

CpG Island Arrays: An Application toward Deciphering Epigenetic Signatures of Breast Cancer¹

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ABSTRACT

CpG island hypermethylation is a frequent epigenetic event in cancer. We have recently developed an array-based method, called differential methylation hybridization (DMH), allowing for a genome-wide screening of CpG island hypermethylation in breast cancer cell lines (T. H-M. Huang *et al.*, *Hum. Mol. Genet.*, 8: 459–470, 1999). In the present study, DMH was applied to screen 28 paired primary breast tumor and normal samples and to determine whether patterns of specific epigenetic alterations correlate with pathological parameters in the patients analyzed. Amplicons, representing a pool of methylated CpG DNA derived from these samples, were used as hybridization probes in an array panel containing 1104 CpG island tags. Close to 9% of these tags exhibited extensive hypermethylation in the majority of breast tumors relative to their normal controls, whereas others had little or no detectable changes. Pattern analysis in a subset of CpG island tags revealed that CpG island hypermethylation is associated with histological grades of breast tumors. Poorly differentiated tumors appeared to exhibit more hypermethylated CpG islands than their moderately or well-differentiated counterparts ($P = 0.041$). This early finding lays the groundwork for a population-based DMH study and demonstrates the need to develop a database for examining large-scale methylation data and for associating specific epigenetic signatures with clinical parameters in breast cancer.

Received 9/23/99; revised 1/15/00; accepted 1/6/00.

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¹ Supported by a gift from Booneslick Trail Quilters Guild, Grant CA-69065 from the National Cancer Institute (to T. H-M. H.), and United States Army Medical Research Command Grant DAMD17-98-1-8214 (to T. H-M. H.).

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INTRODUCTION

The recent development of high-throughput technologies has provided powerful tools for comprehensive genome-wide analysis of gene expression, chromosomal alterations, and nucleotide mutations in cancer. These technologies, including oligonucleotide (1), cDNA (2, 3), and tissue (4) microarrays and serial analysis of gene expression (5), can generate a vast amount of molecular information and may provide comprehensive profiles of genetic changes for different types of cancer cells. Translational research is just beginning to search unique molecular identifiers, *i.e.*, genetic signatures, in clinical tumor specimens. This type of study will pave the way for complementing the histopathological examinations currently in use with molecular tumor diagnosis and classification in the future.

Until recently, such high-throughput technological development has been lacking for another common molecular alteration, *i.e.*, DNA methylation, in the tumor genome. This epigenetic alteration usually occurs by deleting or adding a methyl group in the fifth carbon position of a cytosine located 5' of a guanine known as CpG dinucleotide (6). Loss of cytosine methylation, or hypomethylation, is frequently observed in bulk chromatin or repetitive sequences and may promote gross chromosomal rearrangements (6–8). In contrast, *de novo* cytosine methylation, or hypermethylation, is a regional event that occurs frequently in GC-rich sequences, called CpG islands, located within the 5' regulatory regions of nontranscribed genes (6, 9).

We have recently adapted the microarray-based strategy and developed a novel technique, called DMH,³ for the first time, providing a tool that can efficiently scan the tumor genome for methylation alterations (10). The first part of DMH is the generation of GC-rich tags derived from a human CpG island genomic library, CGI (11). These tags were then arrayed onto solid supports (*e.g.*, nylon membranes). The second part involves the preparation of amplicons, representing a pool of methylated CpG DNA, from tumor or reference samples. Amplicons are used as probes for CpG island array hybridization. The differences in tumor and reference signal intensities on CpG island arrays tested reflect methylation alterations of corresponding sequences in the tumor DNA. DMH was successfully applied to detect specific methylation profiles in a group of breast cancer cell lines, and hypermethylation of CpG island loci was independently confirmed by Southern-based analysis (10). Subsequent pattern analysis of the positive loci revealed potential mechanisms governing aberrant methylation in these cells.

In this study, we determined whether DMH alone can be routinely applied to identify CpG island hypermethylation in

³ The abbreviations used are: DMH, differential methylation hybridization; CGI, CpG island; HBC, hypermethylation in breast cancer; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated.

clinical specimens. We analyzed 28 paired normal and breast tumor specimens with an array panel representing ~2% of total CpG islands in the genome. Methylation data derived from DMH were correlated with pathological parameters in the patients analyzed. Our results suggest that increased CpG island hypermethylation is associated with high-grade tumors. This initial study lays the groundwork for further population-based analysis to examine the epigenotype-phenotype relationship in breast cancer.

MATERIALS AND METHODS

Patient Samples. Breast tumor specimens were obtained from 28 female patients undergoing mastectomies at the Ellis Fischel Cancer Center (Columbia, MO) between 1992 and 1998. Adjacent, normal parenchyma was obtained from the same patient to serve as a normal control. All tumors used in this study were classified as infiltrating ductal carcinomas and were graded based on the Nottingham modified criteria of Bloom and Richardson (12). This tumor-grading method was based on histological features of tubule formation, nuclear pleomorphism, and mitotic activity, and points were assigned for each category accordingly. The overall tumor grade was the sum total of scores between 3 and 9. Tumors with poorly differentiated phenotypes (8–9 points) are likely to have fewer or no tubular structures, irregular and large nuclei, and high mitotic counts. Tumors with moderately (6–7 points) or well-differentiated (3–5 points) phenotypes may have definite tubule formation, moderate outlines of epithelial cell shapes and uniformity of nuclear chromatin, and low mitotic indexes. Patient clinical information is shown in Table 1. High molecular weight DNA was isolated from these specimens using the QIAamp tissue kit (Qiagen).

Amplicon Generation. DMH was performed as described previously (10). Briefly, genomic DNA (0.5–1 μ g) from breast tumor or normal samples was digested with a 4-base TTAA cutter *Mse*I known to restrict DNA into small fragments (<200-bp) but leave CpG islands relatively intact (11). The cleaved ends of the digests were ligated with 0.5 nmol of linkers, H-12/H-24 (H-12, 5'-TAA TCC CTC GGA; and H-24, 5'-AGG CAA CTG TGC TAT CCG AGG GAT), in a buffer containing 400 units of T4 DNA ligase (New England Biolabs) at 16°C. After ligation, repetitive Cot-1 DNA was removed from the ligated products using a subtractive hybridization technique (13). The subtracted DNA was further digested with methylation-sensitive *Bst*UI (cuts CGCG, but not ^mCGCG, ^mC: 5-methylcytosine). This endonuclease was used in the analysis, because >77% of known CpG islands contain *Bst*UI sites (11). Linker-PCR reactions were performed with the pretreated DNAs (~50 ng) in a 20- μ l volume, containing 0.4 μ M H-24 primer, 1 unit Deep Vent (exo⁻) DNA polymerase (New England Biolabs), 5% (v/v) DMSO, and 200 μ M deoxynucleotide triphosphates in a buffer provided by the supplier. The tubes were incubated for 3 min at 72°C to fill in 5' protruding ends of ligated linkers and subjected to 15 cycles of amplification, as described previously (10). Low amplification cycles were used to prevent an overabundance of leftover repetitive sequences generated by PCR. In addition, under this condition all amplification is expected to be in the linear range of the assay, allowing for semiquantitation of dot intensities described later.

Table 1 Clinicopathological information for patients with infiltrating ductal carcinoma of the breast

Patient no.	Age at diagnosis (yr)	Clinical stage ^a	Histological grade	Estrogen receptor	Progesterone receptor
097	57	II	WD	+	-
157	49	II	WD	+	+
025	42	II	MD	-	-
045	44	III	MD	+	-
063	52	III	MD	+	+
067	40	IV	MD	-	-
071	33	II	MD	-	-
083	45	III	MD	+	+
085	64	II	MD	+	+
091	55	II	MD	-	-
095	53	I	MD	+	+
109	66	III	MD	+	+
137	82	I	MD	+	-
187	83	II	MD	+	+
031	60	II	PD	-	-
043	35	III	PD	-	-
047	38	III/IV	PD	-	-
065	42	IV	PD	-	-
089	83	III	PD	+	-
103	67	II	PD	+	-
119	67	III	PD	-	-
121	52	III	PD	-	-
123	38	II	PD	-	-
129	76	II	PD	+	+
135	46	I	PD	-	-
153	50	I	PD	-	-
155	43	—	PD	-	-
167	40	III	PD	-	-

^a Classification according to the TNM system (19).

The amplified products, labeled as normal or tumor amplicons, were purified and ³²P-labeled for array hybridization.

Array Hybridization. *Bst*UI-positive, Cot-1-negative, CpG island clones were prepared from the CGI genomic library and used for 96-well format PCR as described previously (10). PCR products (0.2–1.5 kb) were denatured and dotted in duplicate on nylon membranes using a 96-pin Multi-Print replicator (V & P Scientific). Alignment devices (Library Copier; V & P Scientific) were used in conjunction with the replicator to convert multiple 96-well PCR samples into one recipient of 576 or 1536 dots on a 10 × 12-cm nylon membrane. Membranes were first hybridized with normal amplicons, and autoradiography was conducted using the Molecular Dynamics PhosphorImager. Probes were stripped, and the same membranes or duplicate membranes were hybridized with tumor amplicons and scanned with the PhosphorImager.

Data Analysis. Dot intensities for positive CpG island tags were measured using the volume review protocol of ImageQuant software (Molecular Dynamics). The raw volume data from tumor and normal samples were normalized prior to comparison. This was achieved by ratio determination of the internal control tags. Briefly, two internal control tags with close volume ratios were selected to estimate hybridization differences between paired amplicons. One of these two control tags from each amplicon was further used to calculate a factor for normalization:

$$\text{Normalization factor} = \frac{\text{Normal internal control tag volume}}{\text{Tumor internal control tag volume}}$$

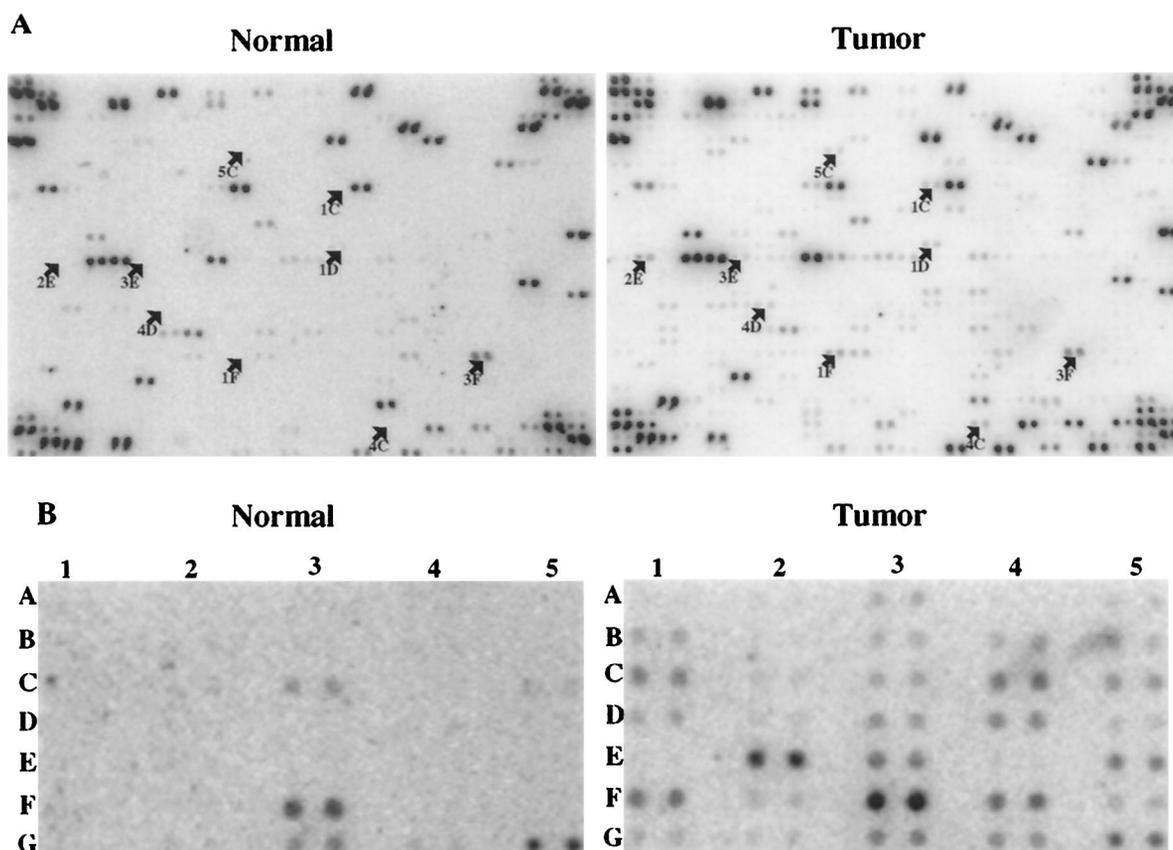


Fig. 1 Representative results of DMH from one patient. The initial screening (A) and the corresponding subarray (B) were shown with some of the hypermethylated clones later dotted on the subarray identified with their X and Y coordinates. PCR products of CpG island tags were dotted onto membranes hybridized first with radiolabeled normal amplicons as described in the text. The same membranes, or duplicate membranes, were later hybridized with tumor amplicons. Each CpG island tag is represented by two parallel dots to differentiate specific hybridization signals from nonspecific background signals, which generally appear as scattered single dots. Five to six sets of positive controls were dotted on the four corners of the arrays to serve as orientation markers and for comparison of hybridization signal intensities.

This factor was applied to normalize tumor tag volumes. For tags with preexisting methylation in normal tissue, the normal tag volume was subtracted from the normalized tumor volume. For tags without preexisting methylation in the normal tags, the normalized tumor volume was used directly. Statistical analyses were performed using the SigmaStat software (version 2.0). The hypermethylation differences among different groups of tumor grades were determined by the unpaired *t* test and by the Mann-Whitney rank sum test when the data failed the normality test. The difference was considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Primary Screening of DMH in Breast Tumors. DMH was initially applied to 28 paired breast tumor and normal samples using an array panel containing 1104 CpG island tags. Fig. 1A shows representative results of DMH screening in paired normal and tumor samples of patient 103. On the basis of the visual inspection, hypermethylated sequences were identified in breast tumors, showing detectable hybridization signals in CpG island tags probed with tumor amplicons but not in the same tags probed with normal amplicons (see examples indicated by arrows). This is because methylated *Bst*UI sites in tumor DNA

were protected from restriction within CpG island sequences, which were then amplified by linker-PCR and hybridized to the corresponding tags. The same sites, however, were unmethylated or partially methylated in normal DNA and were restricted by *Bst*UI; therefore, no hybridization signals were detected in the arrays. Some of these hypermethylated CGI island tags were confirmed in the subsequent secondary screening (Fig. 1B).

In addition, we observed a few CpG island tags that were detected by normal amplicons (*i.e.*, preexisting methylation) but showed greater signal intensities when probed with tumor amplicons (*e.g.*, CpG island tags in the lower right-hand corner in Fig. 1A). These sequences usually exhibited more prominent hybridization signals among all of the tags, likely representing abundant copies of GC-rich ribosomal DNA, as described previously in the cell line study (10). Methylation of ribosomal DNA has been observed previously in normal cells (14) but shown to increase to a greater extent in breast tumors (15). Another possibility is that the increased copy numbers of normally methylated CpG island loci in tumors are attributable to aneuploidy. Excluding this preexisting condition, the extent of hypermethylation in unmethylated CpG islands was quite variable among patients in this group; close to 9% of the tested

Table 2 A list of positive CGI clones isolated by DMH

CpG island clone ^a	Insert size (kb)	GenBank match	Accession number
HBC-17 ^b	0.75		
HBC-19 ^b	0.90	PAX7	AL021528
HBC-24 ^b	1.10	IMAGE:2518953 5' mRNA sequence	AI928953
HBC-25 ^b	0.70		
HBC-27 ^b	0.70		
HBC-33	0.85		
HBC-34	0.95	IMAGE:1113203 3' mRNA sequence	AA604922
HBC-35	1.00		
HBC-36	0.70		
HBC-37	1.00	PAC 163M9	AL021920
HBC-38	0.50	CGI 40c10	Z58446
HBC-39	0.60	EST185442	AA313564
HBC-40	0.95		
HBC-41	0.60	CGI 29h6	Z58110
HBC-42	0.50	CGI 13f7	Z56764
HBC-43	0.80	Genomic clone NH0444B04	AC007392
HBC-44	1.20		
HBC-45	0.50	PAC 29K1	Z98745
HBC-46	0.65	IMAGE:2177671 3' mRNA sequence	AI500696
HBC-47	0.70		
HBC-48	1.50	BAC clone RG300E22	AC004774
HBC-49	0.50	CGI 40c10	Z58447
HBC-50	0.45		
HBC-51	0.70	COL9A1 (alt exon1)	M32133
HBC-52	0.60	IMAGE:2092259 3' mRNA sequence	AI381934
HBC-53	0.80	Genomic clone NH0444B04	AC007392
HBC-54	0.85		
HBC-55	0.80	CAVEOLIN-1 (exon2)	AF095592
HBC-56	0.60		
HBC-57	0.90	GATA-3 (exon1)	X55122

^a HBC clones have been sequenced, and these data and other information are available to the research community on our Web site: www.missouri.edu/~hypermet.

^b The clone was hypermethylated in a study of breast cancer cell lines (10) and in primary breast tumors in this study.

*Bst*UI sites exhibited complete methylation in some breast tumors examined, whereas others had little or no detectable change in the tested sites.

We also noticed that a few tags showed stronger signal intensities when probed with normal amplicons than with tumor amplicons. It is, however, unclear whether these tags represent hypomethylated sequences in the primary tumors analyzed. To our knowledge, specific hypomethylation of normally methylated CpG islands has thus far not been reported in tumors. One possibility is the allelic loss of methylated loci because of chromosomal deletions in tumor cells (9). These tags usually showed stronger hybridization signals and likely contained abundant satellite or repetitive DNA normally methylated but becoming hypomethylated in cancer cells (6, 16). Another possibility is residual normal cells present in tumor specimens, leading to a false-positive identification of hypomethylated sequences. Tissue heterogeneity or contamination in clinical specimens has been a common problem hampering the detection of true genetic or epigenetic alterations in primary tumors. This issue, however, does not apply to the identification of hyper-

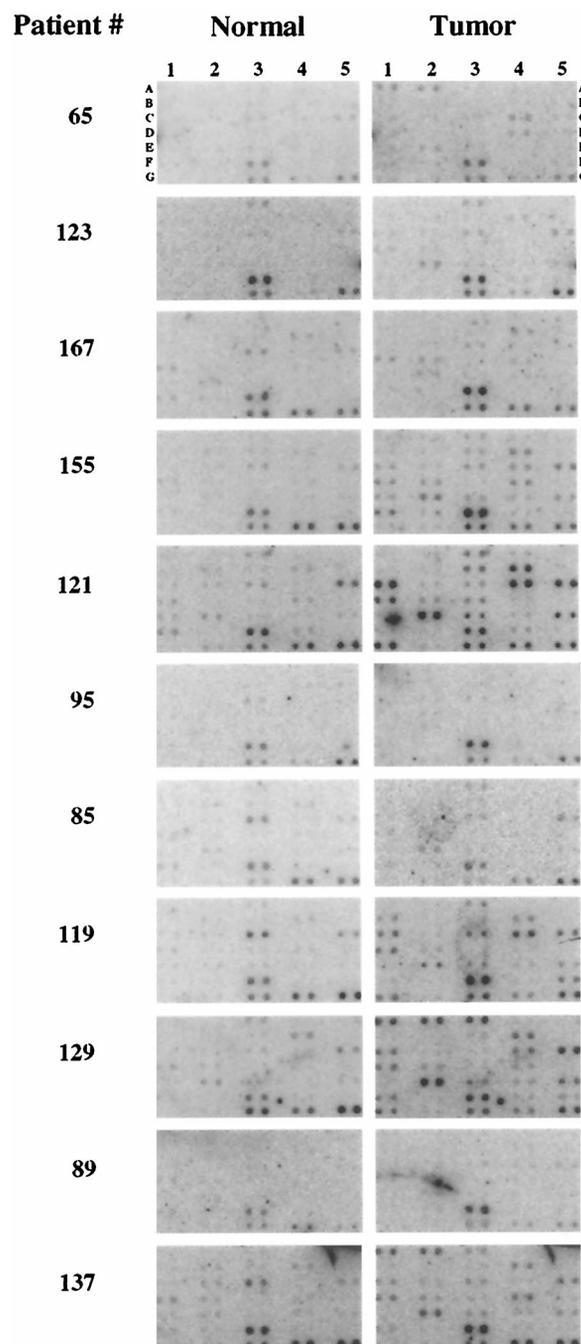
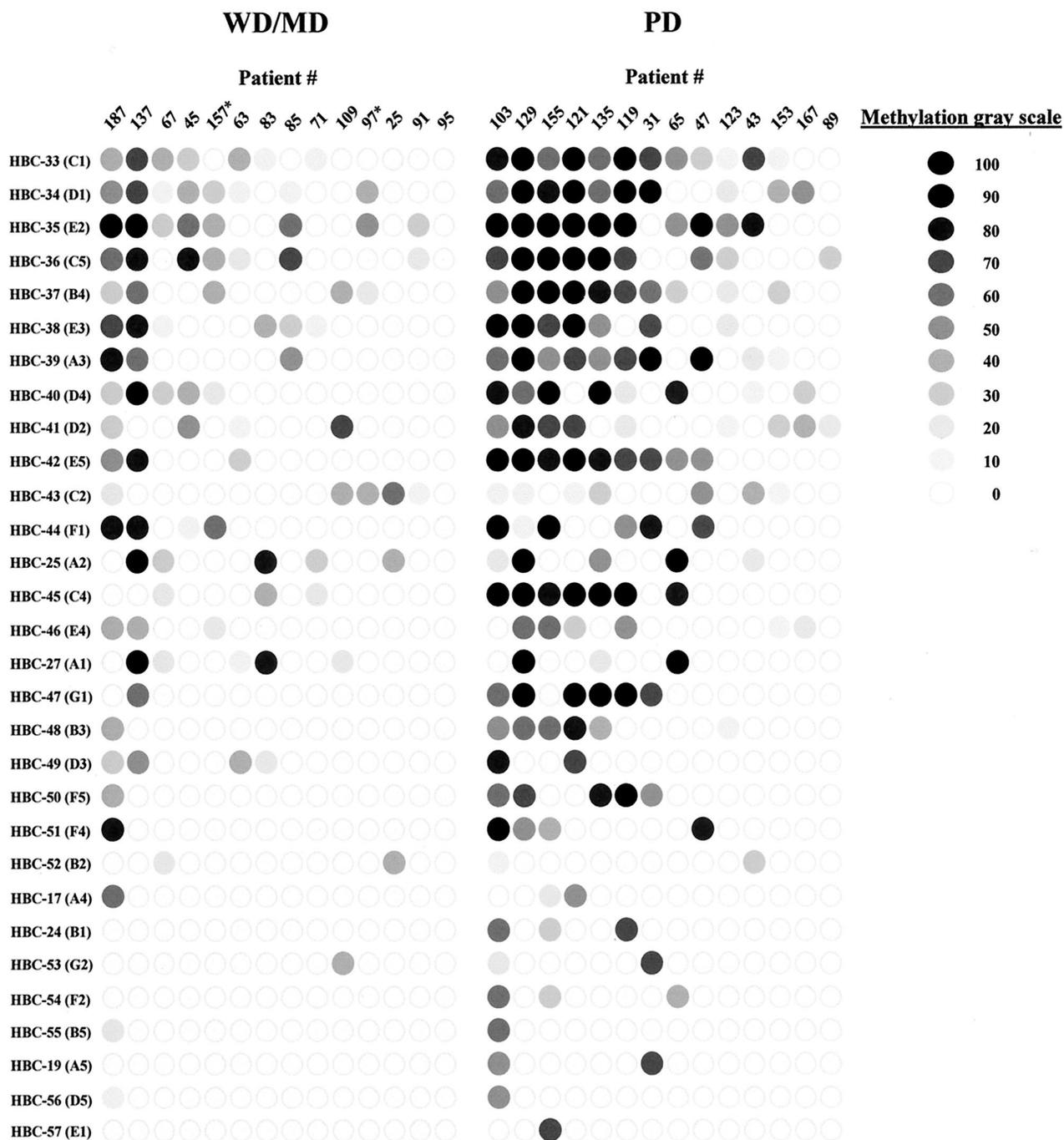


Fig. 2 Identification of hypermethylated CpG island loci by DMH. The 30 CpG island tags shown in this subarray panel were selected from an initial DMH screening of >1000 tags. Five additional tags—coordinates on the X and Y axes are 3C, 3F, 3G, 4G, and 5G—were included as internal controls. CpG island tags were dotted onto membranes in duplicate and probed with radiolabeled amplicons for the normal and breast tumors as indicated. DMH screening from 11 of 28 patients were represented here, and experiments were performed independently twice.

methylated CpG sequences attributable to the gain of additional PCR fragments in tumor amplicons relative to normal amplicons. Because it is less clear whether DMH is suited for the detection of hypomethylated sequences in primary tumors, we



* WD tumors

Fig. 3 Hypermethylation pattern analysis of 30 CpG island loci in 28 primary breast tumors. Methylation gray scale shown at right represents volume percentile generated by ranking hybridization signal intensities of these tested loci as described in the text. Data from primary tumors were presented according to their tumor grades: WD/MD and PD. Within each group, patients were arranged from left to right according to their increased methylation propensities. Thirty CpG island loci (on the left of the panel with their secondary screening coordinates shown in parentheses) were listed from top to bottom according to their increased methylation scales derived from the primary tumors. Five CpG island loci (HBC-17, HBC-19, HBC-24, HBC-25, and HBC-27) were found to be hypermethylated in breast cancer cell lines, as reported previously by our laboratory.

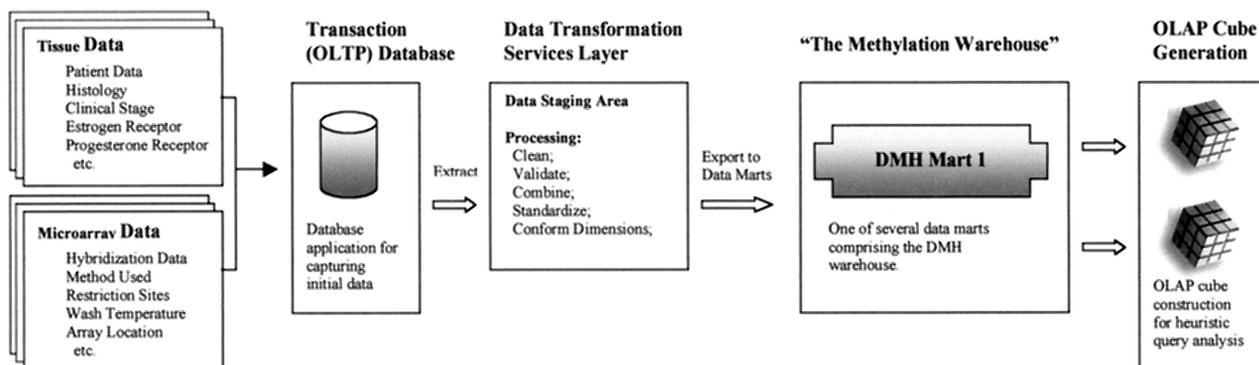


Fig. 4 Diagrammatic representation of the data warehouse used to store and query methylation data. Compared with traditional On-line Transactional Processing (OLTP) relational databases, one can visualize patterns across libraries of experiments more effectively using a data warehouse architecture and an On-line Analytical Processing (OLAP) browser.

focused on the hypermethylation findings in our subsequent analyses.

Sequence Characterization of CpG Island Tags.

Thirty CpG island tags, positive for hypermethylation in the primary screening, were selected for further characterization. DNA sequencing results showed that 9 of these tags contained sequences identical to known cDNAs, *PAX7* (5' end), *Caveolin-1* (exon 2), *GATA-3* (exon 1), and *COL9A1* (exon 1), and 5 expressed sequence tags (AI928953, AA604922, AA313564, AI500696, and AI381934), as shown in Table 2. This finding is consistent with that of Engelman *et al.* (17), where they also observed CpG island methylation in the *Caveolin-1* gene in breast cancer cell lines. Five CpG island tags, HBC-17, HBC-19, HBC-24, HBC-25, and HBC-27, found to be hypermethylated in breast cancer cell lines as reported previously (10, 18), were also identified in this study. The remainder 25 tags were numerically assigned as HBC-33 through HBC-57, following the previous series of studies mentioned above.

Secondary Screening of DMH in Breast Tumors. As shown earlier in Fig. 1B, the 30 CpG island tags were rearranged for secondary DMH screening in the patient group to confirm their hypermethylation status (see representative results in Fig. 2). Five additional tags—coordinates on the X and Y axes are 3C, 3F, 3G, 4G, and 5G—exhibit no hybridization intensity differences among a few of the breast tumors tested in the primary screening were chosen as internal controls. Again, most normal controls showed few or no detectable hybridization signals at the tested loci, whereas the corresponding breast tumors exhibited various degrees of hybridization intensities, reflecting the differences in CpG island hypermethylation.

To semiquantify the methylation differences, hybridization signal intensity for each CpG island tag was measured using the volume review protocol of ImageQuant software as described in "Materials and Methods." From Fig. 2, it is clear that dot intensities of the internal controls sometimes varied among patients or between a patient's paired tumor and normal samples, likely because of tissue heterogeneity or tumor aneuploidy. Therefore, internal control volume ratios were tested, and two with close volume ratios were selected for normalization. The adjusted tumor volumes were used for clinical correlation in this patient group.

CpG Island Hypermethylation and Tumor Grades.

Statistical analysis revealed that CpG island hypermethylation was associated with histological grades of breast tumors ($P = 0.041$). To aid us in visualizing differences in CpG island hypermethylation among different tumor grades, we devised a gray scale by categorizing tumor methylation volumes into percentiles as depicted in Fig. 3. The PD (3) group exhibited more frequent and extensive hypermethylation at the loci tested than their MD/WD (3) counterparts did; half of the 14 PD tumors showed extensive hypermethylation at multiple loci (>10), whereas only 2 of the 14 MD/WD tumors showed hypermethylation at these loci. Moreover, the greatest degrees of differences were seen at loci HBC-42, HBC-45, and HBC-47 that were frequently hypermethylated in PD tumors but not in MD/WD. This result suggests that patients with more advanced disease status are prone to methylation alterations. It should be noted that some of the patients showed little or no changes of methylation at the loci tested. This indicates that progression of some tumors may be independent of this epigenetic event, or the alteration could occur in later stages of tumor development in such patients. No association of hypermethylation with other clinical parameters was found in this study.

The finding of epigenetic differences in breast tumor grades may have an important implication. We can envisage a mechanism whereby, in patients with methylator phenotype, methylated CpG islands could progressively accumulate during tumor development. As a result of CpG island hypermethylation, critical tumor suppressor genes may become silenced, leading to some cells with growth advantage. Clonal expansion as well as an accumulation of more CpG island hypermethylation would further promote the malignant potential of these cells. Therefore, differential methylation patterns observed in various clinical specimens may reflect different stages or types of cancer. In our case, the most common methylation of CpG island loci (*e.g.*, HBC-33, HBC-34, HBC-35, and HBC-36) observed among different tumor grades likely occurs early during tumor development, whereas methylation groups (*e.g.*, HBC-42, HBC-45, and HBC-47) observed preferentially in the PD group, but not in the WD/MD groups, occur in later stages.

This early study in a small panel of breast cancer patients has proven that DMH is useful for surveying changes of meth-

ylation patterns in cancer. This achievement opens up an unprecedented opportunity for full-scale development of DMH for population-based analysis. Because DMH can be readily reconfigured into a high-throughput, microarray-based assay, this makes possible the detection of CpG island hypermethylation at the whole genome level.

Methylation Data Warehouse. With ~45,000 CpG islands in the human genome, deciphering specific epigenetic signatures in primary tumors can be a daunting task. We have developed a data warehouse, an advanced information system that verifies, cleans, and stores large quantities of methylation data generated from high-throughput DMH experiments. We have also developed a model of data visualization and schema to support this large-scale study. Sophisticated visualization tools are needed to improve knowledge transfer to researchers. We have implemented an On-Line Transactional Processing system that captures DMH raw data, which are then processed by a data transformation services layer for population of the data warehouse (Fig. 4). An On-Line Analytical Processing browser has also been implemented to help researchers perform heuristic queries and view patterns among microarray experiments. The data collected can be readily retrieved to analyze patterns of methylation in tumor samples and to correlate those changes with clinicopathological parameters of patients.

In conclusion, our results demonstrate the power of DMH with hybridization arrays to rapidly generate information on patterns of differentially methylated CpG islands that may then be moved on to population-based studies of the epigenotype-phenotype relationship.

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Clinical Cancer Research

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Clin Cancer Res 2000;6:1432-1438.

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