

Changes in CpG Islands Promoter Methylation Patterns during Ductal Breast Carcinoma Progression

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Abstract

Aberrant promoter methylation of several known or putative tumor suppressor genes occurs frequently during carcinogenesis, and this epigenetic change has been considered as a potential molecular marker for cancer. We examined the methylation status of nine genes (*APC*, *CDH1*, *CTNNB1*, *TIMP3*, *ESR1*, *GSTP1*, *MGMT*, *THBS1*, and *TMS1*), by quantitative methylation specific PCR. Synchronous preinvasive lesions (atypical ductal hyperplasia and/or ductal carcinoma *in situ*) and invasive ductal breast carcinoma from 52 patients, together with pure lesions from 24 patients and 12 normal tissues paired to tumor and 20 normal breast distant from tumor were analyzed. Aberrant promoter methylation was detected in both preinvasive and invasive lesions for genes *APC*, *CDH1*, *CTNNB1*, *TIMP3*, *ESR1*, and *GSTP1*. However, hierarchical mixed model and Generalized Estimating Equations model analyses showed that only *APC*,

CDH1, and *CTNNB1* promoter regions showed a higher frequency and methylation levels in pathologic samples when compared with normal breast. Whereas *APC* and *CTNNB1* did not show differences in methylation levels or frequencies, *CDH1* showed higher methylation levels in invasive tumors as compared with preinvasive lesions ($P < 0.04$, Mann-Whitney test with permutation correction). The analysis of *APC*, *CDH1*, and *CTNNB1* methylation status was able to distinguish between normal and pathologic samples with a sensitivity of 67% (95% confidence interval, 60-71%) and a specificity of 75% (95% confidence interval, 69-81%). Our data point to the direct involvement of *APC*, *CDH1*, and *CTNNB1* promoter methylation in the early stages of breast cancer progression and suggest that they may represent a useful tool for the detection of tumor cells in clinical specimens. (Cancer Epidemiol Biomarkers Prev 2009;18(10):2694-700)

Introduction

Breast cancer is the most common neoplastic disease in women, with approximately 1 million new cases diagnosed each year worldwide. Despite advances in early detection and better management, mammary tumors

are still the primary cause of cancer deaths among women (1). The advent of mammography screening has led to an increased detection of preinvasive mammary lesions, and to a better elucidation of the pathologic events that precede the development of invasive breast carcinoma (2, 3). Invasive breast cancer is classified into two main morphologic subtypes, with the ductal type representing about 80% of breast malignancy. Among the breast lesions classified as preinvasive, hyperplasia of the usual type is morphologically and phenotypically heterogeneous, whereas atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS) are homogeneous in cell type and marker expression (4). Retrospective studies indicate that 30% to 50% of the *in situ* carcinomas evolve into invasive tumors within 6 to 10 years after diagnosis, whereas epidemiologic and clinical data are less clear about the role of ADH (2). However, genetic studies based on comparative genomic hybridization and loss of heterozygosity analysis have shown similar chromosomal abnormalities in both ADH and DCIS, suggesting that they may have a similar clonal origin (4).

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There is a compelling body of evidence supporting the relevance of epigenetic mechanisms in the development and progression of cancer (5, 6). In recent years, an increasing number of gene promoters were found aberrantly methylated in tumors. Such DNA methylation mapping has suggested the existence of a unique profile of hypermethylated CpG islands that characterize each neoplasia (5-8). In a previous study we determined the methylation profile in a series of invasive breast cancer and breast benign lesions (9). A similar pattern of methylation distribution was found for all the genes tested with the exception of *CDH1*, which was found methylated in malignant tumors but not in the benign breast lesions (9). These data suggest that methylation of at least some genes could be directly associated with the malignant phenotype.

Several studies analyzed promoter methylation status in preinvasive breast lesions (8, 10-15), but only two of them investigated promoter methylation changes in synchronous preinvasive lesions associated with invasive carcinoma (14, 15). Lehman et al. (14) investigated 16 synchronous DCIS-invasive ductal carcinoma (DCIS-IDC) showing no change in promoter methylation for *p16*, *RASSF1A*, and *14-3-3σ*, and a mixed behavior for *cyclinD2*. In another study, Subramaniam et al. (15) showed changes in methylation frequencies of the *RUNX3* gene promoter in 23 synchronous DCIS-IDC samples as compared with normal tissues.

In the present study we carried out quantitative methylation specific PCR analysis for a panel of genes to characterize methylation pattern in ADH, DCIS, and IDC. All the genes tested were previously identified as methylated in mammary tumors or breast cancer cell lines (*ESR1*, *CDH1*, *APC*, *GSTP1*, *TIMP3*, *MGMT*, *THBS1*, and *TMS1*; ref. 7). The only exception was *CTNNB1*, which encodes *β-catenin*, that was described as methylated only in metastatic gastric cancer and endometrial tumors (16, 17).

Materials and Methods

Cases Selection. Pathologic samples from 76 patients [median age, 48 y; interquartile range (IQR), 44-60] and 20 normal breast tissues distant from tumor (NBAT; median age, 56 y; IQR, 50-63) were obtained as paraffin-embedded samples or fresh-frozen specimens from the Department of Pathology "L. Armani," University of Naples, Italy; the Department of Pathology, IRCCS "Casa Sollievo della Sofferenza," San Giovanni Rotondo, Italy; and the Department of Pathology, IRCCS Oncology Institute, Bari, Italy. Prior written and informed consent was obtained from each patient in accordance with institutional guidelines.

Promoter methylation status was initially determined on 52 patients for whom synchronous preinvasive and invasive lesions and/or normal breast tissue adjacent to tumor (NBAT) were available (Table 1) and 20 NBAT (training set). Additional pure preinvasive and invasive lesions from 24 patients were also tested, and results from this analysis were merged with data from the training set to constitute the validation set. In three cases multiple synchronous lesions were available for each patient (two ADH were analyzed for patient BP23, three DCIS and two IDC were available for patient BP18, and two DCIS were tested for patient BP22). Multiple lesions were located on

Table 1. Breakdown of samples and patients analyzed for methylation status

Samples	Patients
Synchronous lesions	
NBAT+ADH+DCIS+IDC	1
NBAT+ DCIS+IDC	7
NBAT+DCIS	3
NBAT+IDC	1
ADH + DCIS + IDC	8
ADH+IDC	11
ADH+DCIS	4
DCIS+IDC*	17
Pure lesions	
ADH*	1
DCIS*	7
IDC	16
NBDT	20

*For three patients multiple lesions were available.

separate paraffin blocks or at >0.5 cm in distance one from another. Clinicopathologic characteristics of the 61 patients affected by invasive breast cancer (45 synchronous lesions and 16 pure IDC) are shown in Supplementary Table S1.

DNA Extraction and Sodium Bisulphite Modification. Sections (5-μm thick) were cut from paraffin blocks or fresh-frozen specimens, H&E staining was performed, and two pathologists (A. Apicella and F. Zito) reviewed each slide to identify the areas of ADH, DCIS, and IDC, and to exclude cancer cell contamination in normal breast tissues (NBT). Sample specimens were manually dissected under a microscope from 12-μm-thick sections to enrich for areas containing ADH, DCIS, or IDC. The 5-μm-thick H&E-stained sections evaluated and marked by pathologist were used as reference slides. For each specimen, 6 to 70 H&E-stained sections were dissected depending on the dimension of the lesion. Tissues were subsequently placed in xylene and genomic DNA was extracted as described previously (18).

DNA extracted from tumors was subjected to bisulphite treatment, as described previously with minor modification (19). Briefly, 1 μg genomic DNA from each sample was denatured with NaOH (final concentration, 0.2 mol/L) in a total volume of 20 μL at 50°C for 20 min. The denatured DNA was diluted by adding 500 μL of a freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L sodium bisulphite, and incubated at 70°C for 3 h. Bisulphite-modified DNA was purified using a Wizard DNA Clean-Up System (Promega), treated with 0.3 mol/L NaOH at room temperature for 10 min, precipitated with ethanol, resuspended in 120 μL of LoTE [2.5 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8)], and stored at -80°C.

Quantitative Methyl Specific PCR. Bisulphite-modified DNA was used as template for fluorescence-based real-time PCR using a relative quantification method with standard curve to determine methylation levels in pathologic samples and controls (19, 20). In this system two amplification primers as in conventional methyl specific-PCR and a dual-labeled fluorogenic hybridization probe are used (21). One fluorescent dye serves as reporter (FAM) and its emission spectra are quenched by a second fluorescent dye (TAMRA). Primers and probe of the genes of interest were designed to specifically amplify the

bisulphate-modified region containing the putative methylated CpGs, whereas primers and probe of a reference gene (*ACTB*) specifically amplify a bisulphite-modified region not containing CpGs (sequences and annealing temperatures are provided in Supplementary Table S2). Leukocyte DNA from a healthy subject was methylated *in vitro* with excess SssI methyltransferase (New England Biolabs Inc.) to generate completely methylated DNA. Serial dilutions (90-0.009 ng) of this DNA were used to construct a calibration curve for the *ACTB* gene and for the genes of interest and were run parallel with each analysis. The calibration curves for the reference and the target genes were constructed by plotting the log [control *in vitro* methylated leukocyte DNA amount] to the Ct values and calculating the equation of the curve ($y = mx + q$ where m is the slope and q the intercept). The quantity of the unknown samples is then calculated as follows: log [unknown DNA amount] = (Ct values - intercept)/slope (Applied Biosystem User Bulletin 2). These procedures are automatically done on 7900 Sequence detection system by the SDS 2.21 software. The relative level of methylated DNA for each gene in each sample is determined as a ratio of the amount of the target gene to the amount of *ACTB* (reference gene) and then multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest/average value of triplicates of *ACTB* × 1,000).

Fluorogenic PCR was set up in 384-well plates on a 7900 Sequence detector (Applied Biosystems). Each plate included patient DNA samples, positive (*in vitro* methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls, and multiple water blanks. Amplification reactions were carried out in a volume of 20 μ L that contained 3 μ L bisulphite-modified DNA, 600 nmol/L forward and reverse primers, 200 nmol/L probe, 5 U of Platinum Taq polymerase (Invitrogen), 200 μ mol/L each of dATP, dCTP, dGTP, dTTP, and 5.5 mmol/L MgCl₂. Amplifications were carried out using the following conditions: one step at 95°C for 3 min, 50 cycles at 95°C for 15 s, and 60°C for 1 min. Supplementary Table S3 lists the nine genes whose promoters were examined, their proposed functions, and the tumors in which these promoters were shown to be hypermethylated.

Statistical Analysis. For each gene the median and IQR of methylation ratios were determined for each of the study groups. Nonparametric tests were used to analyze data samples because the Kolmogorov-Smirnov and the Shapiro tests led to rejection of the Gaussian distribution assumption for the variables analyzed. For each of the genes cutoff values were determined by drawing the Receiver Operating Characteristic (ROC) curve using methylation levels in normal breast tissues adjacent and distant from tumors ($n = 32$) and pathologic samples (ADH, DCIS, and IDC; $n = 138$). The area under the ROC curve, computed numerically and tested for statistical significance, was assumed as a measure of goodness of the test. Cutoff values were chosen among ROC curves points in order to optimize specificity while fixing a good value of sensitivity. Methylation frequencies were then calculated for each of the genes as the percentage of samples showing methylation levels equal or above the cutoff values.

For the analysis of methylation frequencies and levels, paired samples represented the training data set, whereas the extended data sample represented the validation data

set. Specific statistical models were implemented, because for the paired samples not all possible lesions were present in each patient. Methylation levels were analyzed by using a hierarchical mixed model with unstructured covariance matrix and robust residuals' estimation to account for nonsymmetric distribution (22). In a similar way methylation frequencies above cutoff values in the training data set were analyzed by using a Generalized Estimating Equations logistic model (23). Such type of modeling overcomes missing data values. In both approaches, the methylation levels and frequencies were treated as repeated measures, in the different tissues/lesions, within each subject. *P* values reported in Table 2 refer to the statistical significance of the hierarchical mixed model's coefficients and Generalized Estimating Equations logistic model's coefficients, respectively.

In the validation set the within-gene differences that were likely to exist between groups were checked using the Kruskal-Wallis test. We then applied the Dunn test for multiple comparisons to determine among which groups the previously found differences live. Such test allowed us to merge the sample groups among which non-statistical difference was discovered. Therefore, it was possible to carry out the comparison between preinvasive versus invasive and between NBT and pathological samples (Mann-Whitney test). Differences in methylation frequencies in the validation set were assessed using the χ^2 test. Because our validation set was not an independent data sample, *P* values of the Kruskal-Wallis, Dunn, Mann-Whitney, and χ^2 tests were not computed asymptotically but by adopting a permutation approach with 10,000 resamplings (24). At the first step the test statistics were computed, then at each of the 10,000 resamplings, the methylation data were randomly relabeled and the test statistics were computed for the new permuted dataset. At the final step, empirical *P* values were derived. These are shown on Table 3. Sensitivity and specificity to distinguish between normal and pathologic breast tissues were estimated with their 95% confidence intervals on the basis of cutoff values. Statistical analysis was done using R-project software and all tests were two-sided with significance at $P \leq 0.05$.

Results

Methylation Analysis of Synchronous Breast Lesions (Training Set). We determined the promoter methylation status of nine genes (*ESR1*, *CDH1*, *APC*, *TIMP-3*, *CTNNB1*, *GSTP1*, *MGMT*, *THBS1*, and *TMS1*) in 52 synchronous breast lesions, 12 paired NBAT and 20 unpaired NBAT (Table 2). Aberrant promoter methylation was detected in both normal tissues and primary tumor for genes *APC*, *CDH1*, *CTNNB1*, *ESR1*, *TIMP3*, and *GSTP1*.

Higher methylation levels were shown for *APC*, *CDH1*, *CTNNB1*, and *TIMP3* in pathologic samples as compared with NBAT (Table 2). Approximately 90% of the methylated cases showed *APC*, *CDH1*, and *CTNNB1* aberrant methylation in either all the sequentially analyzed samples or only in invasive phenotypes. A more heterogeneous pattern was seen for *ESR1*, *GSTP1*, and *TIMP3* (Supplementary Fig. S1). Only *CDH1* showed a statistically significant difference in methylation levels in DCIS as compared with IDC.

Table 2. Methylation levels and frequencies above cutoff values in paired normal breast tissue adjacent to tumor, atypical ductal hyperplasia, ductal carcinoma *in situ*, and invasive ductal carcinoma

Genes	NBAT (n = 12)	ADH (n = 24)	DCIS (n = 40)	IDC (n = 45)	P*								
					Multiple comparison [†]	NBAT vs PATH [‡]	NBAT vs ADH	NBAT vs DCIS	NBAT vs IDC	ADH vs DCIS	ADH vs IDC	DCIS vs IDC	PRE [†] vs IDC
APC	0 [‡] (0-0.04)	3.04 [‡] (0-34.0)	4.45 [‡] (0-416.71)	43.64 [‡] (0.14-640.02)	0.003	0.0009	0.06	0.007	0.02	0.31	0.08	0.21	0.12
	1 [§] (8)	8 [§] (33)	15 [§] (38)	24 [§] (53)	0.005	0.09	0.36	0.11	0.009	0.68	0.03	0.005	0.0009
CDH1	0 [‡] (0-3.19)	0 [‡] (0-28.84)	1.11 [‡] (0-10.09)	6.39 [‡] (0.38-38.18)	0.002	0.002	0.008	0.02	0.02	0.23	0.14	0.05	0.09
	1 [§] (8)	7 [§] (29)	12 [§] (31)	21 [§] (47)	0.01	0.10	0.21	0.19	0.03	0.88	0.07	0.005	0.005
CTNNB1	0 [‡] (0-0)	0 [‡] (0-0)	0 [‡] (0-1.45)	0.1 [‡] (0-3.14)	0.16	0.03	0.30	0.79	0.05	0.35	0.07	0.05	0.05
	1 [§] (8)	4 [§] (17)	6 [§] (15)	11 [§] (47)	0.38	0.42	0.59	0.60	0.21	0.96	0.31	0.24	0.15
ESR1	0 [‡] (0-15.2)	0 [‡] (0-9.67)	2.18 [‡] (0-16.22)	0.7 [‡] (0-14.2)	0.17	0.69	0.19	0.61	0.28	0.06	0.06	0.22	0.10
	2 [§] (17)	4 [§] (17)	10 [§] (26)	11 [§] (24)	0.82	0.84	0.79	0.58	0.68	0.47	0.40	0.99	0.49
TIMP3	0 [‡] (0-11.44)	1.65 [‡] (0-29.89)	0.44 [‡] (0-13.48)	2.33 [‡] (0-28.02)	0.17	0.03	0.36	0.98	0.07	0.37	0.19	0.07	0.11
	3 [§] (25)	9 [§] (38)	12 [§] (31)	17 [§] (38)	0.79	0.41	0.57	0.40	0.68	0.99	0.78	0.68	0.69
GSTPI	0 [‡] (0-3.73)	0 [‡] (0-0)	0 [‡] (0-0.54)	0 [‡] (0-1.46)	0.28	0.13	0.68	0.13	0.21	0.12	0.22	0.33	0.27
	2 [§] (17)	3 [§] (12)	7 [§] (18)	11 [§] (24)	0.54	0.91	0.67	0.73	0.59	0.36	0.23	0.70	0.24

*Upper P values refer to statistical analyses done using a hierarchical mixed model with unstructured covariance matrix; lower P values refer to statistical analysis done using a GEE logistic model.

[†]Multiple comparison among NBAT, ADH, DCIS, and IDC. PATH merges the ADH, DCIS, and IDC groups (n = 138); PRE merges the ADH and DCIS groups (n = 76).

[‡]Median value (Interquartile Range, IQR).

[§]Number of samples methylated above cutoff value (% of methylated samples).

Because methylation was detected in both normal and tumor tissues, methylation status was further analyzed by categorizing samples on the basis of cutoff values. No statistically significant differences in methylation levels were found between the NBAT and the NBDT groups; thus

they could be merged in the NBT group (Supplementary Table S4). Methylation levels detected in the NBT group together with data from all pathologic lesions (ADH, DCIS, and IDC) of the training set were used to draw ROC curves (Supplementary Fig. S2). Cutoff values were

Table 3. Methylation levels and frequencies above cut off values of the extended data sample in normal breast tissue, atypical ductal hyperplasia, ductal carcinoma *in situ*, and invasive ductal carcinoma in the extended data sample

Genes	NBT (n = 32)	ADH (n = 26)	DCIS (n = 50)	IDC (n = 62)	P*								
					Multiple comparison [†]	NBT vs PATH [‡]	NBAT vs ADH	NBT vs DCIS	NBT vs IDC	ADH vs DCIS	ADH vs IDC	DCIS vs IDC	PRE [†] vs IDC
APC	0 [‡] (0-0)	5.09 (0-372.23)	4.55 (0-466.0)	89.75 [‡] (0.09-561.50)	0.00001 [§]	0.00001	0.004	0.00001	0.00001	0.55	0.09	0.19	0.08
	3 ^{**} (9)	10 ^{**} (39)	22 ^{**} (44)	34 ^{**} (55)	0.0002	0.0002	0.007	0.0009	0.00009	0.80	0.2	0.22	0.13
CDH1	0 [‡] (0-1.43)	0 [‡] (0-21.37)	0 [‡] (0-10.64)	4.69 [‡] (0-28.44)	0.0005 [§]	0.0009	0.2	0.01	0.0003	0.35	0.03	0.15	0.04
	4 ^{**} (12)	7 ^{**} (27)	15 ^{**} (30)	26 ^{**} (42)	0.03	0.01	0.14	0.08	0.003	0.8	0.19	0.14	0.06
CTNNB1	0 [‡] (0-0)	0 [‡] (0-0)	0 [‡] (0-1.14)	0 [‡] (0-0.87)	0.08 [§]	0.05	0.66	0.1	0.02	0.29	0.09	0.45	0.2
	3 ^{**} (9)	4 ^{**} (15)	7 ^{**} (14)	11 (18)	0.75	0.34	0.72	0.65	0.64	0.29	0.77	0.14	0.55
ESR1	0 [‡] (0-15.48)	2.78 [‡] (0-11.40)	0.68 [‡] (0-14.01)	1.58 [‡] (0-14.21)	0.91 [§]	0.53	0.79	0.66	0.45	0.91	0.70	0.74	0.70
	8 ^{**} (25)	4 ^{**} (15)	12 ^{**} (24)	15 ^{**} (25)	0.95	0.81	0.75	0.59	0.79	0.66	0.72	0.72	0.81
TIMP3	0 [‡] (0-17.69)	2.62 [‡] (0-41.13)	0.23 [‡] (0-13.96)	1.64 [‡] (0-17.48)	0.63 [§]	0.48	0.23	0.86	0.56	0.26	0.46	0.66	0.88
	8 ^{**} (25)	11 ^{**} (42)	16 ^{**} (32)	20 ^{**} (33)	0.69	0.39	0.25	0.71	0.52	0.5	0.5	0.82	0.84
GSTPI	0 [‡] (0-0)	0 [‡] (0-0)	0 [‡] (0-0)	0 [‡] (0-15.24)	0.27 [§]	0.18	0.85	0.35	0.07	0.53	0.16	0.33	0.2
	3 ^{**} (9)	3 ^{**} (11)	8 ^{**} (16)	14 ^{**} (23)	0.31	0.22	0.68	0.41	0.08	0.73	0.24	0.33	0.23

*Upper P values for each gene refer to Kruskal Wallis, Mann Whitney, and Dunn tests as indicated. Lower P values for each gene refer to χ^2 square test; all statistics were computed by adopting a permutation approach with 10,000 resamplings (see Materials and Method).

[†]Multiple comparison among NBT, ADH, DCIS, and IDC; PATH merges the ADH, DCIS, and IDC groups (n = 138); PRE merges the ADH and DCIS groups (n = 76).

[‡]Median value (IQR).

[§]Kruskal Wallis.

^{||}Mann Whitney.

^{||}Dunn.

**No. of samples methylated above cutoff value (% of methylated samples).

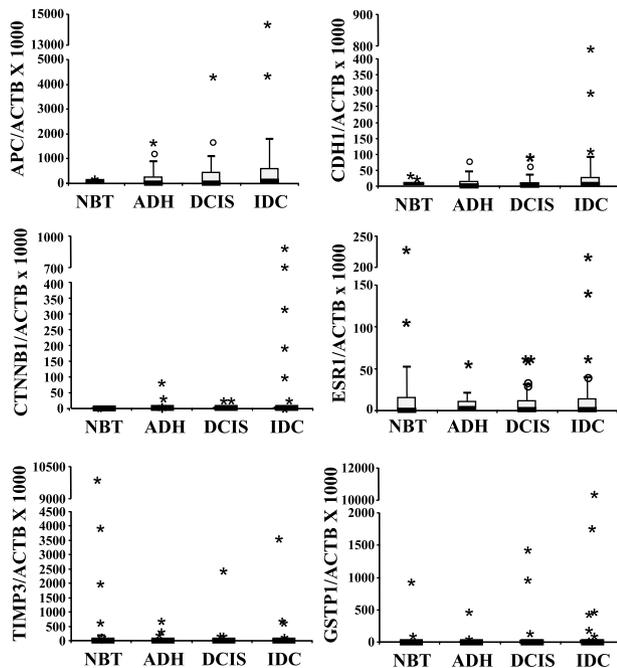


Figure 1. Promoter methylation levels for *APC*, *CDH1*, and *CTNNB1*, *TIMP3*, *ESRI*, and *GSTP1* promoter regions in NBT ($n = 32$), preinvasive lesions ADH ($n = 26$), DCIS ($n = 50$), and IDC ($n = 62$). Methylation levels are expressed as the logarithm ratio of the quantity mean value of the target gene to the quantity mean value of the reference gene (*ACTB*) multiplied by 1,000. Boxes, interquartile range (interval between the 25th and 75th percentile); lines inside boxes, median values; whiskers, interval between the 10th and 90th percentiles; empty circle, outliers values between 1.5 and 3 length upper or down from the interquartile range. *, extreme cases with more than three boxes' length upper or down from the interquartile range.

then chosen on the basis of the ROC curves to optimize specificity while fixing a good value of sensitivity in distinguishing between normal breast tissues and pathologic samples, and were as follows: 22.63 for *APC*, 7.6 for

CDH1, 2.94 for *CTNNB1*, 8.34 for *TIMP3*, 14.3 for *ESRI*, and 6.47 for *GSTP1*.

APC, *CDH1*, and *CTNNB1* showed higher frequencies of methylation in invasive lesions as compared with pre-invasive lesions (Table 2). When DCIS and IDC were compared, a statistically significant difference in methylation frequencies was found for *APC* and *CDH1* but not for *CTNNB1* and *TIMP3* (Table 2).

Methylation Analysis of the Extended Data Sample (Validation Set). To confirm and extend results obtained from the analysis of synchronous lesions, additional pure lesions from 24 patients (Table 1) were analyzed by quantitative methyl specific PCR for promoter methylation of *ESRI*, *CDH1*, *APC*, *TIMP3*, *CTNNB1*, and *GSTP1*. Results from this analysis were merged with data obtained from synchronous lesions and analyzed by using a permutation analysis approach (see Materials and Methods). In total, 32 NBT, 26 ADH, 50 DCIS, and 62 IDC were studied, and the results are shown on Table 3.

The comparison analysis among the NBT, ADH, DCIS, and IDC groups confirmed the existence of differences in methylation levels and frequencies for *APC* and *CDH1* and only changes in methylation levels for *CTNNB1* (Table 3, Fig. 1, Supplementary Fig. S3). No association was found for *TIMP3* either for methylation frequencies or levels. *APC* showed differences in methylation levels for all three pathologic sample groups, *CDH1* showed significant differences in DCIS and IDC, and *CTNNB1* only in IDC (Table 3, Supplementary Fig. S4). No differences were detected between ADH and DCIS, thus the two groups were merged (PRE) and compared with invasive lesions (Table 3). Only *CDH1* showed significant differences in methylation levels in IDC as compared with pre-invasive lesions.

Simultaneous analysis of *APC*, *CDH1*, and *CTNNB1* loci allowed the identification of pathologic samples with a specificity of 75% (95% confidence interval, 69-81) and a sensitivity of 67% (95% confidence interval, 60-74). The addition of *ESRI*, *TIMP3*, and *GSTP1* slightly increases sensitivity but significantly reduces specificity (Table 4).

Correlation of Promoter Aberrant Methylation in Primary Tumors with Clinicopathologic Parameters. In tumor samples no correlations were found between *APC*,

Table 4. Sensitivity and specificity of promoter hypermethylation analysis in distinguish between normal breast and pathologic samples

Gene aberrantly methylated	Sensitivity TP/TP+FN % (95% CI)	Specificity TN/FP+TN % (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
<i>APC</i>	53 (45-60)	91 (88-95)	96 (93-99)	29 (22-36)
<i>CDH1</i>	35 (28-42)	88 (82-92)	88 (83-93)	20 (14-26)
<i>CTNNB1</i>	22 (16-28)	91 (87-95)	92 (88-96)	24 (18-30)
<i>ESRI</i>	24 (17-31)	73 (65-81)	82 (68-82)	22 (15-30)
<i>TIMP3</i>	35 (27-43)	73 (65-81)	75 (75-88)	25 (18-32)
<i>GSTP1</i>	19 (12-26)	90 (85-95)	86 (80-92)	26 (19-34)
<i>MGMT</i>	N/A	N/A	N/A	N/A
<i>THBS1</i>	N/A	N/A	N/A	N/A
<i>TMS1</i>	N/A	N/A	N/A	N/A
At least one of <i>APC</i> , <i>CDH1</i> and <i>CTNNB1</i>	67 (60-74)	75 (69-81)	92 (88-96)	35 (28-42)
At least one of <i>APC</i> , <i>CDH1</i> , <i>CTNNB1</i> , <i>TIMP3</i> , <i>ESRI</i> and <i>GSTP1</i>	69 (62-76)	47 (40-54)	87 (82-91)	23 (17-29)

Abbreviations: 95% CI, 95% confidence Interval; N/A, not applicable; TP, true positive; FN, false negative, TN, true negative.

CDH1, and *CTNNB1* methylation level and protein levels (Estrogen Receptor and Progesteron Receptor status and Mib1/Ki67) or standard clinical/pathologic (age, stage, grade, lymph node status) parameters (data not shown).

Discussion

We investigated the pattern of methylation in a series of preinvasive and invasive breast lesions. Due to the limited amount of DNA in preinvasive lesions we were not able to test all the relevant genes that show significant alterations in breast cancer. Thus, we chose to analyze those genes that were more likely to be involved in progression from preinvasive to invasive phenotype (Supplementary Table S3). Of the nine genes tested, only *APC*, *CDH1*, and *CTNNB1* showed a different methylation pattern in normal breast tissues and pathologic samples. In synchronous lesions NBAT, ADH, and DCIS showed similar methylation patterns for *APC* and *CTNNB1*, whereas statistically significant differences were seen in ADH and DCIS as compared with NBAT for *CDH1*. Similar levels of *CDH1* methylation were detected for ADH as compared with DCIS (Table 2). Although these results may be due to the panel of genes analyzed, they are in agreement with immunophenotypic and genetic data and suggest that atypical hyperplasia should be considered as a well-differentiated or simply small *in situ* carcinoma (4).

The *APC* promoter was methylated above the cutoff value in approximately half of preinvasive and invasive lesions. An increase in methylation levels was also seen between the two pathologic groups but the difference was not statistically significant. *CDH1* was methylated in 30% of the preinvasive lesions and in 42% of the invasive tumors, and methylation levels were significantly higher in IDC as compared with preinvasive lesions.

Although *APC* and *CDH1* methylation were previously reported in approximately 40% to 50% of breast cancers (9, 16, 17, 25-27), this is the first report of *CTNNB1* methylation in breast cancer. *CTNNB1* promoter methylation was recently reported in gastric and endometrial cancers (16, 17). In a series of primary metastatic gastric cancer and cell lines derived from metastasis, loss of expression of β -catenin was related to promoter methylation (16). Sequence analysis of the bisulphate-treated DNA showed heavy CpG methylation of the *CTNNB1* promoter region in a cell line with absent β -catenin expression, and treatment with 5-deoxyazacytidine was able to restore β -catenin expression (16). Moreover, Whitcomb et al. (17) reported methylation at the *CTNNB1* promoter region in 17% of primary endometrial tumors. In mammary tumors, loss of β -catenin expression was shown by immunohistochemistry in both lobular and ductal breast carcinomas (25, 28, 29).

Interestingly, *apc*, *cdh1* and β -catenin play an interaction role in the maintenance of cell-to-cell adhesion, and regulation of cell extracellular matrix interactions (30, 31). The E-cadherin is mainly expressed in epithelial tissues and in normal breast prevalently in the luminal cells. In developmental animal models, E-cadherin expression is temporarily down-regulated when budding lobules invade the stroma (32). Although the cell-to-cell adhesion properties of E-cadherin reside in the extracellular domain, its function depends on the interaction

with catenins and in particular β -catenin, which is responsible for the anchorage of E-cadherin to the actin cytoskeleton (31). The *apc* protein participates in the multiprotein complex involved in the phosphorylation and subsequent ubiquitination of β -catenin (30). Moreover, *APC* terminal C-region can interact with cytoskeleton proteins, contributing to cell migration, proliferation, and adhesion (30). A role in "invasion suppression" for *apc*, *cdh1* and β -catenin would be consistent with our results showing changes in promoter methylation in the switch from preinvasive lesions to invasive breast cancer. We can hypothesize that *APC* and *CTNNB1* methylation would be an early event during breast cancer development, correlated with abnormal proliferation of the breast epithelia. *CDH1* methylation would occur later and is likely to play a more direct role in the loss of cell-to-cell adhesion and the acquisition of invasive properties by the cancer cells. *cdh1* is involved in maintaining cell-to-cell adhesion and is regarded as suppressor of cellular invasion (33). Furthermore, loss of *cdh1* expression in primary tumors has been associated with decreased patient survival (34). Loss of E-cadherin protein expression is most frequent for infiltrating lobular tumor types and is often a biallelic event resulting from any combination of gene promoter hypermethylation, mutation, or allelic loss, whereas ductal histology often presents with varying levels of expression (35, 36). Interestingly, Shinozaka et al. (37) also found high levels of *CDH1* methylation in breast carcinomas of the ductal type. In their study methylation correlated with mRNA expression levels and was more frequent in primary tumors with a more aggressive phenotype. These data are consistent with our findings that methylation level of *CDH1* is significantly higher in IDC as compared with preinvasive lesions and suggest that *CDH1* methylation may represent an important step in the progression of breast carcinomas of the ductal type.

TIMP3, *ESR1*, and *GSTP1* methylation frequency and levels were similar in normal and neoplastic breast tissues from cancer patients. Hu et al. (38) investigated, with a whole genome analysis approach, methylation patterns in epithelial and myoepithelial cells, stromal fibroblasts from normal breast tissue, and *in situ* and invasive breast carcinomas. The analysis showed distinct epigenetic changes in all types of cells, with some genes differentially methylated in normal and neoplastic samples and others showing the same methylation pattern in cells derived from normal and neoplastic tissues (39). Thus, our results could be explained by the presence of similar levels of methylation of *TIMP3*, *ESR1*, and *GSTP1* in cells from normal and cancer specimens.

The detection of breast cancer in the early stages is a key for a successful treatment of the disease. DNA methylation has an advantage over other molecular detection methods (e.g. single gene mutation, microsatellite analysis,) because it can be detected with a very high degree of specificity even in the presence of an excess of unmethylated DNA. A number of studies have reported the ability to detect breast cancer cells by DNA methylation analysis in fine needle aspirations (nipple aspirates and ductal lavages) with variable sensitivity (40-44). We showed that the determination of methylation status of *APC*, *CDH1*, and *CTNNB1* is able to distinguish between normal breast and pathologic samples with a specificity of 75% and a sensitivity of

67%. Thus, it is likely that the combined detection of methylation of *APC*, *CDH1*, and *CTNNB1* could be more informative than other methylation markers in identifying cancer cells in cytologic specimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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