

Featured Article

Hypermethylation in Histologically Distinct Classes of Breast Cancer

Young Kyung Bae,¹ Amy Brown,²
Elizabeth Garrett,³ Daniel Bornman,²
Mary Jo Fackler,³ Saraswati Sukumar,^{2,3}
James G. Herman,³ and Edward Gabrielson^{2,3}

¹Department of Pathology, Yeungnam University College of Medicine, Daegu, South Korea, and ²Department of Pathology and ³Stanley Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland

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 *GSTPI*; 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 *GSTPI*), 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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Edward Gabrielson, Department of Pathology, Johns Hopkins Cancer Center, 417 North Caroline Street, Baltimore, MD 21231. E-mail: egabriel@jhmi.edu.

©2004 American Association for Cancer Research.

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)
<i>RARβ</i>	M: GAACGCGAGCGATTCCGAGT U: GGATTGGGATGTTGAGAATGT	M: GACCAATCCAACCGAAACG U: CAACCAATCCAACCAAAACAA	142 158	56 56
<i>Cyclin D2</i>	M: GGGTCGATCGTGTGGCG U: GAAATATATTAAGGGTGTG	M: ACATAAAACCTCCACGCTCG U: AAAATCCACCAACACAATCA	124 147	60 52
<i>Twist</i>	M: TTCGGATGGGGTTGTTATC U: TTTGGATGGGGTTGTTATTGT	M: AAACGACCTAACCCGAACG U: CCTAACCCAAACAACCAACC	200 193	56 56
<i>ER</i>	M: GTGTATTGGATAGTAGTAAGTTCGTC U: GGTGTATTTGGATAGTAGTAAGTTTGT	M: CGTAAAAAAAACCGATCTAACCG U: CCATAAAAAAAAACCAATCTAACCA	118 120	55 55
<i>E-Cadherin</i>	M: TATCGCGTTTATGCGAGGTC U: TTAATTAGTGGTATGGGGGT	M: CAAATAAACCCGAAACACC U: ATCAAAATCAAACCAAACTAAAAACA	139 147	56 56
<i>BRCA1</i>	M: GGTAAATTTAGAGTTTCGAGAGACG U: GTGGTTAATTTAGAGTTTTCGAGAGATG	M: CTATAATTCGCGCTTTTCC U: CCACACTTTTCCATTACCACA	143 136	58 58
<i>THRβ</i>	M: GGTAATTTGGTTAGAGATCGCGC U: TATTGGTAATTTGGTTAGAGGATTGTGT	M: CACCCCTCCGATCTTACGACG U: CACACCCCTCCAATTCTTACAACA	119 125	56 56
<i>GST-P1</i>	M: TTCGGGGTGTAGCGGCGTC U: GATGTTTGGGGTGTAGTGGTGT	M: GCCCAATACTAAATCACGACG U: CCACCCCAATACTAAATCACAAACA	89 94	59 59
<i>HIN-1</i>	M: GGTACGGTTTTTTACGGTTCGTC U: GGTATGGGTTTTTATGGTTTGT	M: AACTTCTTATACCCGATCCTCG3 U: CAAAACCTTCTTATACCCAATCCTCA	136 136	56 56
<i>RASSF1A</i>	M: GTTGGTATTCGTTGGGCGC U: GGTGTATTGTTGGAGTG	M: GCACCAGTATACGTAACG U: CTACAAACCTTACACACAACA	160 180	56 56
<i>BAX</i>	M: GAGGTAGGTGCGGTTACGTG U: GGTGTTGTGGGGTAGTGGTT	M: AATCACGTAAAAACCCCGCT U: ACCACCTCTCACAAATCCA	102 118	57 57
<i>RB</i>	M: TAGAAGTACGTTCCGGTTCGC U: TTTTGTGGTTGGATGTGGTG	M: CGATACGCGAACTAAAACGC U: ATCTCTCCCAACTCCATT	106 104	57 57

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

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 μ g of purified genomic DNA or 50 μ L 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 *SssI* 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 μ L) 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

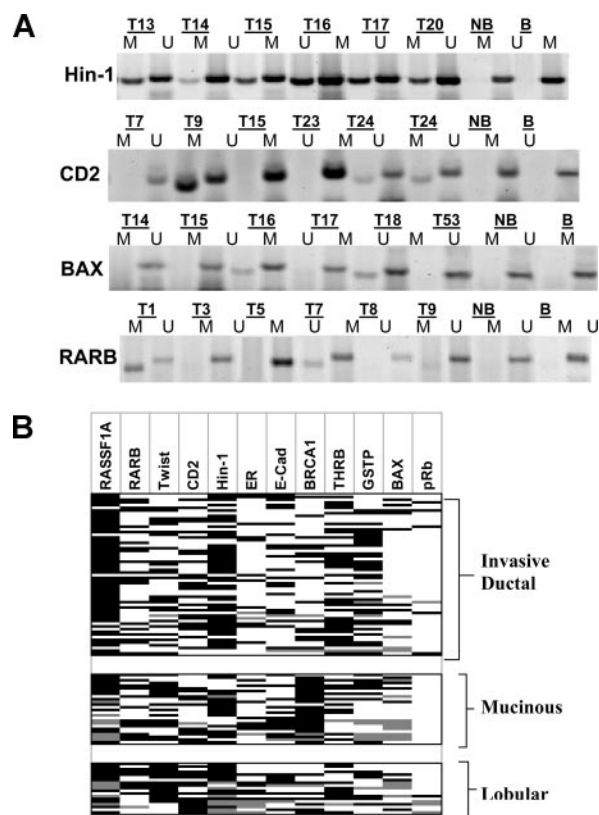


Fig. 1 Methylation of genes in breast cancer. In *A*, representative MSP results are shown for three genes (*Hin-1*, *RARβ*, and *Cyclin D2*) in breast cancer samples (T), normal breast (NB), and peripheral blood leukocytes (B). Lanes labeled with "M" represent reactions using primers specific for bisulfite-treated 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.

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 *H1N1* (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).

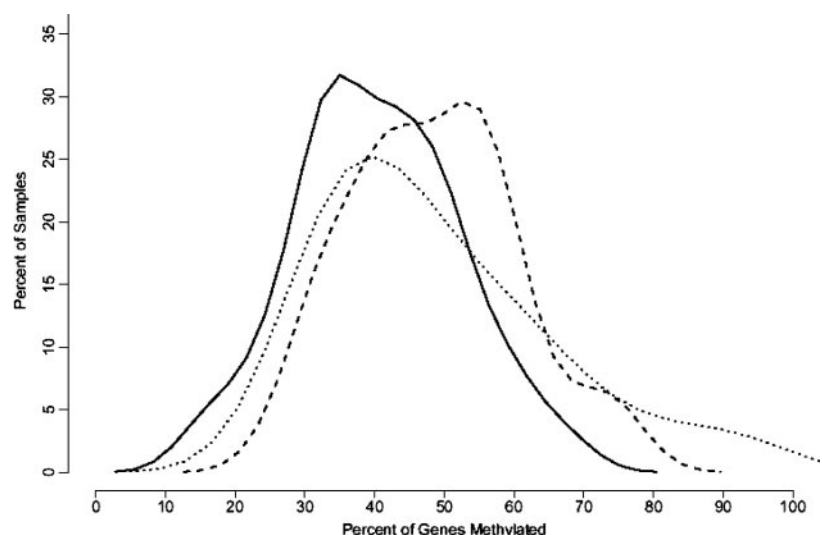
Gene-Specific Patterns of Methylation for Classification of Breast Cancer. We next examined gene-specific patterns of methylation in the three histologic types of breast cancer to determine in principle whether these patterns could be useful for the molecular classification of these cancers. Only one 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.

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).



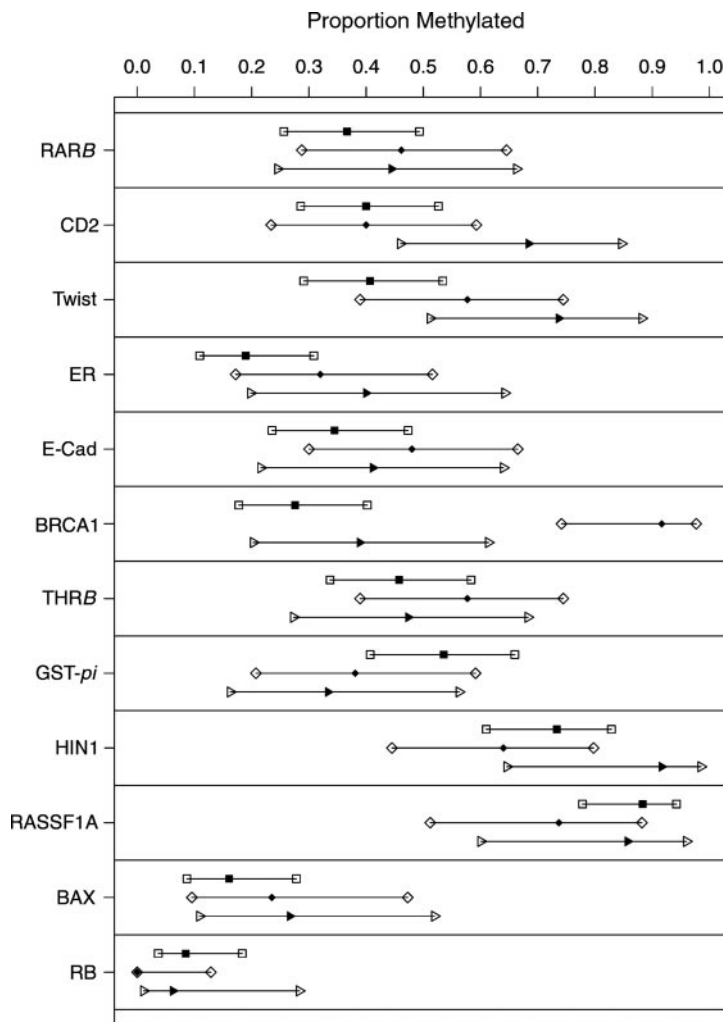


Fig. 3 Gene-specific patterns of methylation in different histologic types of breast cancers. Ductal cancers are designated by *squares*, mucinous cancers by *diamonds*, and lobular cancers by *triangles*. Vertical bars are used to designate means and SDs.

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 methyl-

ation 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

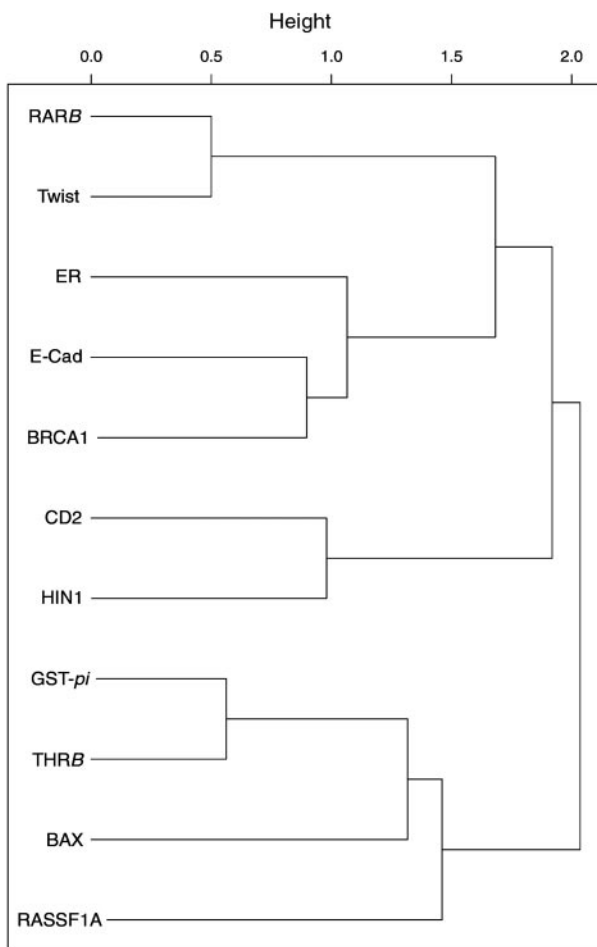


Fig. 4 Dendrogram demonstrating clustering of genes according to patterns of methylation. Odds ratios for coordinate patterns of methylation were calculated using data for all 109 breast cancers analyzed, and dendrogram was generated using agglomerative clustering.

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 patterns 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

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 non-redundant combinations of genes could provide a more parsimonious set of markers for breast cancer detection in biological specimens.

REFERENCES

- Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB, Herman JG. Inactivation of glutathione *S*-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res* 1998;58(20):4515–8.
- Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* (Bethesda) 2000;92(7):564–9.
- Herman JG, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995;55(20):4525–30.
- Evron E, Umbricht CB, Korz D, et al. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res* 2001;61(6):2782–7.
- Graff JR, Herman JG, Lapidus RG, et al. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995;55(22):5195–9.
- Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG. Methylation patterns of the E-cadherin 5'-CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 2000;275(4):2727–32.
- Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 1994;54(10):2552–5.
- Widschwendter M, Berger J, Hermann M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. *J Natl Cancer Inst* (Bethesda) 2000;92(10):826–32.
- Li Z, Meng ZH, Chandrasekaran R, et al. Biallelic inactivation of the thyroid hormone receptor beta1 gene in early stage breast cancer. *Cancer Res* 2002;62(7):1939–43.
- Raman V, Martensen SA, Reisman D, et al. Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature* (Lond.) 2000;405(6789):974–8.
- Burbee DG, Forgacs E, Zochbauer-Muller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* (Bethesda) 2001;93(9):691–9.
- Evron E, Dooley WC, Umbricht CB, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet* 2001;357(9265):1335–6.
- Krop IE, Sgroi D, Porter DA, et al. HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. *Proc Natl Acad Sci USA* 2001;98(17):9796–801.
- Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61(8):3225–9.
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999;96(15):8681–6.
- Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci USA* 2000;97(2):710–5.
- Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;93(18):9821–6.
- McCutcheon A. *Latent class analysis*. Newbury Park: Sage Publications, 1987.
- Garrett ES, Zeger SL. Latent class model diagnosis. *Biometrics* 2000;56(4):1055–67.
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998;95(25):14863–8.
- Fujii H, Anbazhagan R, Bornman DM, Garrett ES, Perlman E, Gabrielson E. Mucinous cancers have fewer genomic alterations than more common classes of breast cancer. *Breast Cancer Res Treat* 2002;76(3):255–60.
- Huiping C, Sigurgeirsdottir JR, Jonasson JG, et al. Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer. *Br J Cancer* 1999;81(7):1103–10.
- Shen CY, Yu JC, Lo YL, et al. Genome-wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. *Cancer Res* 2000;60(14):3884–92.
- Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998;95(12):6870–5.
- Yamashita K, Dai T, Dai Y, Yamamoto F, Perucho M. Genetics supersedes epigenetics in colon cancer phenotype. *Cancer Cell* 2003;4(2):121–31.
- Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* (Lond.) 2000;406(6797):747–52.
- Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001;98(19):10869–74.
- West M, Blanchette C, Dressman H, et al. Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc Natl Acad Sci USA* 2001;98(20):11462–7.
- van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347(25):1999–2009.
- van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* (Lond.) 2002;415(6871):530–6.
- Lehmann U, Celikkaya G, Hasemeier B, Langer F, Kreipe H. Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. *Cancer Res* 2002;62(22):6634–8.
- Fackler MJ, McVeigh M, Evron E, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer* 2003;107(6):970–5.

33. Krop I, Maguire P, Lahti-Domenici J, et al. Lack of HIN-1 methylation in BRCA1-linked and "BRCA1-like" breast tumors. *Cancer Res* 2003;63(9):2024–7.
34. Gonzalgo ML, Liang G, Spruck CH 3rd, Zingg JM, Rideout WM 3rd, Jones PA. Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. *Cancer Res* 1997;57(4):594–9.
35. Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res* 1999;59(10):2307–12.
36. Hayashizaki Y, Hirotsune S, Okazaki Y, et al. Restriction landmark genomic scanning method and its various applications. *Electrophoresis* 1993;14(4):251–8.
37. Costello JF, Fruhwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000;24(2):132–8.
38. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet* 1999;8(3):459–70.
39. Yan PS, Chen CM, Shi H, et al. Dissecting complex epigenetic alterations in breast cancer using CpG island microarrays. *Cancer Res* 2001;61(23):8375–80.
40. Shi H, Yan PS, Chen CM, et al. Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells. *Cancer Res* 2002;62(11):3214–20.