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DNA Methylation Profiling: A New Tool for Evaluating Hematologic Malignancies

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INTRODUCTION

The addition of a methyl group (CH_3) to specific cytosines in mammalian DNA controls early development by regulating gene activity (reviewed in 1–12). DNA methylation is defined as an "epigenetic" modification that results in a heritable change in gene function without altering the primary DNA sequence itself. The requirement for a properly functioning DNA methylation system in mammals is best appreciated when observing the severe consequences that result when this system fails. Mutations in the enzymes controlling methyl group addition, the DNA methyltransferases (DNMTs), cause deficient or nonphysiologic DNA methylation patterns that interrupt normal development. For example, targeted deletion of *DNMT1* in mice is lethal to developing embryos, while naturally occurring *DNMT3B* mutations may cause the ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome in humans (13, 14). Also, deletion of MeCP2, a protein that binds to methylated DNA, results in a Rett syndrome-like neurodevelopmental disorder in mice (15, 16). These findings highlight the importance of DNA methylation in epigenetic control of early mammalian development.

The role of DNA methylation following embryogenesis is not resolved. However, DNA methylation patterns have been determined for numerous genes in multiple types of human malignancy. One rationale for this effort has been that nonphysiologic methylation patterns could promote transformation of normal cells to malignant cells through disruptions in physiologic gene regulatory processes. For example, hypermethylation could lead to aberrant tumor suppressor gene silencing, while hypomethylation could lead to inappropriate activation of developmentally silenced proto-oncogenes, both of which could lead to cancer (17–20). Aberrant DNA methylation could also result from, rather than promote, cellular transformation due to defects in malignant cells that affect methyl-group addition or removal. The accumulated evidence suggests that nonphysiologic DNA methylation probably both causes and results from malignant transformation. Therefore, determining the DNA methylation patterns in tumors has the potential to improve our understanding of the transformation process and possibly impact patient care in the areas of diagnosis, prognosis, and therapeutic interventions. With these goals in mind, this review provides a brief overview of DNA methylation in mammalian development and then focuses on results, technologies, and emerging concepts for surveying global changes in DNA methylation as applied to human hematologic malignancies.

DNA METHYLATION IN MAMMALIAN DEVELOPMENT

DNA methylation patterns are erased and then reestablished in early, preimplantation embryos and in germ cells during two main periods of global epigenetic reprogramming (reviewed in 21). Demethylation of preblastula embryonic DNA erases most inherited gamete methylation, with the major exception of the parent-specific imprinted genes (22, 23). Following implantation, *de novo* and then maintenance DNA methylating activities, provided by *DNMT3A/3B* and *DNMT1* methyltransferase enzymes, respectively, methylate the carbon-5 position of the pyrimidine ring of cytosines predominantly in CpG dinucleotides (reviewed in 24, 25; Fig. 1). Cytosines not within CpG dinucleotides may also be methylated, but at much lower frequencies and little is known about this type of non-CpG DNA methylation (26–32). Also, cytosines located within GC-rich regions of the genome, in so-called CpG islands, typically escape *de novo* and maintenance methylation, with the exception of CpG islands

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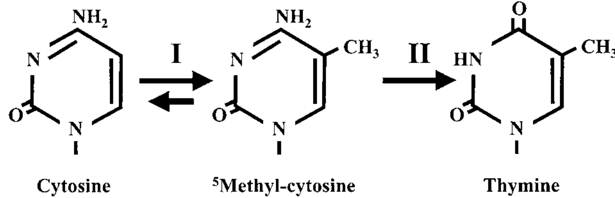

FIG. 1. DNA methylation is linked to tumorigenesis. In step I, a DNA methyltransferase (*DNMT*) enzyme adds a methyl group from *S*-adenosylmethionine to carbon-5 of a cytosine base. When a CpG dinucleotide is unmethylated on both complimentary DNA strands, *DNMT3A/3B* adds the methyl group (*de novo* DNA methylation). However, if one of the two complimentary CpGs is already methylated, then *DNMT1* adds a methyl group to the unmethylated CpG (maintenance methylation). In this way, the DNA methylation system provides heritable control over gene expression after strand separation, DNA replication, and mitosis. Demethylation, through passive lack of maintenance methylation or active removal of a methyl group, can erase this epigenetic mark. In step II, spontaneous deamination of a methylated cytosine creates thymine, linking DNA methylation to the production of new DNA mutations, such as TGA stop codons. This deamination reaction has resulted in a more drastic reduction in the frequency of CpG dinucleotides outside of unmethylated CpG islands than would statistically be expected. Aberrant DNA methylation may therefore silence both alleles of a tumor suppressor gene through CpG island hypermethylation and/or induction of deleterious point mutations.

on inactivated X chromosomes (33, 34). CpG islands are found mainly in promoters and exons of 50 to 60% of all human and mouse genes, including almost all constitutively expressed housekeeping genes (35, 36). Because DNA methylation generally results in gene silencing (see below), the absence of methylation within CpG islands impedes silencing and permits continued expression of many genes that are required throughout development.

Reprogramming of DNA methylation patterns in early embryos appears to be required for embryonic and extraembryonic X-chromosome inactivation in females and may be linked to first decisions controlling subsequent cell fate and distinct lineage development (37–39). Developing germ cells are also globally reprogrammed to reestablish the imprinted methylation pattern that will survive the next round of reprogramming in preimplantation embryos (40–46). Most of the CpG sites that become methylated in early embryos are stably maintained in this state, except for CpG islands and other linked regions of genes that become actively expressed during subsequent extrauterine development (21, 28, 47–49). As such, somatic cells in children and adults may respond to internal developmental and environmental cues through specific, rather than global, changes in DNA methylation. These changes may include specific demethylation, which leads to cell type-specific methylation patterns that potentially regulate tissue-specific gene expression (21, 47–49). However, much work remains to establish the precise role(s) of changes in DNA methylation patterns in postpartum mammalian development.

DNA METHYLATION IN THE SPECTRUM OF EPIGENETICS

A complex interrelationship exists between DNA methylation and additional types of epigenetic (or nonmutational) alterations that modulate gene activity, including changes in histone modifications, chromosome structure, and DNA remodeling complex composition. However, caution must be applied to interpreting results obtained from different biological systems since these interrelationships are at least partially distinct between organisms, suggesting that epigenetic mechanisms discovered in one organism may or may not be applicable to another. For example, yeast, like mammals, contain an emerging histone modification code and yet, unlike mammals, yeast have no demonstrable DNA methylation (50, 51). The filamentous fungus *Neurospora crassa* exhibits histone modifications that appear to direct sites of DNA methylation, while the mechanism(s) that directs methylation patterning in mammals is not yet resolved (52). Also, plants differ from mammals in that they have abundant non-CpG DNA methylation (53, 54).

In general, an association between DNA methylation and gene silencing has been observed (1, 55, 56). Genes that are not expressed are methylated predominantly at CpG dinucleotides and the associated transcription repression is manifest in stable and heritable changes in local chromatin structure (57–59). DNA methylation also helps to inactivate and silence exogenous viral and parasitic genomes and repetitive DNA elements in somatic cells (1, 55, 56). Transcription repression by methylated CpG appears to be mediated either directly, by the inhibition of essential transcription factor binding to methyl-CpG containing DNA motifs, or indirectly, by the recruitment of methyl-CpG-binding proteins that subsequently recruit histone deacetylase (HDAC) containing corepressor complexes to methylated DNA (4, 60–69). These HDAC corepressor complexes generate hypoacetylated histones H3 and H4 that are organized into repressive or so-called closed nucleosome structures across unexpressed or silenced genes. In undermethylated and expressed genes, histones H3 and H4 are acetylated and arranged into local chromatin structures with open nucleosomal organization (57, 58). Additional recent work has revealed an intricate "histone code" of modifications that include, in addition to acetylation, alterations in methylation and phosphorylation of specific histone amino acid residues that may regulate the transcription factor accessibility of specific genes (reviewed in 70).

CpG methylation, histone modifications, and chromatin structures are radically different between expressed versus silenced and unexpressed genes. In contrast to the well-studied features of chromatin structure at these two extremes, the dynamic changes that occur in either activating or silencing genes are not so well resolved (71). The progression of changes in CpG methylation, histone deacetylation, and nucleosome structure (i.e., loss of accessibility to DNase or restriction enzymes) has been analyzed in the silencing of transfected chromosomally integrated reporter genes and exogenous retroviral genomes in infected cells (72–74). Gene silencing in both systems showed progressive increases in CpG methylation and histone deacetylation and decreases in chromatin accessibility over time. Remarkably, in both systems, gene silencing occurred early and preceded the appearance of appreciable CpG methylation (72, 74). Gene silencing at early stages could be reversed by addition of HDAC inhibitors, implying that histone deacetylation precedes extensive CpG methylation (74). However, when CpG methylation had increased to dense methylation patterns at later times in silencing, 5-azacytidine pretreatment to inhibit DNA methyltransferase enzyme activity and decrease DNA methylation was required for HDAC inhibitor reversal of gene silencing (72, 74). These results suggest that increased levels of methyl-CpG fix and stabilize the state of silenced genes and that one function of dense CpG methylation may be to lock genes in a silenced chromatin state, such as heterochromatin.

DNA METHYLATION AND HEMATOLOGIC MALIGNANCIES

DNA methylation patterns have been described for few genes in normal hematopoietic development (47–49). Despite this, the DNA methylation status for a number of genes in sporadic hematologic tumors has been determined (Table 1, with references therein). Most of these genes were originally chosen for study due to their presumed or known role(s) in tumorigenesis, metastasis, or other aspects of malignant progression in hematologic or other cancer types. In addition, some of the investigated genes were chosen because they were found to be aberrantly silenced or expressed in surveys of tumor samples compared to their patterns of expression in the cell types from which they were derived. Some genes were subsequently examined for genetic lesions to correlate with dysregulated expression. However, gene sequences from some individual samples failed to demonstrate loss of heterozygosity, mutations, translocations, or other genetic aberrations, indicating that additional types of cellular control could be responsible for tumorigenic gene silencing or activation. Such results undermined the hypothesis that sporadic hematologic malignancies arose through purely genetic (i.e., primary DNA sequence) alterations. Many of these silenced genes were subsequently investigated with a variety of techniques to establish the presence, and sometimes the extent, of DNA methylation as a source of aberrant silencing or activation. Most of these studies involved examination of one or a few genes in multiple tumor samples of the same histologic subtype and yielded a tantalizing yet incomplete picture of the methylation pattern of the tumor type in question. For example, in hematologic malignancies, hypermethylated genes were detected in multiple fundamental pathways involved in cancer, including cell cycle control (*p15(INK4b), p16(INK4a), RB1, p27(Kip1), p73*), DNA damage repair (*O^6MGMT*), apoptosis inhibition (*DAPK*), tumor invasion/metastasis (*E-cadherin*), and growth factor responses (*ER, EphA3*; Table 1). Furthermore, distinct gene methylation patterns have also been detected in many nonhematologic malignancies, such as those involving the reproductive system. Here, studies have shown that *CD44, EDNRB,* and *PTEN/MMAC1* genes were frequently methylated in prostate cancer, while *HIC1, p16, BRCA1,* and *E-cadherin* were often methylated in sporadic breast cancers (75–82).

Interestingly, using this candidate gene approach, it became evident that DNA methylation patterns for specific genes were either unique to one or several tumor types or shared between different tumor types. The *p15* CDK inhibitor of the cell cycle was among the most common genes showing high levels of methylation in hematologic malignancies, whereas it was rarely if at all methylated in colon, breast, or lung cancers (83). By contrast, methylation of the *p16* CDK cell cycle inhibitor gene was frequently observed in sporadic breast, colon, bladder, prostate, and hematologic cancers (76, 84–88). Importantly, detection of hyper- or hypomethylation of specific genes in distinct cancer types, when correlated with changes in gene expression, potentially provides additional markers for classifying diagnostically difficult cases. Such detection may also provide an opportunity to better predict disease outcome and develop therapies targeted against specific pathway defects based on cancer types. Furthermore, the occurrence of hyper- or hypomethylated genes in common between cancer types suggests coordinated mechanisms of gene dysregulation in malignant transformation and may provide the opportunity to develop broadly active therapeutic reagents. Underscoring this conservation of distinct or shared methylation patterns in tumorigenesis, a recent study showed that methylation patterns in hereditary breast and colon cancer are similar to the patterns identified for specific genes in sporadic breast and colon cancer cases (89).

However, the list of potentially relevant methylated genes in each cancer type is incomplete and the techniques used to determine methylation patterns vary

TABLE 1

Genes That Demonstrate DNA Methylation in Primary Hematologic Tumor Samples

Gene	Tumor type	Protein function	Incidence (%)	Method used[a]	References	Expression
BCR-ABL(Pa)	CML (chronic)	Translocated tyrosine kinase	4/14 (29)	REP	(121)	No
	CML (chronic)		57/93 (61)	SB	(122)	No
	CML (chronic)		1/6 (17)	MSP	(123)	No
	CML (chronic)		19/30 (63)	REP	(124)	No
	CML (accelerated)		3/3 (100)	REP	(121)	No
	CML (accelerated)		9/12 (75)	SB	(122)	No
	CML (accelerated)		2/2 (100)	MSP	(123)	No
	CML (accelerated)		4/4 (100)	REP	(124)	No
	CML (blast)		11/11 (100)	REP	(121)	No
	CML (blast)		4/5 (80)	SB	(122)	No
	CML (blast)		3/3 (100)	MSP	(123)	No
	CML (blast)		2/2 (100)	REP	(124)	No
	CML		17/21 (81)	Ms-SnuPE	(125)	No
CACNAIG	AML	T-type calcium channel	3/23 (13)	MCA	(99)	No
Calcitonin	Adult AML	Peptide hormone	12/17 (71)	BS	(126)	No
DAPK	Lymphoma	Apoptosis	41/53 (77)	MSP	(83, 127)	No
	Leukemia		8/86 (9)	MSP	(83, 127)	No
	Multiple myeloma		20/26 (77)	MSP	(128)	No
E-cadherin	Adult AML	Adhesion	9/13 (69)	BS	(126)	No
	Leukemia		30/75 (40)	MSP	(83)	No
EphA3	ALL (pre-B and T)	Receptor tyrosine kinase	5/5 (100)	BS	(129)	Variable
ER	Adult AML	Estrogen receptor	20/36 (56)	BS	(130)	No
	Adult AML		7/12 (58)	BS	(126)	No
	Childhood ALL		8/9 (89)	SB	(131)	No
	Adult ALL		17/18 (94)	SB	(131)	No
	AML		21/23 (91)	SB	(131)	No
	Chronic CML		3/6 (50)	SB	(131)	No
	Blast CML		9/9 (100)	SB	(131)	No
	Lymphoma		5/8 (63)	SB	(131)	No
GPR37	Adult AML	G-protein-coupled receptor	26/36 (72)	BS	(130)	No
HIC1	AML	Candidate tumor suppressor	6/61 (10)	SB	(91)	No
	NHL		2/8 (25)	SB	(91)	No
	ALL		17/25 (68)	SB	(91)	No
	Chronic CML		3/6 (50)	SB	(91)	No
	Blast CML		8/8 (100)	SB	(91)	No
	Adult AML		10/12 (83)	BS	(126, 132)	No effect
MDR1	Adult AML	Drug efflux	13/36 (36)	BS	(130)	No
MINT1	Adult AML	Protein binding	7/36 (19)	BS	(130)	No
Myf-3(MyoD)	Leukemia and NHL	Muscle transcription factor	115/123 (93)	SB	(133)	No effect
	Adult AML		28/36 (78)	BS	(130)	No
O^6MGMT	Lymphoma	DNA repair	15/61 (25)	MSP	(83, 134)	Silencing
p15(INK4b)	Multiple myeloma	CDK inhibitor 2B	8/12 (67)	SB	(87)	No
	CML		8/34 (24)	Ms-SnuPE	(125)	No
	Adult AML		53/60 (88)	SB and MSP	(83, 135)	No
	Adult AML		24/42 (57)	Modified REP	(136)	Silencing
	Adult AML		6/8 (75)	BS	(137)	No
	Adult AML		11/36 (31)	BS	(130)	Silencing
	Adult AML		13/19 (68)	BS	(126)	No
	Childhood AML		14/29 (48)	SB and REP	(138)	No
	Childhood AML		18/27 (67)	SB and MSP	(83, 135)	No
	Adult ALL		5/7 (71)	SB and MSP	(83, 135)	No
	Childhood B-ALL		12/29 (41)	SB	(139)	Silencing
	Childhood B-ALL		11/23 (48)	SB and MSP	(83, 135)	No
	Childhood T-ALL		6/12 (50)	SB and MSP	(83, 135)	No
	Childhood T-ALL		8/17 (47)	SB	(139)	Silencing
	Childhood T-ALL		17/45 (38)	SB	(140)	Silencing
	MDS		16/32 (50)	SB and REP	(141)	No
	MDS		10/15 (67)	Modified REP	(136)	Silencing
	MDS		17/50 (34)	MSP	(90)	No
	B-NHL		30/47 (64)	REP	(142)	Silencing
	T-NHL		4/9 (44)	REP	(142)	Silencing
	Burkitt's lymphoma		4/8 (50)	SB and MSP	(83, 135)	No
p16(INK4a)	Multiple myeloma	CDK inhibitor 2A	9/12 (75)	SB	(87)	No
	Multiple myeloma		8/16 (50)	MSP	(143)	No
	B-NHL		15/47 (32)	MSP and REP	(142)	Silencing
	B-NHL		20/62 (32)	MSP	(144)	No
	T-NHL		3/20 (15)	SB and REP	(88)	No
	T-NHL		2/9 (22)	MSP and REP	(142)	Silencing
	CNS lymphomas		5/18 (28)	MSP	(145)	Silencing
	B-NHL low grade		4/16 (25)	SB and REP	(88)	No
	B-NHL high grade		6/31 (19)	SB and REP	(88)	No

TABLE 1—*Continued*

Gene	Tumor type	Protein function	Incidence (%)	Method used[a]	References	Expression
	B-NHL high grade		5/6 (83)	SB and MSP	(83, 135)	No
	Burkitt's lymphoma		6/8 (75)	SB and MSP	(83, 135)	No
	Adult AML		6/36 (17)	BS	(130)	No
	Adult AML		5/20 (25)	BS	(126)	No
	Childhood AML		11/29 (38)	SB and REP	(138)	No
p27(Kip1)	CNS lymphomas	CDK inhibitor 1B	2/18 (11)	MSP	(145)	Silencing
p73	ALL	Candidate tumor suppressor	11/35 (31)	MSP	(146)	Silencing
	Burkitt's lymphoma		3/10 (30)	MSP	(146)	No
PITX2	Adult AML	Homeotic gene	31/36 (86)	BS	(130)	No
PTC-A	Adult AML	WNT signaling	6/36 (17)	BS	(130)	No
PTC-B	Adult AML	WNT signaling	4/36 (11)	BS	(130)	No
RB1	CNS lymphomas	Tumor suppressor	2/18 (11)	MSP	(145)	Silencing
SDC4	Adult AML	Surface receptor	22/36 (61)	BS	(130)	Silencing
THBS1	Adult AML	Angiogenesis inhibitor	9/36 (25)	BS	(130)	No

Note. Data from cell lines are excluded from this compilation because cell line methylation patterns may differ dramatically from similarly classified primary sample methylation patterns (147). The last column describes whether gene expression was simultaneously examined with the methylation status for each gene studied. [a] Key: BS, genomic bisulfite sequencing; MSP, methylation-sensitive PCR; SB, Southern blot with methylation-sensitive and -insensitive restriction enzyme digests; REP, digestion with methylation-sensitive or -insensitive restriction enzymes followed by PCR; Ms-SnuPE, methylation-sensitive single nucleotide primer extension; MCA, methylated CpG island amplification.

widely in their sensitivity and information content. For many genes studied, merely the presence of methylation, rather than its extent, has been established. Also, many genes have not been investigated for a correlation between methylation and alterations in gene expression. In fact, for many genes it is unclear if the regions examined for DNA methylation even impact gene expression. Therefore, new methods for determining the breadth of aberrant gene methylation and the impact on gene expression are needed to provide a more complete picture of the cellular pathways involved for each tumor type. In this regard, some ideas and techniques have been borrowed from recently developed massively parallel gene expression screening methods and are being adapted to DNA methylation determination. These new methods provide some of the foundation for evaluation of the "methylome" (or the "epigenome") to determine DNA methylation profiles in normal and tumor samples.

LESSONS FROM GENE EXPRESSION PROFILING

Changes in DNA methylation patterns for multiple genes may occur with the progression of hematologic cancers. Some genes, such as *p15(INK4b),* appear to be methylated early in the development of a tumor and may be part of the process that initiates malignancy (90). Other genes, such as *HIC1,* appear to be methylated late in tumor progression and may be involved in metastases or the evolution of aggressive tumor behavior (91). Methylation changes may have a large or small impact on the expression of individual genes but, when summed across the genome, even small changes in gene methylation may radically affect the expressed gene profile of a cell during malignant transformation. In fact, the importance of even small changes in a single gene's expression cannot be overemphasized. For example, a 50% reduction in the expression of one allele of the *APC* tumor suppressor gene can result in development of familial adenomatous polyposis coli (92). Even the modest number of genes for which changes in DNA methylation have a documented impact on expression levels suggest that aberrant methylation patterns could have as large a role in promoting tumorigenesis as changes in DNA base sequence composition by traditional mutational mechanisms.

A revolution in the methods for diagnosing, classifying, and predicting outcomes of human malignancies is well under way. Traditional techniques such as visual and histologic inspection, immunohistochemistry, cytogenetics, molecular diagnostics, and electron microscopy are being merged with newer molecular tools that rely on detection of gene and protein expression patterns. Array-based gene and protein expression profiling has the potential to detect critical molecular differences between tumors even within traditional tumor categories and thereby promises to yield information for subclassification, customized therapies, and individualized outcome predictions. These array-based survey procedures can simultaneously detect changes in the expression of thousands of genes or proteins and are predicted by many to eventually supercede current methods in detection and management of human malignancies due to their superior information content.

Two distinct hematologic malignancies were among the first human tumor types to be scrutinized using array-based gene expression profiling. This technique accurately replicated the traditional classification of acute lymphoblastic versus acute myeloid leukemias (93). Expression profiling and gene cluster analysis

TABLE 2

Methods for Detecting DNA Methylation

Method name[a]	Some advantages	Some disadvantages	References
NNA	Quantitative whole genome methylation	Cannot determine the DNA sequence context	(30, 97)
SB	Semiquantitative; rapid and relatively easy detection	Sites with MS and MI enzymes only; relatively abundant DNA required; insensitive for low-level DNA methylation; false positive results with partial digestion	(98)
MCA	Increased sensitivity over SB; can isolate novel methylated sequences	Loss of specificity due to: Partial enzyme digestion; poor linker ligation reaction; inefficient subtractive hybridization	(99)
Genomic BS	Detects every methylated cytosine in a sample; may detect low-level DNA methylation	False positives due to chemical non-conversion; Difficult to quantitate extent of methylation at a site	(100, 101)
COBRA	Better quantitative assessment than genomic BS	Sites with MS and MI enzymes only	(104)
MSP	Better quantitative assessment than genomic BS	Surveys mainly CpG-dense regions	(105)
Ms-SNuPE	Semiquantitative for small sample amount	Only a few cytosines can be analyzed simultaneously	(106)
MethyLight	MSP with high-throughput detection	Surveys mainly CpG-dense regions	(107)

[a] Key: NNA, nearest neighbor analysis; MS and MI SB, methylation-sensitive and -insensitive restriction digestion followed by Southern blot; MCA, methylated-CpG island amplification; genomic BS, genomic bisulfite sequencing; COBRA, combined bisulfite restriction analysis; MSP, methylation-specific PCR; Ms-SNuPE, methylation-sensitive single nucleotide primer extension.

was also used to subclassify diffuse large B cell lymphoma (DLBCL), a particularly heterogeneous group of B cell tumors, into those derived from activated versus germinal center B cells (94). This subclassification further stratified DLBCL into aggressive, poor-outcome versus prolonged survival subtypes. A separate subclassification scheme for DLBCL, based on gene expression profiling coupled to a supervised computer learning algorithm, showed that specific genetic pathway alteration rather than cell-of-origin issues was a more accurate predictor of outcome and resulted in improved choices for therapy and patient lifestyle (95). The powerful capabilities and promise of array-based gene and protein expression profiling in hematologic malignancies has been the subject of numerous recent reviews (for an example, see 96).

The spectrum of characteristic and idiosyncratic changes detected by gene and protein array screening in cancer samples implicates defects in the fundamental processes that regulate gene expression and cellular homeostasis. Cause and effect is not established by this screening procedure; rather, a static picture of global expression emerges of the cancerous tissue under examination. At the DNA level, changes in expression are due to genetic and/or epigenetic alterations (Fig. 1). One area of recently increased activity and unknown promise is the discovery of DNA methylation profiles on a candidate gene or genomewide screening basis. Part of the promise of this approach is the potential to expose new types of causation and new gene targets in cancer for which traditional genetic methods have failed or have provided an inadequate or incomplete picture.

DETECTING DNA METHYLATION

Many methods have been developed for investigating patterns of DNA methylation and each has advantages and disadvantages (Table 2). One of the earliest techniques used was a modified nearest neighbor analysis (NNA), which detects residues immediately 3′ of methylated cytosines in whole genomes (30, 97). This analysis is semiquantitative for total genomic cytosine methylation but cannot determine which gene(s) contains the methylated cytosine(s) or its position(s) within the examined DNA. Another early method was use of methylation-sensitive and -insensitive restriction enzyme digestion followed by Southern blot hybridization (98). This analysis has the advantage of yielding semiquantitative methylation data at specific cytosines and is rapid and easy to perform. However, its main disadvantages are that only a relatively small number of CpG and non-CpG dinucleotide positions can be surveyed based upon the availability of sequence-specific methylation-sensitive and -insensitive restriction enzymes and abundant starting DNA is required. Also, infrequent low-level methylation will be missed by Southern blot hybridization, resulting in low sensitivity, and false positives can result from incomplete enzyme digestions. An alternate, recently developed technique that utilizes methylation-sensitive restriction enzyme cutting is methylated-CpG island amplification (MCA; 99). In this method, DNA methylation differences between two samples (i.e., normal and tumor) are determined by methyl-sensitive enzyme cutting followed by a subtractive hybridization between the cut samples. Advantages of this technique

TABLE 3
High-Throughput DNA Methylation Profiling Techniques

Method	Description of technology	Some advantages	Some disadvantages	References
RLGS	MS and MI enzyme digests with 2-D gel detection	Detects unknown genes	Requires MS and MI enzymes	(109–111)
DMH	MS and MI enzyme digests with cDNA array detection	Screens multiple CpG islands	Requires MS and MI enzymes	(115–117)
MSO	MS and PCR coupled to oligonucleotide array detection	Enzyme-independent assay	May miss sparse methylation	(119)
MDMA	BS with multiplex PCR and oligo array detection	Multiplex PCR reactions	Limited choice of primers	(120)

[a] Key: RLGS, restriction landmark genomic scanning; DMH, differential methylation hybridization; MSO, methylation-specific oligonucleotide microarray; MDMA, microarray-based DNA methylation analysis; MS, methylation-sensitive; MI, methylation-insensitive.

are increased sensitivity and the ability to isolate novel methylated sequences, while the main disadvantages are potential loss of specificity from incomplete enzyme digests, poor linker ligation reactions, and inefficiencies in subtractive hybridization.

About 10 years ago, a technique based upon chemical conversion of cytosine, but not methylcytosine, to uracil with sodium bisulfite treatment and base addition was developed (100, 101). Genomic bisulfite sequencing analysis provides relatively high sensitivity and specificity by determining the cytosine methylation pattern and sequence context for both CpG and non-CpG sites and provides the most complete detection of low-level cytosine methylation currently available. However, as originally applied, detection required PCR amplification of bisulfite-converted products followed by cloning and then automated DNA sequencing. Inefficiencies in some of these steps may yield a poor representation of the percentage of methylation for any particular cytosine in a sample. In addition, the overall process is relatively slow and laborious and potential nonconversion, due to poorly performed reactions or interference by sequence-specific structural constraints, may lead to falsely positive results (102).

Over the last few years several modifications of the original bisulfite sequencing protocol have been developed. One modification, called combined bisulfite restriction analysis (COBRA), replaces direct sequencing of PCR-amplified products with restriction digestion at sites that may or may not be modified by bisulfite treatment (103, 104). Again, this is a limited examination of potentially methylated sites but can potentially give a more quantitative estimate of the percentage of methylation at these sites than can sequencing cloned PCR products. Another modification, called methylation-specific PCR (MSP), employs initial bisulfite conversion followed by PCR amplification with primers designed to discriminate between converted and nonconverted cytosines (105). The dependence of MSP on relatively high CpG site densities restricts primer design and regions of analysis essentially to CpG islands throughout the genome. A third modification, called methylation-sensitive single nucleotide primer extension (Ms-SNuPE), utilizes bisulfite-treated DNA followed by a primer extension reaction that incorporates either radioactive cytosine or thymidine based upon conversion or nonconversion of a particular CpG cytosine in a DNA sample (106). The power of this technique is the semiquantitative detection of methylcytosine at a given nucleotide position from small samples without use of PCR or restriction enzymes; its weakness is the restricted number of cytosines that can be simultaneously analyzed and the addition of gel isolation and gel detection procedures. A fourth modification, called MethyLight, combines bisulfite-treated DNA with methylation-specific PCR primers and real-time fluorescence detection to increase the number of CpG cytosines that can be simultaneously surveyed within and between genes in a sample (107). As such, MethyLight is the first attempt to combine powerful genomic bisulfite sequencing technology with high-throughput analysis to develop a more complete picture of the methylation state of a patient sample.

DNA METHYLATION PROFILING IN PATIENT SAMPLES

An ideal technique for determining the DNA methylation profile of a particular sample would include an easy, inexpensive, quick, and reproducible method to survey all of the cytosine residues within the sample. As discussed in the preceding section, no current method ideally combines all of these desirable features. Furthermore, even with an ideal procedure, cellular heterogeneity will continue to remain an obstacle. Illustrating this difficulty, methylation profile analysis of single peripheral blood T cells from a healthy male donor discovered extensive heterogeneity in CpG island methylation for multiple genes throughout the genome (108). Clearly, within any tumor sample individual cells will contain distinct (and perhaps evolving) patterns of DNA methylation that may presage unexpected aggressive behavior compared to the antici-

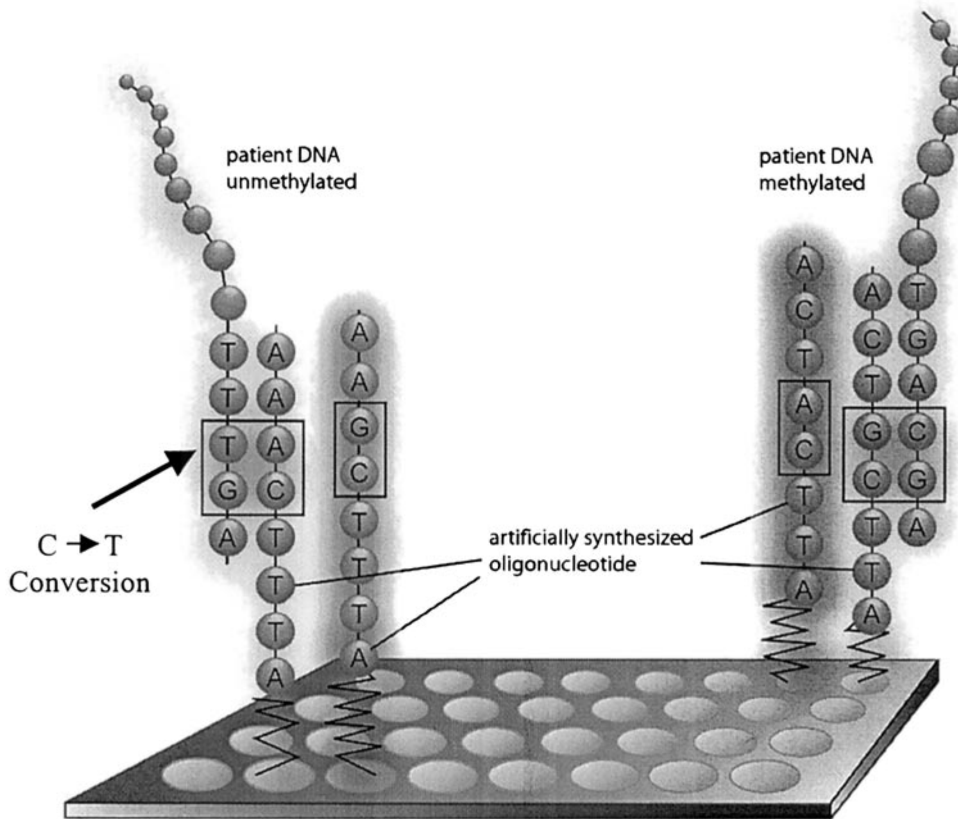

FIG. 2. Microarray-based DNA methylation analysis (MDMA; 120). In this technique, patient samples are treated with bisulfite and base, followed by PCR amplification with fluorescently labeled PCR primers flanking the region to be analyzed. Each CpG position is then surveyed by hybridization of the amplified and tagged patient sample DNA to a glass slide containing arrayed oligonucleotides. The spotted oligonucleotides are synthesized to discriminate between methylated (bisulfite nonconverted) or nonmethylated (bisulfite converted) CpG sites. For example, in the figure on the left an oligonucleotide with a CpG site changed to CpA hybridizes with a C-to-T bisulfite-converted DNA probe, while on the right an oligonucleotide that retains the CpG site hybridizes with its compliment from the methylated, and bisulfite noncoverted, patient sample. In this way, a ratio between methylated and unmethylated CpG dinucleotides from a region of interest in a patient sample can be derived from comparison of the fluorescent intensities between the arrayed spots (figure presented with permission; copyrights by Epigenomics, AG).

pated outcome based on the predominant methylation pattern. Clonal evolution, through genomic, and potentially epigenetic, instability is a key feature of malignant progression. This occurrence will force the examination of numerous samples to validate or refute the notion of a predominant, genomewide methylation pattern that can accurately predict outcome or effectively guide treatment options.

In addition to methodologies that evaluate candidate genes one at a time in a given sample (see Table 2), several genomewide survey techniques have been developed in recent years (Table 3, with references therein). One of the earliest methods, restriction landmark genomic scanning (RLGS), utilizes a methylation-sensitive restriction enzyme digestion followed by isotopic end-labeling and electrophoresis in a one-dimensional tube gel. Then, a second *in situ* digestion with a methylation-insensitive restriction enzyme is performed followed by electorphoresis into an acrylamide gel at a 90° angle to the first gel, creating a two-dimensional profile (or "fingerprint") of genomewide methylation at the methylation-sensitive restriction enzyme cutting site (109–111). The intensity of resultant spots in the 2-D gel is proportional to the amount of cutting, and hence the amount of DNA methylation, that occurred at the end-labeled position in the genome. This technique is also able to detect hemi- or allele-specific gene methylation, as occurs in imprinted sequences (112). Since many CpG islands contain *Not*I (GCGGGCCGC) recognition sites, use of this methylation-sensitive enzyme provides a crude survey of CpG island methylation. The combination of *Not*I and *Eco*RV digests yields 1000 to 3000 DNA fragments from human patient samples, seen as discrete spots in a 2-D gel. To facilitate spot identification, a library can be constructed with the same two restriction enzymes used in patient sample analyses (113). Spiking of a specific end-labeled library clone into a

sample before electrophoresis will enrich the signal intensity at a unique spot in the 2-D gel, thereby identifying the DNA fragment in question (114). Although a powerful technique that is able to detect DNA methylation in numerous known and unknown genes, the main limitations of RLGS are its ability to survey only positions for which methylation-sensitive and -insensitive enzymes exist and the requirement for relatively abundant starting DNA.

A second technique, differential methylation hybridization (DMH), merges array-based technologies pioneered in gene expression profiling with methylation-sensitive restriction enzyme cutting (115–117). Multiple GC-enriched DNA fragments from a human CpG island genomic library (CGI) are stamped in duplicate positions on a nylon membrane (118). The DNA samples (tumor and normal reference) are digested with *Mse*I (TTAA recognition site) and depleted of highly repetitive elements with subsequent linker addition. The linker-ligated samples are then digested with methylation-sensitive *Bst*UI (CGCG recognition site). After this, the tumor and reference DNAs are PCR amplified with primers to the added linkers and radioactively labeled. Hybridization to the stamped nylon membrane is first performed with the reference DNA, followed by stripping and rehybridization with the tumor sample DNA. A comparison of the hybridization intensities for individual spots between reference and tumor samples forms the basis for detection of methylation within the linker-spanned interval. Again, limitations of this technique are that it screens only positions within regions that contain methylation-sensitive and -insensitive restriction enzyme digest sites and relatively abundant starting DNA is required.

Recently, two new restriction enzyme-independent methods for genomewide methylation analysis have been described. Methylation-specific oligonucleotide (MSO) microarray technology merges MS–PCR with detection on oligonucleotide arrays (119). In this procedure, DNA from a sample is sodium bisulfite treated, PCR amplified, and then hybridized to glass slide-arrayed oligonucleotides designed specifically to discriminate between converted and unconverted CpG sites. So far, this procedure has been validated for the GC-rich CpG island of the human estrogen receptor α gene in breast cell line and primary tumor samples (119). It remains unclear how heavily this method will depend on the CpG island sequence being queried and its degree of hypermethylation. In the event that a CpG island has sparse methylation, critical methylated CpG dinucleotides may be missed by this survey.

Most recent is the development of microarray-based DNA methylation analysis (MDMA), a technique which combines genomic bisulfite sequencing with array-based screening and supervised or unsupervised computer learning algorithms to classify tumors (Fig. 2; 120). In this method, fluorescently labeled PCR primers that do not overlap CpG dinucleotides in target genes were used to amplify bisulfite-treated DNA from tumor samples. This was followed by hybridization to oligonucleotides arrayed on glass slides that could discriminate between single bisulfite-converted or nonconverted CpG sites in GC-rich regions of multiple candidate genes. An advantage of this method is that it does not rely on PCR primer ligation or the use of degenerate primers to amplify regions in which CpG dinucleotides may or may not be converted by bisulfite treatment. Another advantage is the ability to simultaneously generate probes in multiplex PCR reactions without biased amplification for converted versus nonconverted sequences. However, this approach is limited by the choice of primers whose design is based on the lack of CpG dinucleotides flanking regions of interest for particular genes. As one proof of principle, MDMA was applied to two classes of acute leukemia patient samples, as was done using gene expression profiling previously (93). Surveying of methylation differences at just five CpG sites yielded a 94% concordance in classifying ALL or AML samples versus standard pathology-based classification schemes (120).

In summary, all of the desirable features, and many of the problems, of each technique used to detect DNA methylation one gene at a time still remain following the marriage of each detection technique with high-throughput methodologies, such as array-based or 2-D gel screening procedures. Because of the significance of DNA methylation patterns in tumor development, behavior, diagnosis, outcome predictions, and treatment decisions, new tools will undoubtedly continue to be developed to accurately assess with increasing detail the methylation state of individual tumor samples. These new technologies are currently being developed in both academia and industry around the world and will need careful evaluation in the coming years.

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