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Hypermethylation in Histologically Distinct Classes of Breast Cancer

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ABSTRACT

Purpose: **A number of different genes are known to be inactivated by aberrant hypermethylation in breast cancer, but it is still unknown to what extent these epigenetic alterations differ according to specific breast cancer phenotypes. We sought to determine whether the extent of hypermethylation or defined profiles of gene hypermethylation are associated with biological characteristics of breast cancers.**

Experimental Design: **We evaluated methylation status of 12 different genes in a series of 109 invasive breast tumors, representing the ductal, lobular, and mucinous histologic subtypes using methylation-specific PCR. Frequencies of methylation were compared across the recognized histologic classes, and multivariate techniques (latent class analysis, factor analysis, recursive partitioning, and hierarchical clustering) were used to seek patterns of methylation for individual genes that distinguish recognized histologic types of breast cancer or define breast cancer phenotypes on a molecular level.**

Results: **All 109 cases studied have aberrant methylation of multiple genes (3 to 10 genes per case), demonstrating that gene hypermethylation is pervasive in breast cancer. Lobular cancers and mucinous cancers, which often have relatively low levels of chromosomal changes, have higher overall frequencies of hypermethylation than ductal cancers (49% in lobular and mucinous *versus* 40% in ductal), but there is a relatively unimodal distribution of methylation frequency for all three histologic types. Only one of the individual genes studied, BRCA1, has a variable frequency of methylation that is significantly dependent on histologic pattern of tumor growth, with a higher frequency of methylation in mucinous cancers than ductal or lobular cancers. Although some trends of histology-specific gene methylation were seen, methylation patterns could not definitively classify breast cancers according to histologic type.**

Conclusions: **Although a more comprehensive hypermethylation profile could potentially be useful for breast cancer classification and understanding the biology of this disease, it appears that the hypermethylation patterns across various forms of breast cancer are less distinct than those between breast cancer and cancers of different tissue origins. Furthermore, the relatively unimodal distribution of methylation frequency for all three histologic types does not support there being a distinct CpG island methylator phenotype for breast cancer.**

INTRODUCTION

Aberrant methylation of CpG islands in gene promoters is a common mechanism for suppressing gene expression in cancer cells. For breast cancer, genes previously reported to be hypermethylated include several genes involved in DNA repair (*BRCA1* and *GSTP1*; refs. 1, 2), cell cycle regulation (*p16^{INK4A}* and *cyclin D2*; refs. 3, 4), cell adhesion (*E-cadherin*; refs. 5, 6), hormone and receptor-mediated cell signaling (*ER*, *RAR*β, and *THR*β; refs. 7–9), regulation of cell transcription (*HOX5A*; ref. 10), or other functions (*RASSF1A*, *Twist*, and *HIN1*; refs. 11–13). Thus, aberrant hypermethylation appears to contribute significantly to the malignant phenotype of breast cancer.

Two important issues not previously addressed by studies of methylation of individual genes in breast cancer are the following: (*a*) whether gene-specific patterns of methylation can distinguish breast cancer phenotypes; and (*b*) whether there is a CpG island methylator phenotype for breast cancer. Both of these possibilities might be expected based on methylation patterns that have been observed in other types of human cancers. For example, unique profiles of methylation for 12 different genes have been found to distinguish 15 different types of human cancer (14), leading us to question whether subsets of breast cancer, which is biologically heterogeneous, could be distinguished in a similar manner. In addition, a distinctively high frequency of methylation has been described for a subset of colorectal cancers (15, 16), leading us to consider the possibility of a similar CpG island methylator phenotype for breast cancer.

In the present study, we addressed these issues, using a candidate gene approach to measure the frequency of aberrant methylation for 12 different genes in a series of 109 different primary breast cancers by methylation-specific PCR (MSP; ref. 17). We included 10 genes that are commonly methylated in breast cancer (*RASSF1A*, *HIN1*, *Twist*, *cyclin D2*, *RAR*β, *THR*β, *E-cadherin*, *ER*, *BRCA1*, and *GSTP1*), as well as two genes that are infrequently methylated in breast cancer (*BAX* and *RB*). By analyzing these genes across a spectrum of breast cancers,

Received 4/6/04; revised 6/8/04; accepted 6/10/04.

Grant support: Korea Science & Engineering Foundation, the National Cancer Institute (Breast Cancer Specialized Programs of Research Excellence Program), and the Department of Defense Breast Cancer Research Program.

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Table 1 Characteristics of breast cancer patients

	Ductal	Mucinous	Lobular
Patient ages (y)			
Mean	50.6	58	56
Range	29–86	40–90	29–83
Tumor grade			
Grade 1	2	14	2
Grade 2	35	15	14
Grade 3	23	1	3
Tumor size			
T ≤ 2 cm	25	16	2
2 cm < T ≤ 5 cm	28	13	14
T > 5 cm	7	1	3
Lymph node metastases			
Positive	32	4	6
Negative	28	26	13

representing well-defined histologic variants, we are able to determine a distribution of methylation frequency for each of these classes of breast cancer. Analysis of a sample of this scale should allow detection of a hypermethylator phenotype, if one exists for breast cancer. Furthermore, analysis of multiple genes for aberrant methylation in a large series of breast cancer cases also allows us to detect nonrandom associations of methylation patterns for different genes, as well as possible shared patterns of methylation that could be used for breast cancer classification.

MATERIALS AND METHODS

Tissue Samples and DNA Isolation. A total of 109 human breast tumor samples was obtained from the Department of Pathology at Johns Hopkins Hospital (Baltimore, MD; n = 57) and from the Department of Pathology at Yeungnam University College of Medicine (Daegu, South Korea; n = 52). All samples were collected in accordance with institutional guidelines for protection of human subjects. These samples consist of 60 invasive ductal cancers (30 Americans and 30 Koreans), 30 mucinous cancers (14 Americans and 16 Koreans), and 19 invasive lobular cancers (13 Americans and 6 Koreans). The patient age, tumor size, tumor grade, and lymph node status for these cancers are summarized in Table 1. Normal breast tissues (n = 8) were obtained from reduction mammoplasty specimens at the Johns Hopkins Hospital from patients of ages 23 to 59 years, and at least four normal breast samples were tested for each of the genes tested in this study. All samples were examined microscopically, and only portions of samples that consisted of at least 75% cancer (or, in the case of controls, normal breast ductal structures) were used in experiments.

DNA extractions from fresh frozen tissues (all 60 ductal cancers, 6 of 30 mucinous cancers, and 5 of 19 lobular cancers) and cancer cell lines were performed using QIAamp Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. We isolated DNA from formalin-fixed, paraffin-embedded tissue sections of the remaining 24 mucinous and 14 lobular cancers by standard methods, using proteinase K digestion and phenol-chloroform extraction. (The use of paraffin-embedded tissue was necessary for these cases because of inadequate numbers of frozen samples available for these histologies.) Although the somewhat poorer quality of DNA from paraffin-embedded tissues affected the success rate of the MSP reactions, we tested several cases where DNA was available from both paraffin sections and fresh frozen sources and confirmed that results from both sets of reactions were identical.

Table 2 PCR primers used for MSP reactions of specific genes analyzed in this study

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)	Annealing temperature (°C)
RARβ	M: GAACGCGAGCGATTCGAGT	M: GACCAATCCAACCGAAACG	142	56
	U: GGATTGGGATGTTGAGAATGT	U: CAACCAATCCAACCAAAACAA	158	56
Cyclin D2	M: GGGTCGATCGTGTTGGCG	M: ACATAAAACCTCCACGCTCG	124	60
	U: GAAATATATTAAAGGGTGTG	U: AAAATCCACCAACACAATCA	147	52
Twist	M: TTTCGGATGGGGTTGTTATC	M: AAACGACCTAACCCGAACG	200	56
	U: TTTGGATGGGGTTGTTATTGT	U: CCTAACCCAAACAACCAACC	193	56
ER	M: GTGTATTTGGATAGTAGTAAGTTCGTC	M: CGTAAAAAAAACCGATCTAACCG	118	55
	U: GGTGTATTTGGATAGTAGTAAGTTTGT	U: CCATAAAAAAAACCAATCTAACCA	120	55
E-Cadherin	M: TATCGCGTTTATGCGAGGTC	M: CAAATAAACCCCGAAACACC	139	56
	U: TTAATTAGTGGTATGGGGGGT	U: ATCAAATCAAACCAAACTAAAACA	147	56
BRCA1	M: GGTTAATTTAGAGTTTCGAGAGACG	M: CTATAATTCCCGCGCTTTTCC	143	58
	U: GTGGTTAATTTAGAGTTTTGAGAGATG	U: CCACACTTTTCCATTACCACA	136	58
THRβ	M: GGTAATTTGGTTAGAGGATCGCGC	M: CACCCCTCCGATTCTTACGACG	119	56
	U: TATTGGTAATTTGGTTAGAGGATTGTGT	U: CACACCCCTCCAATTCTTACAACA	125	56
GST-P1	M: TTCGGGGTGTAGCGGCGTC	M: GCCCCAATACTAAATCACGACG	89	59
	U: GATGTTTGGGGTGTAGTGGTGTT	U: CCACCCCAATACTAAATCACAACA	94	59
HIN-1	M: GGTACGGGTTTTTTACGGTTCGTC	M: AACTTCTTATACCCGATCCTCG3	136	56
	U: GGTATGGGTTTTTTATGGTTTGTT	U: CAAAACTTCTTATACCCAATCCTCA	136	56
RASSF1A	M: GTTGGTATTCGTTGGGCGC	M: GCACCACGTATACGTAACG	160	56
	U: GGTTGTATTTGGTTGGAGTG	U: CTACAAACCTTTACACACAACA	180	56
BAX	M: GAGGTAGGTGCGGTTACGTG	M: AATCACGTAAAAACCCCGCT	102	57
	U: GGTGTTGTGGGGTAGTGGTT	U: ACCACCTCTCACCAAATCCA	118	57
RB	M: TAGAAGTACGTTCGGGTCGC	M: CGATACGCGAACTAAAACGC	106	57
	U: TTTTGTGGTTGGATGTGGTG	U: ATCCTCTCCCAACTCCCATT	104	57

Abbreviations: M, methylated primers; U, unmethylated primers.

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Sodium Bisulfite Treatment and MSP. Analysis of the methylation status was studied by sodium bisulfite modification of DNA and subsequent MSP as described previously (17). Briefly, 1 μg of purified genomic DNA or 50 μL of DNA extract were denatured by NaOH and modified by sodium bisulfite. DNA samples were purified using Microcon YM-30 centrifugal filter devices (Millipore) according to manufacturer's instructions, again treated with NaOH, precipitated with ethanol, and resuspended in water. PCR reactions were then performed in parallel using primers specific for either methylated or the modified unmethylated DNA, and these PCR products were separated by agarose gel electrophoresis. Primer pairs (listed in Table 2) were purchased from Invitrogen. These primer sequences were selected based on previously published data for methylation of these genes in breast cancer, except for *BAX* and *RB*. For these two genes, at least four different combinations of methylation-specific primers were tested to interrogate the CpG island of the gene promoter region. Results are shown for the primer sets selected as most sensitive.

Human MDA-MB-231 and MCF-7 breast cancer lines, normal breast DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs), and DNA from peripheral blood mononuclear cells of apparently healthy individuals were used as controls for methylated or unmethylated reactions. Blank controls (*i.e.,* without DNA) were also amplified with each series of PCR reactions. PCR products (10 μL) were loaded on 2% agarose gel mixed with GelStar nucleic acid stain solution (Cambrex). Polymerized gels were then imaged directly on a Typhoon scanner (Amersham BioSciences) to detect fluorescent bands.

Statistical Analysis. Comparisons of proportions (of samples with methylation) across cancer types were performed using Fisher's exact test, and exact confidence intervals were calculated for proportions. Some cases had limited quantities or marginal quality of DNA, resulting in the inability to complete assays for all genes (a total of 66 of 1208 attempted assays). For decision-based analyses, these missing values of methylation status were substituted with the calculated overall prevalence of methylation of that particular gene. Imputing values allow us to use samples in our analysis that have one or more missing values in a conservative way (*i.e.,* our inferences will be biased toward the null) while considering that methylation of some genes are more prevalent than others. After these adjustments to the data, we summed the methylation values for each sample, obtaining the methylation frequencies. Randomized permutations for missing reactions were also tested and found not to affect conclusions. *t* tests and Wilcoxon rank-sum tests were used for testing significance of differential methylation frequencies of genes among different groups. Two-sided tests were used, with the α level set to 0.05. To compare the distribution of methylation frequency across cancer types, smooth density plots were estimated and rescaled.

To detect possible common patterns of methylation related to cancer histology, a latent class analysis (18) was performed. The latent class model approach, which is similar to a k-means clustering, is useful for analysis of binary data. This model was fit using a Bayesian marker chain Monte Carlo approach using WinBugs software, assuming two and three classes (19). Hierarchical clustering of genes and samples was performed using GeneCluster (20). The similarity metric used was a centered correlation because although the data are binary, Pearson correlation is still a valid measure of similarity. Recursive partitioning and principal components analyses were performed using the statistical package R. For both of these approaches, all genes were included in the analyses.

RESULTS

Prevalence of Methylation in Different Histologic Patterns of Breast Cancer. A total of 12 gene promoter regions was analyzed for methylation of CpG islands in 109 cases of infiltrating breast cancer (including 30 cases of mucinous cancer, 19 cases of lobular cancer, and 60 cases of ductal cancer) and in normal breast tissues from 8 reduction mammoplasty specimens. All of the breast cancer cases had hypermethylation of multiple genes (at least three), whereas in normal breast tissues, methylation of these genes was undetectable or was observed at levels significantly lower than those scored as positive in the tumor samples. These results demonstrate the

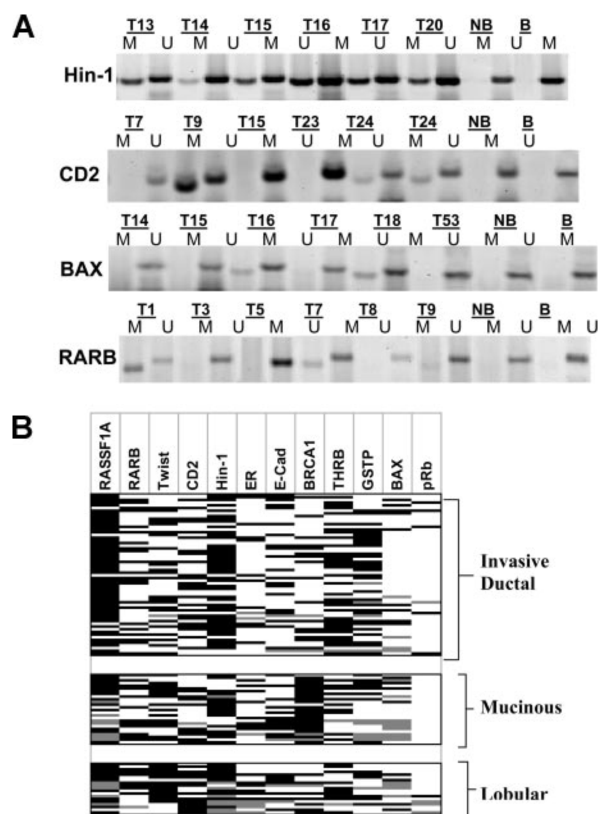

Fig. 1 Methylation of genes in breast cancer. In *A*, representative MSP results are shown for three genes (*Hin-1*, *RAR*β, and *Cyclin D2*) in breast cancer samples (T), normal breast (NB), and peripheral blood leukocytes (B). Lanes labeled with "M" represent reactions using primers specific for bisulfite-treated DNA product with methylated CpG sites, and lanes labeled "U" represent reactions using primers specific for DNA products with unmethylated CpG sites. In *B*, all data for 109 breast cancers is summarized. Breast cancer cases are separated by histologic diagnoses (ductal, lobular, or mucinous). ■ designate methylated genes, □ designate unmethylated genes, and designate unsuccessful or inconclusive reactions.

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pervasiveness of aberrant CpG island methylation (*i.e.,* hypermethylation) in breast cancer.

Among the genes tested, those most frequently methylated in breast cancer are *RASSF1A* (85% of cases) and *HIN1* (73% of cases). *BAX* (19% of cases) and *RB* (6% of cases), two genes that have not been previously reported to be methylated in breast cancer, are least frequently methylated among the 12 genes selected. Examples of MSP results and a summary of methylation for all genes in all cases of cancer are shown in Fig. 1.

Examining the frequency distribution for numbers of genes methylated in each case allows us to determine whether the three histologic variants have different levels of methylation and whether there is any evidence for a subset of breast cancers with distinctly high frequencies of gene hypermethylation. As shown in Fig. 2, the mean frequency of methylation is significantly greater for mucinous and lobular cancers than for ductal cancers (40% in ductal *versus* 49% in the other two groups; P testing that the means are different = 0.01). However, as seen in Fig. 2, there is significant overlap of these distributions, and the overall level of methylation will probably not be useful as a classification tool. The distribution in numbers of genes methylated per case has a relatively normal, unimodal distribution for each of the three histologic categories of breast cancer, suggesting that there is not a distinct CpG island methylator phenotype in breast cancer.

We also compared the frequency of methylation for breast cancers from American and Korean women, two demographically distinct populations and found no significant differences with regard to overall frequency of gene methylation or frequency of methylation for any specific gene. Furthermore, we found no correlation between overall methylation frequency or gene-specific methylation and patient age, tumor size, or lymph node status (data not shown).

Gene-Specific Patterns of Methylation for Classification of Breast Cancer. We next examined gene-specific patterns of methylation in the three histologic types of breast cancer to determine in principle whether these patterns could be useful for the molecular classification of these cancers. Only one gene, *BRCA1*, showed a significantly different frequency of methylation among the different histologic types of breast cancer (Fig. 3). Twenty-two of the 24 cases of mucinous cancer (92%) studied had methylation of BRCA1, whereas only 7 of 18 (39%) of lobular cancers and 16 of 58 (28%) ductal cancers had methylation of this gene ($P < 0.001$). This finding corroborates previous observations of high frequencies of *BRCA1* methylation in mucinous cancers made with a smaller number of cases (2), and collectively, these data suggest that *BRCA1* methylation is characteristic of mucinous breast cancer.

The finding of differential frequencies of methylation among different histologic forms of breast cancer for this one gene (BRCA1) provides an indication that gene-specific patterns of methylation could provide a basis for breast cancer classification. To further explore the possible use of methylation patterns for breast cancer classification, we tested decision-based analysis methods for ability to (*a*) recognize gene methylation patterns that segregate cancers according to well-defined histologic categories and (*b*) define different classes of cancer that cannot be readily defined by histologic appearance.

The application of divisive and agglomerative hierarchical clustering methods led to multiple small clusters of cases that had no readily apparent biological similarities such as common histologic growth patterns (results not shown). Latent class analyses of the methylation markers, where we assumed two or three underlying classes of cancer, did not produce classes that were well separated, and the classes did not appear to be related to histology. This is not surprising because of the moderate sample size (*i.e.,* latent class analyses usually require relatively large numbers of samples to produce meaningful results). Principal components analysis and factor analysis were also performed to see if one or a few factors would show differences across histologic subtypes, but discriminatory ability was poor and percentage of variance explained by the first four factors was low (53%). Lastly, a classification tree was applied to the data using all 11 genes. This approach was moderately successful, with 70% of samples correctly classified based on the best fitting algorithm.

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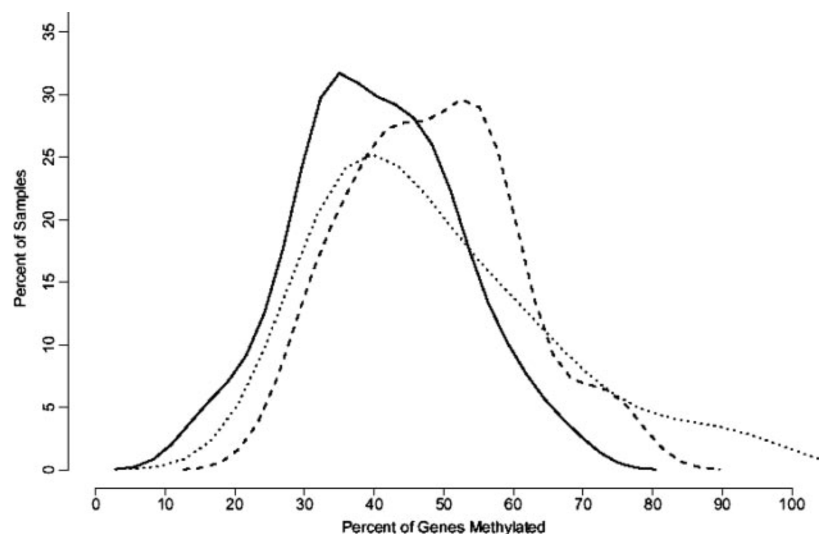

Fig. 2 Distribution of genes methylated per case (expressed as a percentage). Separate graphs are plotted for ductal breast cancer (*solid line*), lobular cancer (*line with long dashes*), and mucinous cancer (*line with short dashes*).

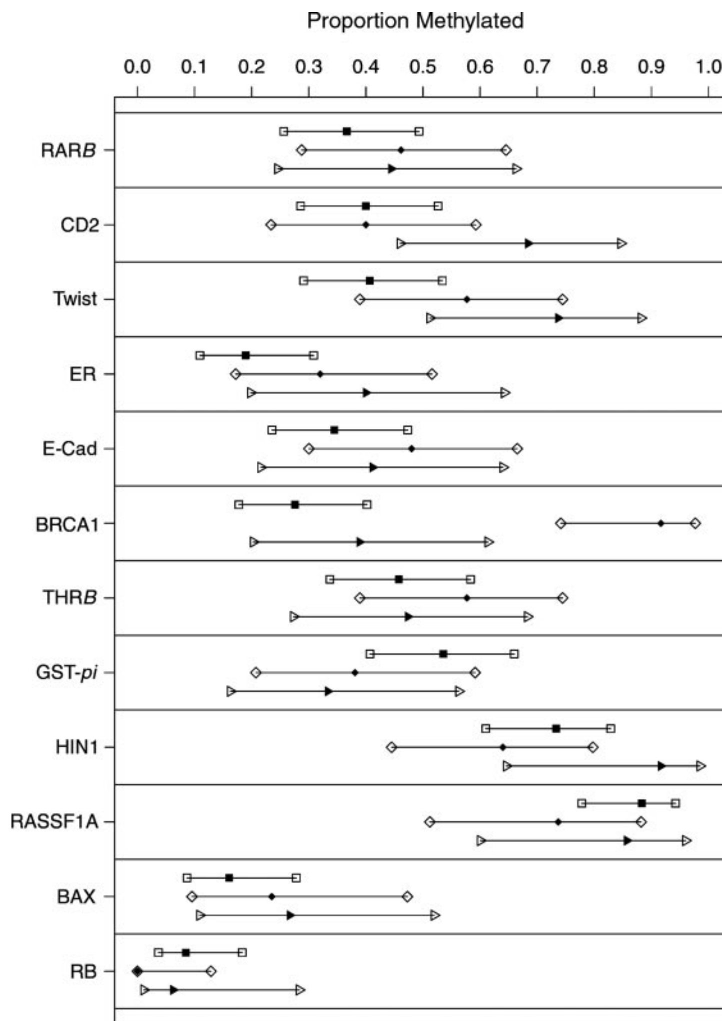

Fig. 3 Gene-specific patterns of methylation in different histologic types of breast cancers. Ductal cancers are designated by *squares*, mucinous cancers by *diamonds*, and lobular cancers by *triangles*. *Vertical bars* are used to designate means and SDs.

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Correlations among Different Genes for Methylation in Breast Cancers. Recognizing strong associations in patterns of molecular alterations among various genes could provide important clues for recognizing functional relationships between specific genes. In addition, recognizing sets of genes that are methylated in concert has implications with regard to the use of sequence-specific methylation for detection of cancer. For example, genes that are mutually hypermethylated at a high frequency are likely to be redundant as markers for detection of cancer-specific hypermethylation, whereas genes that are reciprocally methylated in cancers could be used effectively in combination for cancer detection. Using hierarchical clustering, we identified significant similarity in the case-specific patterns of methylation for genes as shown in Fig. 4. *RAR*β and *twist* and *GSTP1* and *THR*β most notably show similarity of methylation patterns.

DISCUSSION

To broadly examine the frequency and distribution of gene hypermethylation in breast cancer, we analyzed candidate genes with MSP, a method that measures alleles with specific methylation patterns. For this technique, primers are designed to amplify sequences that are either completely methylated at a series of CpG sites (typically three to five) or unmethylated at all CpG sites. Although alleles with dense methylation, involving all of the critical CpG sites, generate positive reactions, this method does not measure alleles that are methylated at only a portion of the critical sites of the primer sequences. Thus, MSP undoubtedly underestimates the extent of hypermethylation for a gene in any particular sample. Although this method is not quantitative, this approach is effective for surveying the spectrum of aberrant methylation in breast cancer and determining to what extent these epigenetic alterations might characterize specific breast cancer phenotypes.

Remarkably, our study of gene hypermethylation in breast cancer included a number of mucinous cancers, which have significantly fewer chromosomal alterations than ductal breast cancer (21) and lobular cancers, which also tend to have fewer chromosomal alterations than ductal cancers (22, 23). Mucinous cancers and lobular cancers have slightly (but significantly) higher overall frequencies of methylation, suggesting that levels of epigenetic alterations may be reciprocal to the levels of levels

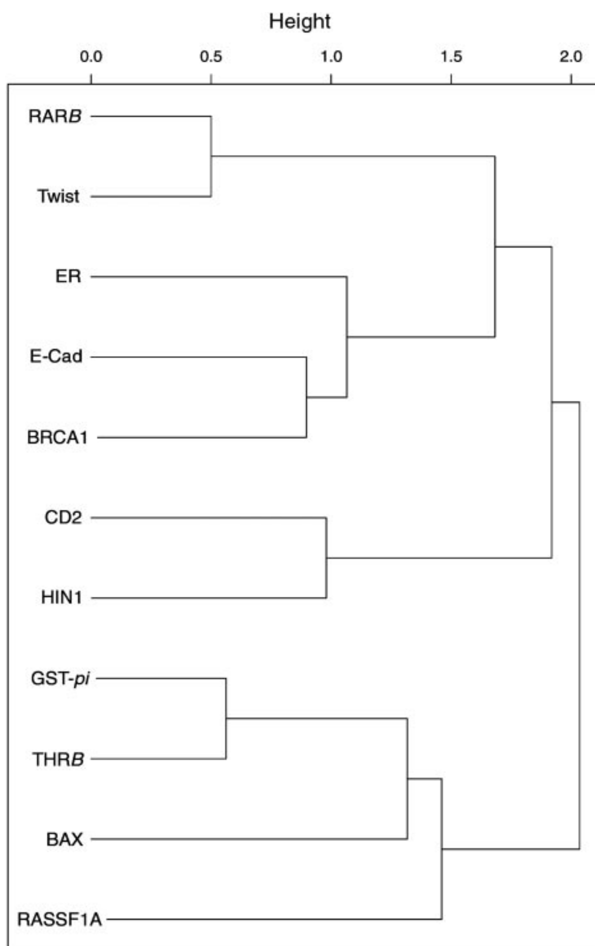

Fig. 4 Dendogram demonstrating clustering of genes according to patterns of methylation. Odds ratios for coordinate patterns of methylation were calculated using data for all 109 breast cancers analyzed, and dendrogram was generated using agglomerative clustering.

of genomic structural changes among the various types of breast cancer.

One major question addressed in this project concerns the possibility of a CpG island methylator phenotype for breast cancer. It has been previously reported that a distinctive subset of colorectal cancers with microsatellite instability have higher frequencies of concordant methylation of specific genes such as p16, thrombospondin-1, insulin-like growth factor II, hypermethylated in cancer-1, and T-type calcium channel gene (*CACNA1G*; refs. 15, 16). These cancers also have fewer chromosome structural alterations and lack the chromosomal instability seen in most colorectal cancers but characteristically have microsatellite instability associated with methylation of *MLH1* (15, 24). However, this concept of a methylator phenotype of colorectal cancer has been recently challenged by data showing a continuous distribution pattern of CpG island somatic hypermethylation for six different genes in colorectal cancers, including cases with microsatellite instability (25).

Although types of breast cancer that have relatively low levels of chromosomal alterations (*i.e.,* mucinous and lobular cancers) also have somewhat higher frequencies of gene methylation, we also find that the distribution and the magnitude of differences do not warrant a designation of CpG island methylator phenotype for any subset of breast cancer. In fact, the normal Gaussian distribution of methylation frequency for these 12 genes argues against such a phenotype.

Another question that we addressed in the analysis of this data was the possibility that methylation patterns could be useful for molecular classification of cancers. There is currently a major emphasis to classify breast cancers on a molecular level, using platforms such as gene expression arrays (26–30), because conventional histology is often inadequate for predicting biological behavior of specific cancers. Cancers of different tissue histogenesis have remarkably unique profiles of promoter hypermethylation (14), and therefore, we reasoned that the variable methylation among breast cancers should be examined for patters that could provide an alternative or complementary tactic to classify breast cancers.

To approach this issue, we first compared the methylation patterns of well-defined classes of breast cancer (mucinous and lobular cancers) to each other and to ductal cancers for a proof-of-principle study. Notably, mucinous and lobular cancers have distinctive histologic and clinical characteristics, whereas the term ductal carcinoma, also known as carcinoma of no distinctive type, refers to a heterogeneous group of cancers with diverse histology and biology. Only one individual gene in our panel, *BRCA1*, shows a frequency of methylation that is significantly different among the various histologic categories, corroborating a previous observation made with a smaller number of mucinous breast cancer cases (17). There are other recent reports, however, of differences in methylation of specific genes across histologic classes of breast cancer, including methylation of death-associated protein kinase preferentially in lobular cancers (31) and more frequent methylation of twist in ductal cancer compared with lobular cancer (ref. 32; a result that is not confirmed in our set of breast cancers). In addition, it has been recently reported that *HIN1* is uncommonly methylated in cancers of *BRCA1* mutation patients (33). These findings collectively suggest that methylation patterns of selected genes could contribute to the molecular classification of breast cancer.

A potentially more robust approach than gene-specific patterns of methylation for cancer classification involves decision-based analysis algorithms to seek patterns that involve combinations of multiple genes. Selected iterations of divisive and agglomerative clustering algorithms and a latent class analysis algorithm, with predefined numbers of categories, were applied, but these algorithms did not improve our ability to correctly classify the breast cancers by histology, as compared with using the methylation status of *BRCA1* alone.

Our study was limited not only by the sample size but also by a relatively modest number of genes tested in our candidate gene approach. These factors, in turn, limit the precision of the model and result in some potential instability of the solution. Analysis of more breast cancer cases and consideration of additional genes could help to define classes of breast cancer based on patterns of aberrant gene promoter methylation. Consideration of more genes could be facilitated by the use of

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several recently described techniques that have been largely applied to discovery of new genes with differential methylation such as methylation-sensitive arbitrarily primed PCR (34), methylated CpG island amplification (35), restriction landmark genome scanning (36, 37), and differential methylation hybridization methods, including CpG island microarrays (38–40). Notably, however, the striking differences in hypermethylation profiles that characterize cancers arising from different tissues (14) were readily detected using the same candidate gene approach used in the present study. Therefore, it appears likely that differences in gene hypermethylation profiles among different classes of breast cancers will not be as robust as differences between breast cancer and other types of cancer.

Finally, our study allowed us to compare different genes to each other for patterns of methylation across a spectrum of breast cancers. A consistent pattern of coordinated methylation of two or more genes could suggest that these genes have complementary functions, rather than overlapping functions, in breast cancer biology. Although the functional significance of concordant gene methylation remains unclear, such findings have practical significance related to the use of methylation markers for the detection of cancer in ductal lavage fluids or other biological specimens. In particular, multiple genes that show parallel profiles of methylation are likely to be redundant as markers for cancer detection, whereas nonredundant combinations of genes could provide a more parsimonious set of markers for breast cancer detection in biological specimens.

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