%) by the Claus model. For comparison, 35 of 55 (65%) of unaffected women had an absolute 5-year Gail risk <1.7%.

Breast Epithelial Cell Sampling. Random periareolar FNA was done in the upper outer quadrant of each breast, using a 25-gauge needle after infiltration of 1% lidocaine. Material from the first needle pass was directly smeared onto glass slides, which were immediately fixed in 95% isopropyl alcohol and later stained for cytologic assessment using the Papanicolaou method. A second sample, obtained from the same site, was fixed in CytoLyt solution (Cytyc Health Corporation, Boxborough, MA) for DNA extraction. FNA samples from patients with an incident breast cancer were obtained during surgery and included the cancer, ipsilateral normal breast, and contralateral normal breast. For the ipsilateral breast, samples were taken from the quadrant opposite the cancer. For patients with prior breast biopsies, care was taken to sample glandular breast tissue well away from the biopsy cavity. All benign samples were taken near the areola, where terminal ductal-lobular units are most abundant. In each case, Papanicolaou-stained smears were evaluated to confirm the presence of tumor or benign breast cell clusters. Samples that did not contain epithelial cells were excluded from analysis. Cytologic classification of the FNA samples from the unaffected women included atypia in 3 of 55 (5%), typical epithelial hyperplasia in 31 of 55 (56%), apocrine metaplasia in 6 of 55 (11%), and normal epithelium only in 15 of 55 (27%).

Genomic DNA Isolation. Samples were coded at the time of collection and evaluated in a blinded fashion. Samples from the right and left breasts were pooled for the first 31 unaffected risk-assessed patients but processed separately for all subsequent patients. Genomic DNA was prepared from FNA cell pellets by proteinase K digestion [250 μg/mL proteinase K (Invitrogen, Carlsbad, CA), 8.75 mmol/L EDTA, 8.75 mmol/L Tris, 0.5% SDS]
for 36 to 48 hours at 37°C followed by addition of NaCl (250 mmol/L), three phenol chloroform (1:1) extractions, and ethanol precipitation.

**Methylation-Specific PCR.** Sodium bisulfite treatment of genomic DNA was done as previously described (14, 15). Since all the FNA samples contained normal cells, DNA recovery after sodium bisulfite treatment was verified by methylation-specific PCR using primers for unmethylated APC (16). Primers for detecting the presence of methylated APC (promoter 1A), H-cadherin, RASSF1A, RAR\(\beta\)2, and cyclin D2 have been previously described (15–19). Annealing temperatures, MgCl\(2\) concentrations (mmol/L) and the number of PCR cycles are as follows: unmethylated APC, 59°C/2.0/35; methylated APC, 64°C/2.0/40; methylated RASSF1A, RAR\(\beta\)2, and H-cadherin, 64°C/1.5/40; and methylated cyclin D2, 55°C/1.5/40. All PCR reactions were done in a Dyad Thermal Cycler (MJ Research, Inc., Waltham, MA) using Hot Star Taq (Qiagen, Germantown, MD). Genomic DNA from HCC1954, a breast ductal carcinoma cell line available through American Type Culture Collection (Manassas, VA), was used as a positive control for the methylated alleles whereas genomic DNA from normal peripheral blood lymphocytes and water blanks were used as negative controls. PCR products were loaded onto a 2.5% agarose gel, electrophoresed and visualized under UV illumination (Fig. 2). The validity of methylation-specific PCR measurements done on FNA samples has been previously reported (20).

In order to compare the methylation status of each gene by patient, the methylation results were combined for the benign samples taken from each breast. If a methylated PCR product was detected in either benign breast, the methylation status of that gene was scored as positive for that patient. Due to the limited quantity of DNA obtained by FNA, all markers were not analyzed for some samples. The methylation results from benign breast tissue for 10 cancer patients and eight risk-assessed patients were only known for one breast. These patients were included in our analysis in Fig. 3.

**Statistical Analysis.** Comparisons between matched samples from cancer patients were analyzed using McNemar’s test. Differences between means were compared using independent \(t\) tests, whereas proportions were compared using \(\chi^2\) and Levy’s multiple comparison or Fisher’s exact tests as appropriate. The goodness of fit \(\chi^2\) test was used to assess the significance of double methylation of APC and RASSF1A in the same patients. Although multiple comparisons were made in Table 2, no adjustments have been made. Significance level was set at 0.05.

**RESULTS**

**Frequency of Methylation in Breast Tumors and Benign Breast Samples.** For RAR\(\beta\)2, APC, and H-cadherin, methylation frequency was highest for the cancer samples, intermediate for benign breast samples from cancer patients, and lowest for samples from unaffected women [RAR\(\beta\)2: 43%, 32%, and 9% (\(P = 0.002\)); APC: 57%, 33%, and 26% (\(P = 0.04\)); H-cadherin: 36%, 28%, and 17% (\(P = 0.21\)); Fig. 3A, B and C]. The frequency of RASSF1A methylation was similar for benign breast samples from cancer patients (29%) and unaffected women (37%), but was 59% for tumor cells (\(P = 0.10\)). Two or more TSG were methylated in 78% of the tumor samples compared with only 40% of the benign breast samples from

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**Fig. 1** Frequency of methylation in invasive breast cancer samples, benign breast tissue samples from cancer patients and samples from unaffected risk assessed patients. RAR\(\beta\)2 (A), APC (B), H-cadherin (C), cyclin D2 (D), RASSF1A (E), and \(\geq 2\) Methylated Genes (F). Error bars, 95% confidence intervals. Significant differences (\(P < 0.05\)): \(\ddagger\), cancer tissue versus benign tissue from cancer patients; \(\dagger\), benign tissue from risk patients versus benign tissue from cancer patients; *, benign tissue from risk patients versus cancer tissue.

**Fig. 2** Distribution of unaffected cases by breast cancer risk index. Risk index is the breast cancer risk for each individual relative to the risk for age- and race-matched women in the general population. High risk is defined as greater than or equal to twice the general population risk. A separate risk index has been calculated for the Gail, Claus, and BRCAPRO models.
cancer patients (P = 0.025) and 24% of samples from unaffected women (P < 0.001; Fig. 3F). Of note, 22% of the unaffected patients showed methylation of both APC and RASSF1A. This is significantly higher than the 10% rate that would have been expected if methylation of these genes occurs independently (P = 0.005).

The cyclin D2 promoter was methylated in 13 of 23 (57%) of tumor samples, none of the benign breast samples from cancer patients, and 1 of 53 (2%) samples from unaffected women (Fig. 3D). This unaffected patient had methylation of APC, RASSF1A, and H-cadherin in both her breasts, but only the right breast was methylated for cyclin D2. Since methylation of the cyclin D2 promoter was only previously found in breast tumor samples, both breasts were resampled. Upon repeat sampling, we could not detect any cyclin D2 methylation in either breast. Cells from the left breast were categorized as cytologically atypical, whereas those from the right showed typical hyperplasia only. MRI detected no abnormalities in either breast.

In order to compare TSG methylation frequencies with calculated breast cancer risk, we have reported the methylation frequencies by patient rather than by breast. However, many investigators have published TSG methylation frequencies from tumor samples and normal breast tissue adjacent to the cancers. In order to compare our results with published data, we also analyzed the methylation frequencies for 17 tumors that had matched ipsilateral normal breast tissue results. The methylation frequencies for cancer and paired ipsilateral breast tissues were as follows: APC, 47% and 18% (P = 0.06); RASSF1A, 53% and 18% (P = 0.03); H-cadherin, 47% and 6% (P = 0.02); cyclin D2, 52% and 0% (P = 0.008); and RARβ2, 31% and 13% (P = 0.38). These results are comparable to previously published reports for all five genes analyzed (16, 17, 21–24).

**Promoter Region Methylation by Calculated Breast Cancer Risk.** TSG methylation did not correlate with cellularity of the FNA samples, cytologic diagnosis of the FNA samples, or the histology of prior breast biopsies but was highly correlated with calculated breast cancer risk in women unaffected with breast cancer. Based on Gail model calculations, 5 of 10 (50%) high-risk women showed methylation of ≥2 genes as compared with 6 of 41 (15%) lower risk women (P = 0.03, Table 2). More specifically, these high-risk women methylated APC or RASSF1A more frequently than women at lower risk [5 of 9 (56%) versus 9
of 44 (20%), \( P = 0.04 \) and 7 of 10 (70%) versus 12 of 41 (29%), \( P = 0.03 \), respectively]. A similar correlation was not observed between TSG methylation frequency and breast cancer risk calculated using the BRCAPRO and Claus family history models. In fact, breast cancer risk calculated using the family history calculated using the BRCAPRO and Claus family history models.

In his initial report, Gail defined three relative risks (RR) that can be calculated from the five Gail risk factors (age, age at menarche, age at first live birth, family history of breast cancer, and number of biopsies; ref. 6). Age at menarche has its own RR, but age is combined with the number of breast biopsies to generate the second RR, and age at first live birth is combined with the number of first-degree relatives (mothers and sisters) with breast cancer to generate the third RR. The RR for age combined with number of breast biopsies correlated with \( \text{APC} \) and \( \text{RASSF1A} \) methylation, but the other RR did not (\( P = 0.002 \) and 0.02, respectively; Fig. 4). Classifying age as \(< 50 \) or \( \geq 50 \) and number of biopsies as \( 0, 1, 2, \) or \( > 2 \) as Gail did shows that the number of biopsies, but not age, is highly correlated with the frequency of \( \text{APC} \) or \( \text{RASSF1A} \) methylation [For biopsies, \( \text{APC} \): 4 of 36 (13%) for 0 biopsies, 4 of 9 (44%) 1 biopsy, 3 of 3 (100%) 2 biopsies, and 3 of 4 (75%) for \( > 2 \) biopsies, \( P = 0.0001 \); \( \text{RASSF1A} \): 9 of 34 (26%), 4 of 8 (50%), 3 of 3 (100%) and 2 of 3 (66%), respectively, \( P = 0.02 \); and for \( < 50 \) or \( \geq 50 \) years of age, \( \text{APC} \): 11 of 41 (27%) and 3 of 12 (25%) \( P = 1 \) and \( \text{RASSF1A} \): 14 of 39 (36%) and 5 of 12 (42%), \( P = 0.76 \), respectively].

**DISCUSSION**

Promoter region methylation of TSG is common in many solid cancers (25–30). We have shown that, in general, promoter region hypermethylation of TSG occurs most frequently in breast cancer followed by benign tissue from cancer patients and at the lowest frequency in benign tissue from unaffected women. Consistent with other studies, we have shown that some TSG are frequently methylated in benign tissue and cancer (\( \text{APC} \), \( \text{RASSF1A} \), \( \text{RAR} \beta 2 \), and \( \text{H-cadherin} \)), whereas others are frequently methylated in cancer but rarely in benign breast (\( \text{cyclin D2} \); ref. 31).

The \( \text{RASSF1A} \) promoter is frequently methylated in benign breast tissue. In microdissected breast tissue, Lehmann et al. (32) showed that the \( \text{RASSF1A} \) promoter was methylated in all epithelial hyperplasia and papilloma samples and in 83% of ductal carcinoma \( \text{in situ} \) samples. Similar methylation frequencies have also been reported in SV40-infected mesothelial cells (33) and cirrhotic liver (26) but at a much lower frequency in intestinal metaplasias of the stomach (34). We have shown that benign breast samples from 70% of unaffected women at high risk for breast cancer (Gail risk index \( \geq 2 \)) had methylated \( \text{RASSF1A} \) promoters compared with only 29% from women at low or intermediate risk for breast cancer. \( \text{RASSF1A} \) influences the G1-S cell cycle checkpoint by regulating the level of cyclin D1 protein (35). Methylolation of \( \text{RASSF1A} \) leads to accumulation of cyclin D1 and may represent one mechanism for over riding cell cycle control under conditions of increased cell cycle pressure.

In preneoplastic conditions, promoter hypermethylation of \( \text{APC} \) has been detected at a low frequency in benign colorectal adenomas (36), but at a much higher frequency in chronic gastritis, intestinal metaplasia, and gastric adenomas (25). As with \( \text{RASSF1A} \) promoter methylation, women at high risk for breast cancer (as determined by the Gail model) had a higher frequency of \( \text{APC} \) promoter methylation (56%) compared with women with low or intermediate risk (20%).

Loss of \( \text{APC} \) expression causes an inappropriate elevation of \( \beta \)-catenin in the nucleus where it binds to Tcf4/Lef family members. This complex has been shown to stimulate transcription of genes involved with cell cycle control such as \( \text{c-myc} \) and
others have shown that hypermethylation occurs frequently in breast cancer. We and relevant for sporadic breast cancer. Indeed, the correlation of Our results suggest that epigenetic TSG silencing may be most (loss of heterozygosity) rather than epigenetic silencing (39, 40). and as such TSG silencing is frequently related to DNA deletions somatic dominant breast cancer susceptibility genes. Most familial but risk calculated using the family history models (Claus and Gail model. 

Fig. 4 Frequency of APC and RASSF1A promoter methylation in benign breast samples from unaffected women by deconstructed Gail model relative risks. For the age and number of biopsies relative risk (C), the frequency of APC methylation was significantly different between women with a relative risk of 1 and women with relative risks 1.1-1.9 ($P = 0.03$) or $>2$ ($P = 0.01$). The number of patients in each RR group is designated within each bar. FLB, first live birth. 

cyclin D1 and cause hyperplasia and adenocarcinomas in mammary fat pads (37, 38). Overexpression of cyclin D1 related to loss of APC and RASSF1A expression may represent a biological consequence of the risk factors accounted for by the Gail model. 

Breast cancer risk calculated by the Gail model was highly correlated with TSG methylation in benign breast epithelium, but risk calculated using the family history models (Claus and BRCA1) was not. The family history models are designed to identify family histories consistent with the inheritance of autosomal dominant breast cancer susceptibility genes. Most familial breast cancer syndromes are related to alterations in DNA repair and as such TSG silencing is frequently related to DNA deletions (loss of heterozygosity) rather than epigenetic silencing (39, 40). Our results suggest that epigenetic TSG silencing may be most relevant for sporadic breast cancer. Indeed, the correlation of APC and RASSF1A methylation in random breast samples with biopsy history may suggest a field change that predisposes breast epithelium to form the mammographic and palpable abnormalities that lead to benign breast biopsies and ultimately to breast cancer. 

Down-regulation of RARβ2 expression caused by promoter hypermethylation occurs frequently in breast cancer. We and others have shown that ~43% of invasive breast cancers have RARβ2 promoter methylation (20, 21, 24, 41). However, this is the first study that has shown significant RARβ2 methylation in normal breast tissue from cancer patients (32%) but minimal methylation in normal breast tissue from unaffected patients (9%). Most other studies found no methylation in normal breast tissue; but in some instances, it is unclear whether the normal tissue came from unaffected or cancer patients (21, 24, 42). However, Bovenzi et al. (41) did report RARβ2 methylation in three of eight benign tissue samples from cancer patients, which is consistent with our results. 

H-cadherin, a glycosylphosphatidylinositol-anchored cell surface protein involved in cell-cell recognition (43–45), is frequently methylated in prostate, lung, colon, and breast tumors; in corresponding benign or preneoplastic samples, the frequency is decreased at least two fold (23, 27, 46–49). However, the methylation frequency in hepatocellular carcinoma and corresponding non-malignant samples was the same (50). We found H-cadherin methylation more frequently in benign breast cancer (32%) than from risk-assessed women (17%); however, the difference was not statistically significant. 

Cyclin D2 transcripts and protein levels are decreased in ~50% of primary breast carcinomas and this correlates with promoter hypermethylation of the cyclin D2 gene (17). Of the five genes we analyzed, this was the only gene that showed no methylation in the ipsilateral or contralateral benign controls from our cancer patients. Others have also detected cyclin D2 promoter methylation in microdissected ductal carcinoma in situ samples but not in epithelial hyperplasia or papilloma samples lending further evidence that methylation of cyclin D2 is a late event in malignant transformation (32). 

In summary, cyclin D2 methylation seems to occur later in breast epithelial cell transformation and may be a exploited in the future as a marker for early detection of breast cancer, whereas RARβ2, APC, and RASSF1A promoter methylation occurs frequently in benign breast epithelium and is associated with epidemiologic markers of increased breast cancer risk. More specifically, RARβ2 methylation is correlated with a personal history of breast cancer, whereas APC and RASSF1A methylation is associated with calculated breast cancer risk particularly in women with prior benign breast biopsies. This may suggest a field change in benign breast epithelium that could be exploited for tissue-based individualized breast cancer risk stratification. 

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