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Special Article

CpG Methylation Analysis—Current Status of Clinical Assays and Potential Applications in Molecular Diagnostics

A Report of the Association for Molecular Pathology

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Methylation of CpG islands in gene promoter regions is a major molecular mechanism of gene silencing and underlies both cancer development and progression. In molecular oncology, testing for the CpG methylation of tissue DNA has emerged as a clinically useful tool for tumor detection, outcome prediction, and treatment selection, as well as for assessing the efficacy of treatment with the use of demethylating agents and monitoring for tumor recurrence. In addition, because CpG methylation occurs early in preneoplastic tissues, methylation tests may be useful as markers of cancer risk in patients with either infectious or inflammatory conditions. The Methylation Working Group of the Clinical Practice Committee of the Association of Molecular Pathology has reviewed the current state of clinical testing in this area. We report here our summary of both the advantages and disadvantages of various methods, as well as the needs for standardization and reporting. We then conclude by summarizing the most promising areas for future clinical testing in cancer molecular diagnostics. ***(J Mol Diagn 2009, 11:266–278; DOI: 10.2353/jmoldx.2009.080125)***

CpG-island methylation of gene promoter regions plays a major role in regulation of gene expression. CpG islands have been defined as genomic regions with a minimum of 200 bp, with % G+C greater than 50 and with observed/expected CpG ratio above 60%.[1] More recently, studies have further defined CpG islands as regions of DNA greater than 500 bp with a G+C equal to or greater than 55% and observed CpG/expected CpG of 0.65.[2] In actively transcribed genes the CpG sites in CpG islands of promoter regions are unmethylated, whereas increased cytosine methylation in the island CpG sites is associated with reduced gene expression and possible gene silencing.

Gene regulation by CpG methylation is involved in a large spectrum of biological processes, from development to aging, including inflammatory and infectious diseases, and cancer. The availability of molecular tech-

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Standard of practice is not being defined by this article, and there may be alternatives.

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niques to evaluate the methylation status of CpG islands in cancer related genes has prompted an explosion of studies in this area, and CpG methylation tests are emerging as clinically useful tests. CpG hypermethylation is critical to silencing of the expression of tumor suppressor genes, such as those that encode CDKN2B (p15), CDKN2A (p16), and *O*-6-methylguanine-DNA methyltransferase (MGMT), as well as globally regulating differentiation programs in many tumor types. The levels of CpG methylation have thus been used to subclassify tumors,[3] predict response to chemotherapeutic agents that are metabolized or antagonized by cellular enzymes regulated by promoter methylation,[4] and to assess the effects of methylating and demethylating therapies. In tumors in which CpG methylation silencing of particular suppressor genes is highly prevalent, the levels of such methylated DNA in blood or body fluids may be indicative of the presence of cancer cells or their circulating DNA.[5,6]

In this report we compare the current techniques and methodological considerations for assessing DNA CpG methylation and summarize the current status of CpG methylation testing with emphasis on neoplasia. Specific sections cover: 1) current methods for clinical testing of CpG methylation and the decision-making criteria for assay selection and validation requirements; 2) applications of CpG methylation testing for cancer detection, prognosis, and monitoring using tumor tissue, cell-free plasma and serum, and cytological and other biological samples; and, 3) the potential use of methylation interference and monitoring of CpG methylation status for prediction of tumors related to bacterial and viral infections.

To promote standardization in clinical reporting, we have used Human Genome Organization (HUGO) gene nomenclature throughout the text (*http://www.genenames.org/index.html*). A table listing the standard gene names used in this document with the common names is provided in Table 1.

Table 1. HUGO Gene Nomenclature Committee-Approved Symbols for Genes Discussed in the Text

Symbol	Common name
APC	adenomatous polyposis coli
CACNA1G	calcium channel, voltage-dependent, T type alpha-1G subunit
CADM1	cell adhesion molecule 1 (IGSF4)
CCND2	cyclin D2
CDH1	cadherin 1 (E-cadherin)
CDH13	cadherin 13 (H-cadherin)
CDKN2A	cyclin-dependent kinase inhibitor 2A (p16)
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15)
CRABP1	cellular retinoic acid binding protein 1
DAPK1	death-associated protein kinase 1
DNMT	DNA methyltransferase
ESR1	estrogen receptor alpha
FHIT	fragile histidine triad
FRBP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor, MDGI)
GSTP1	glutathione S-transferase pi
HIC1	hypermethylated in cancer 1
HSD17B4	hydroxysteroid (17-beta) dehydrogenase 4
HSIL	high-grade squamous intraepithelial lesion
IGF2	insulin-like growth factor 2
LATS1	large tumor suppressor, homolog 1
LATS2	large tumor suppressor, homolog 2
LINE	long interspersed nucleotide element
MGMT	O-6-methylguanine-DNA methyltransferase
MYOD1	myogenic differentiation 1
NEUROG1	neurogenin 1
PGR	progesterone receptor
PSA	prostate specific antigen
RARB	retinoic acid receptor beta
RASSF1	RAS association (RalGDS/AF-6) domain family 1
RUNX3	runt-related transcription factor 3
SFRP1	secreted frizzled-related protein 1 (SARP2)
SOCS1	suppressor of cytokine signaling 1
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2 (HPP1, hyperplastic polyposis 1)
TWIST1	twist homolog 1

Current Methods Used for CpG Methylation Testing

Analysis of CpG methylation requires some method of discriminating between the methylated and unmethylated DNA sequences, usually following PCR amplification of targeted sequence(s). Post-PCR detection techniques routinely used to differentiate methylated and unmethylated DNA include capillary electrophoretic separation, dideoxynucleotide sequencing,[7] pyrosequencing,[8] mass spectrometry,[3,9] high performance liquid chromatography,[10] and array hybridization.[11–17]

Technical Considerations in the Bisulfite Conversion Step

The majority of methods for methylation analysis begin with the conversion by sodium bisulfite of unmethylated cytosine to uracil (and then to thymine following *in vitro* DNA synthesis). By contrast, methylated cytosines are largely protected from this conversion process (Figure 1). Bisulfite treatment thus creates different sequences in methylated and unmethylated fragments, which can be detected by a variety of techniques.

However, the effects of bisulfite treatment on DNA are harsh and difficult to control and often result in significant DNA degradation of up to 85% to 95% of target sequences.[18] This reduction in DNA template can greatly affect assay performance, including introducing PCR bias in amplification of sequences.[19] Furthermore, the stability of bisulfite-treated DNA is reduced due to nucleotide mispairing and incomplete complementarity. Therefore, in this initial step, one needs to optimize the conditions required for full bisulfite conversion of unmethylated cytosine to uracil and yet minimize the degradative effects of this treatment on DNA.

Although there are minimal data on the effects of temperature and time of storage on the stability of bisulfite-treated DNA, most laboratories analyze bisulfite-converted DNA soon after conversion to minimize further DNA degradation. Until more data are available, ultra-low temperature storage conditions (−70°C or below) should be used if converted DNA must be stored before analy-

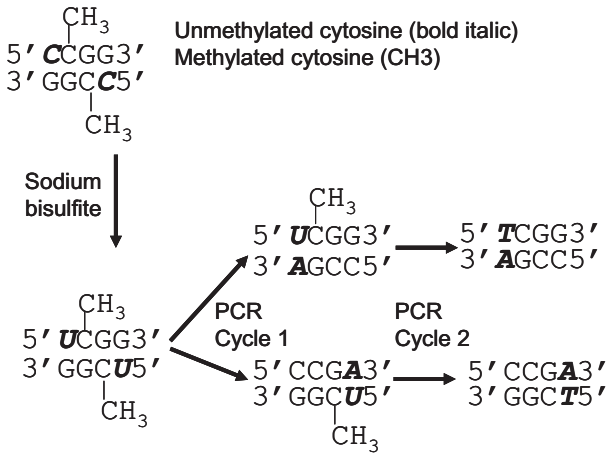

Figure 1. Bisulfite modification of DNA for methylation assays. Bisulfite modification converts unmethylated cytosine to uracil, while methylated cytosine is not modified. After PCR, uracil is replaced by thymine on the newly synthesized DNA strands.

sis. Published detailed studies to determine the effect of storage of bisulfite-treated DNA are needed. It is also advisable to include appropriate controls to validate the results obtained with such DNA.[20] Another approach to minimize DNA loss has been to perform bisulfite conversion of DNA directly in tissue lysates.[21] This method may be particularly useful for smaller samples. In practice, small samples are those that yield limited microgram amounts of DNA, such as tissue samples that are few millimeters in size, as are those obtained as endoscopic or needle biopsies. Alternatively, to decrease loss of DNA during bisulfite treatment, isolated DNA can be immobilized on nylon[22] or in agarose.[23] While complicated, bisulfite conversion can be reproducible and can be reliably used for quantitative analysis of DNA methylation (see below).[21]

Qualitative and Quantitative CpG Methylation Detection after Bisulfite Conversion

There is a wide variety of PCR-based detection methods,[24] including those in which the sequence differences between bisulfite-converted and unconverted cytosines are incorporated into the primers used for amplification, so-called methylation-specific PCR (MSP). Examples of MSP are represented in Figure 2. Alternatively, in methylation-independent PCR, the primer sequences do not function to differentiate methylated and unmethylated DNA, but rather they are detected by another method. The advantages and disadvantages of each of the techniques are compared in Table 2.

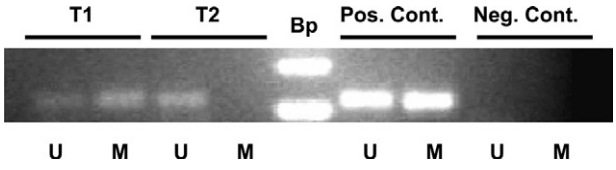

Figure 2. Methylation of the *MLH1* gene CpG island promoter region detected by methylation specific PCR (MSP). After sodium bisulfite conversion of genomic DNA from colon cancer tumor samples (T1 and T2) or positive control DNA (Pos. Cont.), PCR was performed with the primer pair specific for methylated *MLH1* DNA (M) or with the primer pair specific for the unmethylated *MLH1* sequence (U). The negative control is a PCR reaction without DNA template. The DNA size ladder (Bp) is indicated. The presence of a PCR product in the lanes labeled (M) indicates the presence of CpG methylation in the sample T1 and in the positive control. For sample T2 no MLH1 CpG methylation is detected.

Originally described in 1996,[20] MSP provides a sensitive method for detecting minimal levels of a methylated target in a sample; however, in its classical format it is nonquantitative and cannot distinguish between low and high levels of a methylated target sequence. By contrast, combining real-time PCR probes with MSP, as in the MethyLight assay, one can achieve a quantitative assessment of the level of DNA methylation of a targeted sequence.[25,26] Real-time SYBR-GREEN MSP is another quantitative MSP method that permits direct application of primers designed for nonquantitative MSP in the real-time quantitative assay. With all MSP-related quantitative assays, there is a risk of nonspecific annealing of primers, which can completely invalidate the readout.[27–30] Therefore, one should carefully design the primers and probes used for PCR amplification and should correlate methylation status with gene expression or function (such as by quantitative reverse transcription PCR or immunohistochemical evaluation of gene expression).

Methods for detecting CpG methylation after methylation-independent PCR using bisulfite-modified DNA include combined bisulfite restriction analysis,[31] pyrosequencing,[8,32–34] matrix-assisted laser desorption/ionization-time of flight,[9] and high performance liquid chromatography.[10,35,36]

CpG Methylation Detection without Bisulfite Conversion: Use of Methylation-Sensitive Restriction Enzymes

Methylation-sensitive restriction endonucleases are also routinely used to discriminate methylated and unmethylated CpG sites.[37–39] The technical bases of combined methylation-sensitive restriction enzymes and PCR for detection of CpG methylation are illustrated in Figure 3. One advantage of restriction enzyme-based analysis over bisulfite treatment methods is that it does not require modification of DNA sequences, which makes downstream analysis relatively simple, and avoids target DNA damage. Several approaches have been developed for simultaneous analysis of CpG methylation in multiple sites of selected genes[11,40] or in the whole genome.[41–44] Use of restriction enzymes for methylation assessment has certain constraints. The analysis is limited by the availability of restriction sites within the fragment of interest. Another limitation is that it provides "all-or-none" readouts that do not depend on the number of accessible restriction sites within the fragment, producing identical results regardless of whether one or all sites are unmethylated. Finally, there is a possibility of incomplete digestion, which will produce false-positive results.

Table 2. Comparison of Quantitative MSP and MIP Assays for Quantitative Assessment of CpG Methylation

	Methylation-specific quantitative PCR (MethyLight, etc.)	Methylation-independent PCR (for subsequent pyrosequencing, mass spectrometry, COBRA, etc.)
Basic characteristics	Quantification during PCR	Quantification after PCR; not specific for the methylated or unmethylated sequences
Paraffin-embedded tissue	Usable	Usable
Precision	Good	Good, especially at high-level methylation
Accuracy	Good	Good, especially at high-level methylation
Monitoring of complete bisulfite conversion	By amplification of a non-CpG genomic reference*	By the presence of non-CpG cytosine in templates that should be completely converted
Genomic reference to measure the amount of input bisulfite-converted DNA	Necessary*	Unnecessary (measuring both methylated and unmethylated sequences)
Resolution	Lower; block of CpG sites coincident with primer and/or probe sequences	Very high (single nucleotide level)
Analytical sensitivity	Very high (1% methylated sequence)	2% to10% methylated sequence (depending on subsequent detection method)
PCR design	Easy for high density CpG sites (applicable to most CpG islands)	Easy when there is a small CpG island abutted by CpG sparse areas. CpG sites within PCR primers must be limited
Closed system versus opening of PCR tubes	PCR tubes always closed	Opening of PCR tubes usually necessary
Samples for standard curves	Necessary	Unnecessary
PCR bias	Specific for methylated sequence by definition	Need to minimize PCR bias between methylated and unmethylated sequences

*Some variants of real-time PCR assays do not require a genomic reference.

Multiplex Detection Methods

A variety of microarray-based techniques have been developed that allow for simultaneous testing of multiple CpG sites in bisulfite-treated or native DNA.[11–17] This approach is particularly important when a uniform and standardized platform is needed for analysis of multiple genes such as those panels used for diagnosis or prognosis stratification of cancer specimens. The clinical applications of microarray-based techniques have yet to be determined; however, differential methylation hybridization using restriction-enzyme based approaches has been applied for selection of hypermethylated sites in colorectal,[45] ovarian,[46] and breast cancers,[40,47,48] and may be effective for prediction of drug response.[15]

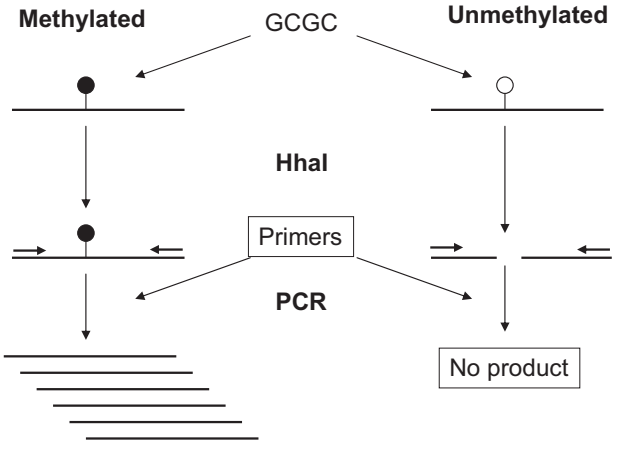

Figure 3. Methylation-sensitive restriction enzymes for detection of CpG Methylation without bisulfite conversion. DNA is first treated with HhaI methylation-sensitive restriction endonuclease and then used for PCR. When the CpG locus being amplified is not methylated, HhaI cleaves its restriction site, resulting in lack of PCR amplification; whereas, if it is methylated, the HhaI restriction sites are protected from restriction enzyme digestion, allowing for PCR amplification.

Selection of Methods for Testing Methylation of Specific CpG Islands

The decision on which method(s) to use for CpG methylation clinical testing will be based on the goals of the testing and required assay performance.[49] Several factors can influence the choice: 1) how many CpG sites (genes, promoters, etc) will be tested in each sample; 2) the anticipated heterogeneity of 'normal' and 'abnormal' cells within samples; and 3) the amount and quality of starting tissue, cells, or DNA material in each sample. If using archived pathology samples, fixative and embedding medium may significantly impact the method of choice.

A clear understanding of the assay goal(s) is essential for successful CpG methylation assay design. A CpG methylation assay used as a surrogate marker of gene expression must target CpG site(s) that are important for gene regulation. This information can be obtained from current databases and previously published papers, but a validation study should be performed to correlate methylation data with loss of protein expression or mRNA levels.

A number of online tools can help in primer/probe selection including MethPrimer,[50] (*http://www.urogene.org/methprimer*, the University of California at Santa Cruz genome browser, McArdle CpG analyzer, and Methyl PrimerExpress (Applied Biosystems, Foster City, CA). When reporting CpG sites used in an assay, it is important to indicate the template source (ie, GenBank accession number) and the coordinates of the examined DNA

Table 3. Reporting Recommendations for a CpG Methylation Assay

Pre-analytic
Clinical indication (e.g., rule out HNPCC)
Tissue source (tumor, aspirate, urine cytology), including fixative, if known
Correlated immunohistochemical or molecular result, if for gene expression or MSI correlation
Analytic
Methods employed, including description of sensitivity and/or other controls
Description of gene(s) and region(s) interrogated using standardized nomenclature
Quantitative or qualitative result
Post-analytic
Comment if methylation at interrogated CpGs is known to correlate with gene silencing
Limitations on detection accuracy or sensitivity, such as sample quality or unusual sample source

segment (see Weisenberger et al[51] for guidelines). For bisulfite-based techniques, highlighting the location of the primer and, if pertinent, probe sequences within the bisulfite-converted sequence is recommended. For methylation-sensitive restriction enzyme-based techniques, a map of the examined region and the number of restriction sites assessed by the assay should be included.

For gene expression applications, quantitative assays are preferred when homogeneous samples are available. Since low levels of CpG methylation detected in tumor samples may not correlate directly with silencing of gene expression,[21,52] quantitative methods can allow the use of cutoff-values established through a validation study with a comparison technique (eg, immunohistochemistry and quantitative reverse transcription PCR).

In contrast, CpG methylation assays that detect characteristic tumor-related genomic changes may require detection of any level of abnormal methylation as a correlative biomarker of the neoplastic process. Nonquantitative MSP may be useful for these applications, especially when quantitative levels would have little value due to variable sample composition, such as may occur with very small biopsies or cytologic specimens. Finally, genome-wide comparative analysis of CpG methylation patterns in normal tissues and tumors may require microarray approaches,[53] although validation requirements for such techniques are inherently complex and not yet well-established.

Elements of Assay Reporting, Validation, and Quality Control in CpG Methylation Assays

The essential elements of a clinical report for CpG methylation testing are summarized in Table 3. In all circumstances, the assay validation and reporting requirements need to be considered in light of the goals of testing. If CpG methylation is assessed as a surrogate marker for gene silencing (or loss of function) then assays must be validated by comparing methylation status of the selected CpG(s) with observed levels of RNA or protein expression. Discordant false-negative (ie, loss of expression of an unmethylated gene) or false-positive results (ie, intact expression of a methylated gene) may be to due to unusual biology of the examined gene (eg, in cases when methylation increases expression,[54,55]) alternative mechanisms of gene silencing, or technical issues (eg, heterogeneity of the cellular constituents, incorrect sampling, or selection of a less informative CpG site). Alternatively, when CpG methylation data are being used as correlative biomarkers, including their use as diagnostic markers[5] or as markers for the CpG island methylator phenotype (CIMP),[56] correlation with gene expression is not always apparent, since a positive methylation status may not correlate with loss of gene expression examined by methods such as immunohistochemistry.

Regardless of the application, validation of qualitative clinical assays such as MSP still requires establishment of the dynamic range and analytic sensitivity of the assay. Use of parallel quantitative techniques such as MethyLight can provide such data.[21,57] To establish assay precision and provide ongoing quality control, availability of well-characterized controls is essential, but these have proven difficult to standardize. Completely unmethylated fragments can be easily recovered from cloned or PCR-amplified DNA. Completely methylated fragments can be made from unmethylated DNA after treatment with *Sss*I methylase. A heterogeneous control with a pre-determined ratio of fully methylated and fully unmethylated DNA can be made by mixing *Sss*I-treated and untreated fragments. This control, however, cannot be considered partially methylated because each fragment is either methylated or unmethylated; currently there is no acceptable procedure to make partially methylated control samples.

The most important consideration in interpretation and reporting of CpG methylation analyses in neoplastic tissues is the heterogeneity of clinical samples. Spurious results might be explained by the scantiness of neoplastic cells or by the presence of too many non-neoplastic cells (eg, lymphocytes, fibroblasts, stromal cells, etc). This is particularly problematic for cytologic samples, where tumor cells can be severely degenerated or significantly diluted by the background of numerous inflammatory cells, benign reactive cells, and microorganisms.

Heterogeneity of the CpG methylation profiles of the neoplastic cells related to clonal evolution, differentiation state, or histological grade may also skew results. When a portion of the sample is selected for analysis, the extent of errors associated with observer-dependent tissue sampling is difficult to predict. The heterogeneity issues remain for cell-free plasma DNA as well, but they are defined by the nature of the specimen and not by observer-dependent selection of starting material. The influence of this heterogeneity on test interpretation is also unknown.

Finally, DNA sample quality issues can greatly influence assay results. Cytologic materials, which are liquid-based and obtained fresh or fixed with nonformalin fixatives, represent good quality samples, whereas formalin-fixed tissues sections can show much greater variation in DNA quality.

Applications of CpG Methylation Testing in Neoplastic Disorders

There has been increased recognition that tumor-associated epigenetic changes play an important role in the initiation and progression of human cancers. Below, we review reported applications of CpG methylation analysis in detection, classification, and monitoring treatment response of various human cancers.

Classification of Colorectal Cancer

The most common clinical application for CpG methylation testing in colorectal neoplasia is as part of the work-up of hereditary non-polyposis colorectal cancer (HNPCC/Lynch syndrome),[58] which produces microsatellite instability (MSI) by germline mutation of one of several DNA mismatch repair genes. Tumors resulting from HNPCC can be distinguished from most cases of the MSI high (-H) subset of sporadic colorectal cancer by absence of CpG methylation of the *MLH1* promoter, which characterizes most cases of sporadic MSI-H colorectal cancer.[51,58–64] However, assessment of *MLH1* methylation by itself is probably not adequate to distinguish between all sporadic colon cancers and HNPCC-associated MSI-H cancers, since methylation of *MLH1* can been seen as a "second hit" in individuals with a germline *MLH1* mutation.[65] Another limitation is that there are rare cases of heritable germline *MLH1* methylation (epimutation), which can be a cause of hereditary MSI-H colorectal cancer mimicking HNPCC/Lynch syndrome.[66]

The CpG island methylator phenotype (CIMP), defined as widespread promoter CpG island methylation, has been established as a unique epigenetic phenotype in colorectal cancer that is correlated with *MLH1* methylation and MSI phenotype.[51,62,67] CIMP-positive colorectal tumors have a distinct clinical, pathological, and molecular profile. Typically, they are associated with older age, proximal tumor location, female gender, poor differentiation, *BRAF* mutations, wild-type *TP53*, inactive WNT/β-catenin, stable chromosomes, and high-level LINE-1 methylation, independent of MSI status.[62,67–71] Particularly, CIMP status may help distinguish sporadic and HNPCC-related tumors with MSI, because most sporadic MSI-H colon cancers exhibit CIMP, while this is typically not seen in HNPCC-associated cancers.[59,62,72–74] Recent studies have suggested the existence of *KRAS* mutation-associated CIMP (CIMP2 or CIMP-low), separate from CIMP-negative (CIMP-0), and *BRAF* mutation-associated CIMP (CIMP1 or CIMP-high).[75–77] Additional studies support a molecular difference between CIMP-low, CIMP-negative, and CIMP-high in colorectal cancer.[78–80] A recent study suggested that all sporadic MSI-H tumors were explained by CIMP and *MLH1* methylation,[51] while other studies have suggested that there may be a subset of sporadic MSI-H tumors that do not exhibit *MLH1* methylation and/or CIMP.[62,81]

Observed differences may be due to the fact that the panel of CpG markers and method of assessment for categorizing CIMP are not yet standardized. Use of quantitative MethyLight technology and evaluation of a new panel of four to eight CpG islands, including *RUNX3, CACNA1G, IGF2, MLH1, NEUROG1, CRABP1, SOCS1,* and *CDKN2A*, may be the most promising approach.[51,56] Currently, it is probably best to regard CIMP as we regard the p.V600E *BRAF* mutation, which is also commonly seen in sporadic MSI colon cancers and is only rarely seen in HNPCC-associated tumors: the presence of either CIMP or the p.V600E mutation is strong evidence that an MSI-H tumor is sporadic, while the absence of both of these findings indicates that the tumor could be either HNPCC-associated or sporadic.

Determination of CIMP status may also be useful in evaluating the prognosis of colon cancer. A relationship of CIMP with prognosis of microsatellite stable colon cancers has been reported. While previous studies have either found no relationship or a very small relationship,[60,82] one study demonstrated a poor prognosis associated with CIMP in microsatellite stable tumors, but not in MSI-H tumors.[83] *BRAF* mutations have also been associated with poor prognosis in microsatellite stable tumors, although in the same study, no effect was seen on the good prognosis of MSI-H tumors.[82] Since microsatellite stable tumors with *BRAF* mutations are usually very heavily methylated,[75] it is possible that the relationship between prognosis and *BRAF* is actually a relationship between prognosis and high levels of methylation. A different CIMP panel that only detects extensive methylation may show such a relationship with prognosis. Future studies are necessary to resolve this question.

Tumor Progression in Esophageal Carcinoma

The stepwise progression to esophageal adenocarcinoma involves an initial stage of intestinal metaplasia (Barrett's esophagus), followed by low-grade and high-grade dysplasia, and finally adenocarcinoma. Shulmann et al characterized the CpG methylation status of 10 genes (*HPP1*, *RUNX3*, *RIZ1*, *CRBP1*, *3-OST-2*, *APC*, *TIMP3*, *P16*, *MGMT*, *P14*) by real-time quantitative MSP.[84] Their studies demonstrated that hypermethylation of *P16*, *RUNX3*, and *HPP1* in Barrett's esophagus or low-grade dysplasia may represent independent risk factors for the progression of Barrett's esophagus to high-grade dysplasia or adenocarcinoma.

Diagnosis of Biliary and Pancreatic Malignancies on Cytologic Specimens

Due to its often cryptic location, early detection of cholangiocarcinoma is paramount in improving clinical management and patient's survival. Yang et al have shown that concurrent methylation of multiple CpG islands is a hallmark for cholangiocarcinoma.[85] Using a panel of 12 tumor suppressor genes, they reported that DNA methylation profiles accurately differentiated malignant cells from reactive cells in biliary brushings.[86] Similarly, Watanabe et al[87] found that aberrant methylation of *SFRP1* (SARP2) was seen in 79% of pancreatic carcinoma and 56% of malignant intraductal papillary mucinous neoplasms, but was rarely seen in

chronic pancreatitis and healthy controls. Hypermethylation of *SFRP1* in pancreatic juice may be a highly sensitive and useful marker in differentiating pancreatic carcinoma from chronic pancreatitis.[87]

Diagnosis and Outcome Prediction in Breast Cancer

Abnormal CpG methylation in breast cancer has been found in the promoters and first exons of genes, including *ESR1* (estrogen receptor α),[88,89] *PGR* (progesterone receptor),[90] *FRBP3* (MDGI, mammary-derived growth inhibitor),[91] *CALCA* (calcitonin),[92] *MUC1,* [93] and known proto-oconcogene *HRAS,* [94] and tumor suppressor *CDKN2A*[95] genes. The first systematic screen to detect all abnormally methylated genes used a differential methylation hybridization approach[11] and identified multiple methylated fragments in cultured tumor cells and in breast cancer tumors,[12] including transcribed domains of ribosomal DNA.[96]

Detection of abnormal CpG methylation specific for breast cancer can be done using fine needle aspirates,[97] nipple aspirate fluid,[98] and ductal lavage,[99] as reviewed by Dua et al.[100] MSP was reported to have high analytical specificity and moderate analytical sensitivity (100% and 67%, respectively) for diagnosis of malignancy when three genes (*RARB, RASSF1*, and *CCND2*) were analyzed in fine needle aspirate samples.[101] Fackler et al[102] evaluated methylation profiles of nine CpG islands in ductal lavages from 37 cancer patients undergoing mastectomy. A cumulative methylation index had an analytical sensitivity of 71% and specificity of 83% in the detection of cancer cells, compared with an analytical sensitivity of 33% and specificity of 99% by cytomorphology alone. This study provides proof-of-principle by showing the advantages of using methylation analyses to query cytologic specimens and indicates its potential use in diagnosis and risk stratification.[102]

Other studies have found methylation of the *CDH1* gene to be associated with breast tumor invasion and lymph node infiltration,[103,104] and methylation of *LATS1* and *LATS2* has been associated with aggressive cancer.[105] Nevertheless, currently, there are insufficient data to determine the clinical usefulness of methylation tests for diagnosis and prognosis of breast cancer so additional studies are warranted.

Progression in Cervical Carcinoma

The progression from precursor squamous intraepithelial lesions to cervical carcinoma requires additional genetic and epigenetic alterations that have not been characterized fully. Gustafson et al examined aberrant promoter methylation of 15 tumor suppressor genes using a multiplex, nested-MSP approach in 11 high-grade squamous intraepithelial lesions, 17 low-grade squamous intraepithelial lesions, and 11 negative tissues from liquid-based cervical cytology samples.[106] Aberrant promoter methylation of *DAPK1* and *CADM1* (IGSF4) occurred at a high frequency in high-grade squamous intraepithelial lesions and was absent in low-grade squamous intraepithelial lesions and negative samples. Also, the mean number of methylated genes was significantly higher in high-grade squamous intraepithelial lesions, as compared with low-grade squamous intraepithelial lesions and negative samples.[106] Aberrant *CDKN2A* (p16) methylation was significantly higher in invasive cervical cancers (61%) as compared with high-grade squamous intraepithelial lesions (20%) or normal cytologic specimens (7.5%).[107] DNA methylation profiling will likely add a new dimension in the application of molecular biomarkers for prediction of disease progression and risk assessment in cervical squamous lesions, but again others studies are warranted.

Diagnosis of Urothelial Carcinoma in Urine Cytology

Urine cytology is the initial method used for screening of bladder urothelial carcinoma. Although high-grade urothelial carcinoma can be readily detected in urine cytology, cytologic detection of low-grade papillary urothelial carcinoma in urine is challenging due to the overlapping cytomorphologic features with benign reactive processes. Wang et al,[108] using a panel of nine CpG islands, found that concurrent methylation of three or more CpG islands can differentiate low-grade papillary urothelial carcinoma lesions from benign/reactive urothelium in urine. The analytical sensitivity to detect low-grade urothelial carcinoma by DNA methylation profiling was 80% in comparison with 13% by cytology alone.[108] These studies demonstrate that analysis of methylation profiling in certain cytologic specimens can be a useful ancillary tool in facilitating early and accurate detection of urothelial cancer cells.

Predicting Response to Chemotherapy: MGMT Profiling in Glioblastoma and Lymphoma

MGMT is a DNA repair enzyme that is frequently methylated in human cancers, including glioblastoma and diffuse large B-cell lymphoma. MGMT functions to repair O^6-methylguanine DNA adducts generated by both endogenous and exogenous exposure to alkylating agents.[109–111] Repair of O^6-methylguanine is critical to prevent accumulation of G>A transition mutations in important growth regulatory genes, including *KRAS* and *TP53*.[112] CpG islands within the promoter and coding region of *MGMT* are aberrantly hyper- or hypomethylated, respectively, resulting in transcriptional repression.[79,113–117] Loss of MGMT expression and/or *MGMT* promoter methylation are associated with a worse prognosis in several tumor types,[118–120] possibly due to an increased mutation rate.

Since unrepaired O^6-methylguanine signals apoptosis,[121] low MGMT expression would be expected to predict an improved clinical response to chemotherapeutic alkylating agents. Thus, *MGMT* promoter methylation status can impact the degree of signaling for apoptosis following alkylating agent therapy. In glioblastoma multiforme, loss of MGMT expression predicts greater efficacy of treatment with temozolimide and other alkylating

agents. Several studies have shown a compelling direct correlation between *MGMT* promoter methylation and drug response that translates into increased overall patient survival.[122–125] Consequently, *MGMT* promoter methylation analysis using MSP is being used in the clinical laboratory to predict outcome and response to therapy in glioblastoma. *MGMT* promoter methylation also predicts improved outcome in patients with diffuse large B-cell lymphoma treated with the alkylating agent cyclophosphamide.[126] In addition to predicting drug response, *MGMT* promoter methylation is an independent predictor of better outcome in glioblastoma and diffuse large B-cell lymphoma.[124,127]

CpG Methylation Profiling of Free DNA in Body Fluids as a Screening Tool

Tumor cells that are undergoing necrosis or apoptosis release fragments of genomic DNA, which may enter the circulation or be released in the urine or stool where they can be used as biomarkers for the diagnosis, staging, or post-treatment monitoring of cancer. There is tremendous variability in the amount and half-life of cell-free DNA released into the circulation[128,129]; however, the ease of obtaining serial serum or plasma has stimulated tremendous interest in the potential utility of detecting tumor-associated methylated DNA in such samples.

Several studies have addressed whether CpG methylation of tumor biomarkers in serum cell-free DNA is in fact correlated with tumor status. Bastian et al evaluated circulating serum cell-free DNA CpG methylation of *GSTP1*, which is hypermethylated in prostate cancer.[130] They found that circulating cell-free DNA with *GSTP1* hypermethylation was not detected in the serum of men with a negative prostate biopsy but was detected in 12% with clinically localized disease and in 28% with metastatic cancer. Detection of hypermethylated *GTSP1* DNA in serum was the most significant predictor of increased prostate specific antigen levels.[130]

Using MethyLight MSP, Muller et al analyzed 215 serum samples from patients with cervical or breast cancer to identify multigene associated CpG methylation changes. In cervical cancer, hypermethylation of three genes (*MYOD1*, *CDH1*, and *CDH13*) in pretreatment sera was significantly associated with a poor disease outcome.[131] Methylation of a similar set of genes (*RASSF1, ESR1, APC, HSD17B4,* and *HIC1*) selected from a panel of 39 genes in serum was found to be informative for prediction of metastasis, with *APC* and *RASSF1* being the most important.[132]

Koyanagi et al studied the association between DNA methylation of *RASSF1* and *RARB* in circulating tumor cells in peripheral blood of melanoma patients with response to biochemotherapy (a treatment modality that includes biological agents such as interferon and interleukin-2).[133] Patients with methylated *RASSF1* and *RARB* showed a significantly poorer response to biochemotherapy, shorter time to progression, and lower overall survival.[133]

Grady et al studied CpG methylation of *MLH1* promoter DNA in the serum of patients with microsatellite unstable colon cancers.[134] In a panel of sera from 19 colon cancer cases, methylation of *MLH1* was detected in sera in three out of nine patients whose primary tumors harbored *MLH1* methylation. The assay proved 33% analytically sensitive and 100% specific.[134]

Detection of hypermethylated DNA in stool samples has been proposed as a screening tool for colorectal cancer.[135,136] Lenhard et al analyzed promoter methylation of *HIC1* in stools of patients with colorectal cancer or adenomas.[136] They found that 97% of samples had amplifiable DNA and *HIC1* was methylated in 42% of colorectal cancer patients and 31% of patients with adenomas, and was not methylated in normal samples. Belshaw et al[135] compared methylation of a panel of CpG islands using MSP and combined bisulfite restriction analysis and found similar methylation frequencies of *ESR1* and *MGMT* between tumor tissue samples and fecal DNA from the same patients.

The above reported data identify potential clinical applications of CpG methylation testing; however, future prospective studies are required to validate these findings and to refine guidelines for clinical practice.

Monitoring Treatment Response to Demethylating Agents

One of the most promising clinical applications for CpG methylation analysis is in monitoring the response to demethylating agents. 5-aza-2′-deoxycytidine/decitabine (Dacogen) and azacitidine (Vidaza) are agents approved by the U. S. Food and Drug Administration for treatment of myelodysplastic syndrome. They function by reversing hypermethylation of tumor suppressors, including the cell cycle regulator p15. Demethylating agents also have variable activity in a wide variety of other tumor types, especially in combination with other agents.

Several clinical studies have now used CpG methylation profiling of pre- and post-treatment blood samples to monitor the therapeutic effects of demethylating agents. The effects of these drugs on both global methylation (eg, LINE repeats) and the CpG methylation of specific target genes have been studied. In a phase I/II study of decitabine in acute myelogenous leukemia/myelodysplastic syndrome, transient and reversible decreases in the level of DNA methylation at LINE and *CDKN2B* (p15) promoter were observed by a quantitative pyrosequencing assay over a 10-day course of treatment.[137] Transcriptional up-regulation of *CDKN2B* (p15) was observed in parallel with decreases in CpG methylation. Lower pretreatment levels of *CDKN2B* (p15) promoter methylation were correlated with clinical responses to decitabine. Changes in the levels of CpG methylation following treatment were modest (shifts of 10% to 20%) strongly indicating the need for reproducible quantitative assays for monitoring methylation levels.[137] As discussed above, such techniques include real-time PCR (eg, MethyLight) and pyrosequencing methodologies.[138]

Given the current wide use of demethylating agents in myelodysplastic syndrome and myeloid leukemias, *CDKN2B* (p15) methylation assays have the potential to be used up-front to predict which patients will respond

to demethylation therapies. However, given the ability to monitor response in these tumors based solely on blood counts, empirical use of demethylating agents in the absence of pretreatment testing may well continue. If demethylating therapy becomes common in solid tumors where treatment response is more difficult to assess, blood monitoring of re-expression of blood proteins, such as fetal hemoglobin due to CpG demethylation, may serve as a useful surrogate marker of drug response.[139]

CpG Methylation and Inflammatory and Infectious Diseases Related to Cancer Development

Viruses and CpG Methylation

Some viruses appear to use methylation to regulate expression of their own viral genes as well as host cellular genes. Diseased tissues that harbor viruses might, therefore, be responsive to therapies that alter methylation patterns.[140–143] For example, Epstein-Barr virus, which is associated with selected histological subtypes of lymphomas and carcinomas, may repress certain viral genes (nuclear antigens EBNA 1-6, and latent membrane proteins LMP 1 and 2) in an effort to elude immune destruction.[144,145] In other examples, hepatocellular carcinomas appear to silence certain tumor suppressor genes in the presence of heptatitis B virus infection,[146] and HPV appears to use methylation to exert its effects on viral and cellular gene expression.[147] JC virus T antigen expression is also associated with widespread CpG methylation referred to as CIMP in colorectal cancer.[148] Dysregulation of DNA methyltranferases may be responsible, at least in part, for the effects of viruses on host gene promoter methylation.[146,149] The first protein ever shown to bind to and activate a methylated promoter was a virally encoded factor, demonstrating that viruses have evolved mechanisms to overcome methylation to their selective advantage.[150] To the extent that host cellular methylation patterns are altered in virus-specific ways, it may be possible to use expression patterns or methylation patterns to identify virus-related subclasses of cancers. Furthermore, a promising novel targeted therapeutic strategy involves demethylation/activation of viral gene expression in a way that triggers immune recognition and destruction of virally infected tumor cells with little adverse effect on uninfected normal cells.

Bacterial Infection and CpG Methylation

In contrast to CpG methylation in tumors, the CpG methylation status of genes in non-neoplastic tissues has received little attention.[151] However, several studies have shown that CpG island hypermethylation of genes known to be methylated in cancers can be detected in the non-neoplastic tissues.[152–154] One of the most remarkable examples of methylation in non-neoplastic tissue is the hypermethylation of multiple CpG islands in the mucosal tissues of patients with inflammatory conditions, such as chronic gastritis associated with *Helicobacter pylori* infection and inflammatory bowel diseases (ulcerative colitis and Crohn's disease), conditions with increased risk of cancer development.

Increased CpG methylation of several genes has been identified in the gastric mucosa of patients with *H. pylori* gastritis, reviewed by Gologan et al.[151] Chan et al[155] demonstrated that *CDH1* (E-cadherin) methylation was more frequent in the gastric mucosa of patients with *H. pylori* infection as compared with those without. Another study,[154] where the methylation status of several genes was examined, reported that CpG methylation was up to 303-fold higher in *H. pylori*-positive than in *H. pylori*-negative gastric mucosal tissue. *MLH1* CpG methylation in gastric epithelial cells associated with reduced RNA and protein levels of *MLH1* were reported after exposure of gastric cells to *H. pylori* organisms.[156] Studies to-date have not provided conclusive evidence regarding the potential role of CpG methylation in inflammatory cells present in the gastric mucosa of *H. pylori* gastritis.

The potential implications of these reported findings are two-fold: first, CpG methylation may become useful in clinical practice to determine the risk of gastric cancer, and second, demethylating agents by restoring the CpG methylation levels in the gastric mucosa may become useful in cancer chemoprevention. Prospective studies for these potential applications of CpG methylation associated with *H. pylori* gastritis and other inflammatory diseases are warranted.

Summary

There are numerous promising clinical applications of CpG methylation testing for tumors and preneoplastic lesions. However, if CpG methylation testing is to become routine in clinical molecular diagnostics, there is a critical need for cross-laboratory comparisons of different methodologies, for the development of standardized quality control materials for assays and for the adoption of standard reporting formats. While significant advances have been made in the analysis of methylation patterns in various clinical tissues and other samples, with the exception of *MGMT* in gliomas, the selection of optimal gene targets for prognostic methylation panels in specific tumor types remains to be established. Genome-wide methylation screens are currently identifying new marker gene panels for prognosis and therapy-response predictors in other tumors. Application of such techniques in carefully controlled clinical trials, where favorable numbers of samples can be compared with patient outcomes data, is an essential component for the development of clinically meaningful targets for CpG methylation analysis. Comparative studies are clearly essential to further advancement of this field, therefore, we urge researchers to identify and define the CpG sites analyzed in published reports by listing DNA sequences and/or describing the location of the tested CpG(s) in relation to the transcriptional start site. Such advances will be instrumental in attaining clinically valuable and reliable CpG methylation assays for molecular diagnosis for the years to come.

References

1. Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. J Mol Biol 1987, 196:261–282
2. Takai D, Jones PA: Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 2002, 99:3740–3745
3. Ehrich M, Nelson M, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor C, Field J, van den Boom D: Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 2005, 102: 15785–15790
4. Avramis V, Mecum R, Nyce J, Steele D, Holcenberg J: Pharmacodynamic and DNA methylation studies of high-dose 1-beta-D-arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. Cancer Chemother Pharmacol 1989, 24:203–210
5. Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, Wood T, Jeronimo C, Rosenbaum E, Stern J, Yu M, Trink B, Kiviat NB, Sidransky D: Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J Clin Oncol 2006, 24:4262–4269
6. Leung WK, To KF, Man EP, Chan MW, Bai AH, Hui AJ, Chan FK, Sung JJ: Quantitative detection of promoter hypermethylation in multiple genes in the serum of patients with colorectal cancer. Am J Gastroenterol 2005, 100:2274–2279
7. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 1992, 89:1827–1831
8. Mikeska T, Bock C, El-Maarri O, Hubner A, Ehrentraut D, Schramm J, Felsberg J, Kahl P, Buttner R, Pietsch T, Waha A: Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. J Mol Diagn 2007, 9:368–381
9. Schatz P, Dietrich D, Schuster M: Rapid analysis of CpG methylation patterns using RNase T1 cleavage and MALDI-TOF. Nucleic Acids Res 2004, 32:e167
10. Matin MM, Baumer A, Hornby DP: An analytical method for the detection of methylation differences at specific chromosomal loci using primer extension and ion pair reverse phase HPLC. Hum Mutat 2002, 20:305–311
11. Huang TH, Perry MR, Laux DE: Methylation profiling of CpG islands in human breast cancer cells. Hum Mol Genet 1999, 8:459–470
12. Yan PS, Perry MR, Laux DE, Asare AL, Caldwell CW, Huang TH: CpG island arrays: an application toward deciphering epigenetic signatures of breast cancer. Clin Cancer Res 2000, 6:1432–1438
13. Yan P, Efferth T, Chen H, Lin J, Rodel F, Fuzesi L, Huang T: Use of CpG island microarrays to identify colorectal tumors with a high degree of concurrent methylation. Methods 2002, 27:162–169
14. Bibikova M, Lin Z, Zhou L, Chudin E, Garcia E, Wu B, Doucet D, Thomas N, Wang Y, Vollmer E, Goldmann T, Seifart C, Jiang W, Barker D, Chee M, Floros J, Fan J: High-throughput DNA methylation profiling using universal bead arrays. Genome Res 2006, 16:383–393
15. Cheng YW, Shawber C, Notterman D, Paty P, Barany F: Multiplexed profiling of candidate genes for CpG island methylation status using a flexible PCR/LDR/Universal Array assay. Genome Res 2006, 16:282–289
16. Fukasawa M, Kimura M, Moritas S, Matsubara K, Yamanaka S, Endo C, Sakurada A, Sato M, Kondo T, Horii A, Sasaki H, Hatada I: Microarray analysis of promoter methylation in lung cancers. J Hum Genet 2006, 51:368–374
17. Schumacher A, Kapranov P, Kaminsky Z, Flanagan J, Assadzadeh A, Yau P, Virtanen C, Winegarden N, Cheng J, Gingeras T, Petronis A: Microarray-based DNA methylation profiling: technology and applications. Nucleic Acids Res 2006, 34:528–542
18. Grunau C, Clark SJ, Rosenthal A: Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acid Research 2001, 29:E65
19. Warnecke PM, Stirzaker C, Melki JR, Millar DS, Paul CL, Clark SJ: Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. Nucleic Acids Res 1997, 25:4422–4426
20. 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, 93:9821–9826
21. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, Makrigiorgos GM, Weisenberger DJ, Laird PW, Loda M, Fuchs CS: Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006, 8:209–217
22. Wang Y, Zheng W, Luo J, Zhang D, Zuhong L: In situ bisulfite modification of membrane-immobilized DNA for multiple methylation analysis. Anal Biochem 2006, 359:183–188
23. Kerjean A, Vieillefond A, Thiounn N, Sibony M, Jeanpierre M, Jouannet P: Bisulfite genomic sequencing of microdissected cells. Nucleic Acids Res 2001, 29:E106–E106
24. Laird PW: The power and the promise of DNA methylation markers. Nat Rev Cancer 2003, 3:253–266
25. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW: MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000, 28:E32
26. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW: CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res 1999, 59:2302–2306
27. Akey DT, Akey JM, Zhang K, Jin L: Assaying DNA methylation based on high-throughput melting curve approaches. Genomics 2002, 80:376–384
28. Thomassin H, Kress C, Grange T: MethylQuant: a sensitive method for quantifying methylation of specific cytosines within the genome. Nucleic Acids Res 2004, 32:e168
29. Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR: A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. Nucleic Acids Res 2004, 32:e125
30. Cottrell SE, Distler J, Goodman NS, Mooney SH, Kluth A, Olek A, Schwope I, Tetzner R, Ziebarth H, Berlin K: A real-time PCR assay for DNA-methylation using methylation-specific blockers. Nucleic Acids Res 2004, 32:e10
31. Xiong Z, Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997, 25:2532–2534
32. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R: Sensitive and quantitative universal pyrosequencing methylation analysis of CpG sites. Biotechniques 2003, 35:146–150
33. Uhlmann K, Brinckmann A, Toliat MR, Ritter H, Nurnberg P: Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. Electrophoresis 2002, 23:4072–4079
34. Tost J, Dunker J, Gut IG: Analysis and quantification of multiple methylation variable positions in CpG islands by pyrosequencing. Biotechniques 2003, 35:152–156
35. EL-Maarri O, Herbibiaux U, Walter J, Oldenburg J: A rapid, quantitative, non-radioactive bisulfite-SNuPE-IP RP HPLC assay for methylation analysis at specific CpG sites. Nucleic Acids Research 2002, 30:E25
36. Deng D, Deng G, Smith MF, Zhou J, Xin H, Powell SM, Lu Y: Simultaneous detection of CpG methylation and single nucleotide polymorphism by denaturing high performance liquid chromatography. Nucleic Acids Res 2002, 30:E13
37. Waalwijk C, Flavell RA: DNA methylation at a CCGG sequence in the large intron of the rabbit beta-globin gene: tissue-specific variations. Nucleic Acids Res 1978, 5:4631–4634
38. Shen CK, Maniatis T: Tissue-specific DNA methylation in a cluster of rabbit beta-like globin genes. Proc Natl Acad Sci USA 1980, 77:6634–6638
39. Singer-Sam J, Grant M, LeBon JM, Okuyama K, Chapman V, Monk M, Riggs AD: Use of a HpaII-polymerase chain reaction assay to study DNA methylation in the Pgk-1 CpG island of mouse embryos at the time of X-chromosome inactivation. Mol Cell Biol 1990, 10:4987–4989
40. Melnikov AA, Scholtens DM, Wiley EL, Khan SA, Levenson VV. Array-based multiplex analysis of DNA methylation in breast cancer tissues. J Mol Diagn 2008, 10:93–101.
41. Hayashizaki Y, Hirotsune S, Okazaki Y, Hatada I, Shibata H, Kawai J, Hirose K, Watanabe S, Fushiki S, Wada S, Sugimoto T, Kobayakawa K, Kawara T, Katsuki M, Shibuya T, Mukai T: Restriction landmark

genomic scanning method and its various applications. Electrophoresis 1993, 14:251–258
42. Plass C, Shibata H, Kalcheva I, Mullins L, Kotelevtseva N, Mullins J, Kato R, Sasaki H, Hirotsune S, Okazaki Y, Held WA, Hayashizaki Y, Chapman VM: Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLGS-M. Nat Genet 1996, 14:106–109
43. Toyota M, Ho C, Ahuja N, Jair KW, Li Q, Ohe-Toyota M, Baylin SB, Issa JP: Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999, 59:2307–2312
44. 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, 57:594–599
45. Estecio MR, Yan PS, Ibrahim AE, Tellez CS, Shen L, Huang TH, Issa JP: High-throughput methylation profiling by MCA coupled to CpG island microarray. Genome Res 2007, 17:1529–1536
46. Wei SH, Balch C, Paik HH, Kim YS, Baldwin RL, Liyanarachchi S, Li L, Wang Z, Wan JC, Davuluri RV, Karlan BY, Gifford G, Brown R, Kim S, Huang TH, Nephew KP: Prognostic DNA methylation biomarkers in ovarian cancer. Clin Cancer Res 2006, 12:2788–2794
47. Chen CM, Chen HL, Hsiau TH, Hsiau AH, Shi H, Brock GJ, Wei SH, Caldwell CW, Yan PS, Huang TH: Methylation target array for rapid analysis of CpG island hypermethylation in multiple tissue genomes. Am J Pathol 2003, 163:37–45
48. Melnikov AA, Gartenhaus RB, Levenson AS, Motchoulskaia NA, Levenson Chernokhvostov VV: MSRE-PCR for analysis of gene-specific DNA methylation. Nucleic Acids Res 2005, 33:e93
49. Kagan J, Srivastava S, Barker P, A. S., Belinsky S, Cairns P: Towards clinical application of methylated DNA sequences as cancer biomarkers: a Joint NCI's EDRN and NIST workshop on standards, methods, assays, reagents, and tools. Cancer Res 2007, 67:4545–4549
50. Li L, Dahiya R: MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002, 18:1427–1431
51. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW: CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006, 38:787–793
52. Toyooka KO, Toyooka S, Maitra A, Feng Q, Kiviat NC, Smith A, Minna JD, Ashfaq R, Gazdar AF: Establishment and validation of real-time polymerase chain reaction method for CDH1 promoter methylation. Am J Pathol 2002, 161:629–634
53. Brena RM, Morrison C, Liyanarachchi S, Jarjoura D, Davuluri RV, Otterson GA, Reisman D, Glaros S, Rush LJ, Plass C: Aberrant DNA methylation of OLIG1, a novel prognostic factor in non-small cell lung cancer. PLoS Med 2007, 4:e108
54. Kominato Y, Hata Y, Takizawa H, Tsuchiya T, Tsukada J, Yamamoto F: Expression of human histo-blood group ABO genes is dependent upon DNA methylation of the promoter region. J Biol Chem 1999, 274:37240–37250
55. Kelavkar UP, Harya NS, Hutzley J, Bacich DJ, Monzon FA, Chandran U, Dhir R, O'Keefe DS: DNA methylation paradigm shift: 15-lipoxygenase-1 upregulation in prostatic intraepithelial neoplasia and prostate cancer by atypical promoter hypermethylation. Prostaglandins Other Lipid Mediat 2007, 82:185–197
56. Ogino S, kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS: Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007, 9:305–314
57. Coleman WB, Rivenbark AG: Quantitative DNA methylation analysis: the promise of high-throughput epigenomic diagnostic testing in human neoplastic disease. J Mol Diagn 2006, 8:152–156
58. Samowitz W: The CpG island methylator phenotype in colorectal cancer. J Mol Diagn 2007, 9:281–283
59. 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, 96:8681–8686
60. Hawkins N, Norrie M, Cheong K, Mokany E, Ku SL, Meagher A, O'Connor T, Ward R: CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002, 122:1376–1387
61. Whitehall VL, Wynter CV, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, Jass JR: Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. Cancer Res 2002, 62:6011–6014
62. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, Wolff RK, Slattery ML: Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005, 129:837–845
63. Grady WM: CIMP and colon cancer gets more complicated. Gut 2007, 56:1498–1500
64. Teodoridis JM, Hardie C, Brown R. CpG island methylator phenotype (CIMP) in cancer: causes and implications. Cancer Lett 2008, 268:177–186
65. Deng G, Bell I, Crawley S, Gum J, Terdiman JP, Allen BA, Truta B, Sleisenger MH, Kim YS: BRAF mutation is frequently present in sporadic colorectal cancer with methylated hMLH1, but not in hereditary nonpolyposis colorectal cancer. Clin Cancer Res 2004, 10:191–195
66. Hitchins MP, Wong JJ, Suthers G, Suter CM, Martin DI, Hawkins NJ, Ward RL: Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med 2007, 356:697–705
67. Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner G, Weisenberger DJ, Campan M, Laird PW, Loda M, Fuchs CS: CpG island methylator phenotype (CIMP) of colorectal cancer is best characterized by quantitative DNA methylation analysis and prospective cohort studies. Gut 2006, 55:1000–1006
68. Goel A, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer RJ, Goldberg R, Bertagnolli MM, Boland CR: The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology 2007, 132:127–138
69. Samowitz WS, Slattery ML, Sweeney C, Herrick J, Wolff RK, Albertsen H: APC mutations and other genetic and epigenetic changes in colon cancer. Mol Cancer Res 2007, 5:165–170
70. Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Meyerhardt JA, Fuchs CS, Ogino S: Correlation of beta-catenin localization with cyclooxygenase-2 expression and CpG island methylator phenotype (CIMP) in colorectal cancer. Neoplasia 2007, 9:569–577
71. Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS: LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG methylator phenotype in colorectal cancer. Int J Cancer 2008, 122:2767–2773
72. Toyota M, Issa JP: The role of DNA hypermethylation in human neoplasia. Electrophoresis 2000, 21:329–333
73. Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA: BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004, 53:1137–1144
74. McGivern A, Wynter CV, Whitehall VL, Kambara T, Spring KJ, Walsh MD, Barker MA, Arnold S, Simms LA, Leggett BA, Young J, Jass JR: Promoter hypermethylation frequency and BRAF mutations distinguish hereditary non-polyposis colon cancer from sporadic MSI-H colon cancer. Fam Cancer 2004, 3:101–107
75. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS: CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006, 8:582–588
76. Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP: Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci USA 2007, 104:18654–18659
77. Ogino S, Goel A: Molecular classification and correlates in colorectal cancer. J Mol Diagn 2008, 10:13–27
78. Ogino S, Kawasaki T, Kirkner GJ, Ohnishi M, Fuchs CS: 18q loss of heterozygosity in microsatellite stable colorectal cancer is correlated with CpG island methylator phenotype-negative (CIMP-0) and inversely with CIMP-low and CIMP-high. BMC Cancer 2007, 7:72
79. Ogino S, Kawasaki T, Kirkner GJ, Suemoto Y, Meyerhardt JA, Fuchs CS. Molecular correlates with MGMT promoter methylation and silencing support CpG island methylator phenotype-low (CIMP-low) in colorectal cancer. Gut 2007, 56:1564–1571

80. Kawasaki T, Ohnishi M, Nosho K, Suemoto Y, Kirkner GJ, Meyerhardt JA, Fuchs CS, Ogino S: CpG island methylator phenotype-low (CIMP-low) colorectal cancer shows not only fewer methylated CIMP-high-specific CpG island, but also low-level methylation in individual loci. Mod Pathol 2008, 21:245–255
81. Oliveira C, Westra JL, Arango D, Ollikainen M, Domingo E, Ferreira A, Velho S, Niessen R, Lagerstedt K, Alhopuro P, Laiho P, Veiga I, Teixeira MR, Ligtenberg M, Kleibeuker JH, Sijmons RH, Plukker JT, Imai K, Lage P, Hamelin R, Albuquerque C, Schwartz S, Jr., Lindblom A, Peltomaki P, Yamamoto H, Aaltonen LA, Seruca R, Hofstra RM: Distinct patterns of KRAS mutations in colorectal carcinomas according to germline mismatch repair defects and hMLH1 methylation status. Hum Mol Genet 2004, 13:2303–2311
82. Samowitz W, Sweeney C, Herrick J, Albertsen H, Levin T, Murtaugh M, Wolff R, Slattery M: Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005, 65:6063–6069
83. Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ: Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. J Clin Oncol 2003, 21:3729–3736
84. Schulmann K, Sterian A, Berki A, Yin J, Sato F, Xu Y, Olaru A, Wang S, Mori Y, Deacu E, Hamilton J, Kan T, Krasna MJ, Beer DG, Pepe MS, Abraham JM, Feng Z, Schmiegel W, Greenwald BD, Meltzer SJ: Inactivation of p16. RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk Oncogene 2005, 24:4138–4148
85. Yang B, Guo M, Herman J, Clark D: Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am J Pathol 2003, 163:1101–1107
86. Yang B, House M, Guo M, Herman J, Clark D: Promoter methylation profiles of tumor suppressor genes in intrahepatic and extrahepatic cholangiocarcinoma. Mod Pathol 2005, 18:412–420
87. Watanabe H, Okada G, Ohtsubo K, Yao F, Jiang P, Mouri H, Wakabayashi T, Sawabu N: Aberrant methylation of secreted apoptosis-related protein 2 (SARP2) in pure pancreatic juice in diagnosis of pancreatic neoplasms. Pancreas 2006, 32:382–389
88. Falette NS, Fuqua SA, Chamness GC, Cheah MS, Greene GL, McGuire WL: Estrogen receptor gene methylation in human breast tumors. Cancer Res 1990, 50:3974–3978
89. 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, 54:2552–2555
90. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa JP, Davidson NE: Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 1996, 2:805–810
91. Huynh H, Alpert L, Pollak M: Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. Cancer Res 1996, 56:4865–4870
92. Hakkarainen M, Wahlfors J, Myohanen S, Hiltunen MO, Eskelinen M, Johansson R, Janne J: Hypermethylation of calcitonin gene regulatory sequences in human breast cancer as revealed by genomic sequencing. Int J Cancer 1996, 69:471–474
93. Zrihan-Licht S, Weiss M, Keydar I, Wreschner DH: DNA methylation status of the MUC1 gene coding for a breast-cancer-associated protein. Int J Cancer 1995, 62:245–251
94. Kass DH, Shen M, Appel NB, Anderson DE, Saunders GF: Examination of DNA methylation of chromosomal hot spots associated with breast cancer. Anticancer Res 1993, 13:1245–1251
95. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995, 55:4525–4530
96. Yan PS, Rodriguez FJ, Laux DE, Perry MR, Standiford SB, Huang TH: Hypermethylation of ribosomal DNA in human breast carcinoma. Br J Cancer 2000, 82:514–517
97. Jeronimo C, Costa I, Martins MC, Monteiro P, Lisboa S, Palmeira C, Henrique R, Teixeira MR, Lopes C: Detection of gene promoter hypermethylation in fine needle washings from breast lesions. Clin Cancer Res 2003, 9:3413–3417
98. Krassenstein R, Sauter E, Dulaimi E, Battagli C, Ehya H, Klein-Szanto A, Cairns P: Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. Clin Cancer Res 2004, 10:28–32
99. Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, Soito AB, Hung DT, Ljung B, Davidson NE, Sukumar S: Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001, 357:1335–1336
100. Dua RS, Isacke CM, Gui GP: The intraductal approach to breast cancer biomarker discovery. J Clin Oncol 2006, 24:1209–1216
101. Pu RT, Laitala LE, Alli PM, Fackler MJ, Sukumar S, Clark DP: Methylation profiling of benign and malignant breast lesions and its application to cytopathology. Mod Pathol 2003, 16:1095–1101
102. Fackler M, Malone K, Zhang Z, Schilling E, Garrett-Mayer E, Swift-Scanlan T, Lange J, Nayar R, Davidson N, Khan S, Sukumar S: Quantitative multiplex methylation-specific PCR analysis doubles detection of tumor cells in breast ductal fluid. Clin Cancer Res 2006, 12:3306–3310
103. Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, Taback B: Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. Clin Cancer Res 2005, 11:2156–2162
104. Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR: CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. BMC Cancer 2006, 6:48
105. Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y, Noguchi S: Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. Clin Cancer Res 2005, 11:1380–1385
106. Gustafson K, Furth E, Heitjan D, Fansler Z, Clark D: DNA methylation profiling of cervical squamous intraepithelial lesions using liquid-based cytology specimens: an approach that utilizes receiver-operating characteristic analysis. Cancer 2004, 102:259–268
107. Feng Q, Balasubramanian A, Hawes S, Toure P, Sow P, Dem A, Dembele B, Critchlow C, Xi L, Lu H, McIntosh M, Young A, Kiviat N: Detection of hypermethylated genes in women with and without cervical neoplasia. J Natl Cancer Inst 2005, 97:273–282
108. Wang L, Weber D, Deeds D, Biscotti C, Yang B. DNA methylation profiling distinguishes low grade papillary urothelial carcinoma from reactive urothelia in urine. Mod Pathol 2006, 19:74A (supplement)
109. Bartsch H: Studies on biomarkers in cancer etiology and prevention: a summary and challenge of 20 years of interdisciplinary research. Mutat Res 2000, 462:255–279
110. Georgiadis P, Samoli E, Kaila S, Katsouyanni K, Kyrtopoulos SA: Ubiquitous presence of O6-methylguanine in human peripheral and cord blood DNA. Cancer Epidemiol Biomarkers Prev 2000, 9:299–305
111. Kyrtopoulos SA: DNA adducts in humans after exposure to methylating agents. Mutat Res 1998, 405:135–143
112. Margison GP, Povey AC, Kaina B, Santibanez Koref MF: Variability and regulation of O6-alkylguanine-DNA alkyltransferase. Carcinogenesis 2003, 24:625–635
113. Danam RP, Howell SR, Brent TP, Harris LC: Epigenetic regulation of O6-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins. Mol Cancer Ther 2005, 4:61–69
114. Soejima H, Zhao W, Mukai T: Epigenetic silencing of the MGMT gene in cancer. Biochem Cell Biol 2005, 83:429–437
115. von Wronski MA, Brent TP: Effect of 5-azacytidine on expression of the human DNA repair enzyme O6-methylguanine-DNA methyltransferase. Carcinogenesis 1994, 15:577–582
116. Wang Y, Kato T, Ayaki H, Ishizaki K, Tano K, Mitra S, Ikenaga M: Correlation between DNA methylation and expression of O6-methylguanine-DNA methyltransferase gene in cultured human tumor cells. Mutat Res 1992, 273:221–330
117. Fox EJ, Leahy DT, Geraghty R, Mulcahy HE, Fennelly D, Hyland JM, O'Donoghue DP, Sheahan K: Mutually exclusive promoter hypermethylation patterns of hMLH1 and O^6-methylguanine DNA methyltransferase in colorectal cancer. J Mol Diagn 2006, 8:68–75
118. Hayashi H, Yazawa T, Okudela K, Nagai J, Ito T, Kanisawa M, Kitamura H: Inactivation of O^6-methylguanine-DNA methyltransferase in human lung adenocarcinoma relates to high-grade histology and worse prognosis among smokers. Jpn J Cancer Res 2002, 93:184–189
119. Komine C, Watanabe T, Katayama Y, Yoshino A, Yokoyama T,

Fukushima T: Promoter hypermethylation of the DNA repair gene O^6-methylguanine-DNA methyltransferase is an independent predictor of shortened progression free survival in patients with low-grade diffuse astrocytomas. Brain Pathol 2003, 13:176–184
120. Matsukura S, Miyazaki K, Yakushiji H, Ogawa A, Harimaya K, Nakabeppu Y, Sekiguchi M: Expression and prognostic significance of O^6-methylguanine-DNA methyltransferase in hepatocellular, gastric, and breast cancers. Ann Surg Oncol 2001, 8:807–816
121. Kaina B, Ochs K, Grosch S, Fritz G, Lips J, Tomicic M, Dunkern T, Christmann M: BER, MGMT, and MMR in defense against alkylation-induced genotoxicity and apoptosis. Prog Nucleic Acid Res Mol Biol 2001, 68:41–54
122. Esteller M, Risques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG: Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 2001, 61:4689–4692
123. Hegi ME, Diserens AC, Godard S, Dietrich PY, Regli L, Ostermann S, Otten P, Van Melle G, de Tribolet N, Stupp R: Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 2004, 10:1871–1874
124. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R: MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005, 352:997–1003
125. Paz MF, Yaya-Tur R, Rojas-Marcos I, Reynes G, Pollan M, Aguirre-Cruz L, Garcia-Lopez JL, Piquer J, Safont MJ, Balana C, Sanchez-Cespedes M, Garcia-Villanueva M, Arribas L, Esteller M: CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res 2004, 10:4933–4938
126. Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, Rossi D, Gloghini A, Vitolo U, Carbone A, Baylin SB, Herman JG: Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. J Natl Cancer Inst 2002, 94:26–32
127. Ohno T, Hiraga J, Ohashi H, Sugisaki C, Li E, Asano H, Ito T, Nagai H, Yamashita Y, Mori N, Kinoshita T, Naoe T: Loss of O^6-methylguanine-DNA methyltransferase protein expression is a favorable prognostic marker in diffuse large B-cell lymphoma. Int J Hematol 2006, 83:341–347
128. Invernizzi P, Biondi ML, Battezzati PM, Perego F, Selmi C, Cecchini F, Podda M, Simoni G: Presence of fetal DNA in maternal plasma decades after pregnancy. Hum Genet 2002, 110:587–591
129. Silva JM, Garcia JM, Dominguez G, Silva J, Miralles C, Cantos B, Coca S, Provencio M, Espana P, Bonilla F: Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. Ann Surg Oncol 2002, 9:71–76
130. Bastian PJ, Palapattu GS, Lin X, Yegnasubramanian S, Mangold LA, Trock B, Eisenberger MA, Partin AW, Nelson WG: Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate-specific antigen recurrence following radical prostatectomy. Clin Cancer Res 2005, 11:4037–4043
131. Muller H, Fiegl H, Widschwendter A, Widschwendter M: Prognostic DNA methylation marker in serum of cancer patients. Ann NY Acad Sci 2004, 1022:44–49
132. Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C, Widschwendter M: DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res 2003, 63:7641–5
133. Koyanagi K, Mori T, O'Day S, Martinez S, Wang H, Hoon D: Association of circulating tumor cells with serum tumor-related methylated DNA in peripheral blood of melanoma patients. Cancer Res 2006, 66:6111–6117
134. Grady WM, Rajput A, Lutterbaugh JD, Markowitz SD: Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Cancer Res 2001, 61:900–902
135. Belshaw NJ, Elliott GO, Williams EA, Bradburn DM, Mills SJ, Mathers JC, Johnson IT: Use of DNA from human stools to detect aberrant CpG island methylation of genes implicated in colorectal cancer. Cancer Epidemiol Biomarkers Prev 2004, 13:1495–1501
136. Lenhard K, Bommer GT, Asutay S, Schauer R, Brabletz T, Goke B, Lamerz R, Kolligs FT: Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. Clin Gastroenterol Hepatol 2005, 3:142–149
137. Garcia-Manero G, Kantarjian H, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda W, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa J: Phase I/II study of the combination of 5-aza-2′ -deoxycytidine with valproic acid in patients with leukemia. Blood 2006, 108:3271–3279
138. Mund C, Hackanson B, Stresemann C, Lubbert M, Lyko F: Characterization of DNA demethylation effects induced by 5-Aza-2′-deoxycytidine in patients with myelodysplastic syndrome. Cancer Res 2005, 65:7086–7090
139. Ruter B, Wijermans PW, Lubbert M: DNA methylation as a therapeutic target in hematologic disorders: recent results in older patients with myelodysplasia and acute myeloid leukemia. Int J Hematol 2004, 80:128–135
140. Tao Q, Young L, Woodman C, Murray P: Epstein-Barr virus (EBV) and its associated human cancers–genetics, epigenetics, pathobiology and novel therapeutics. Front Biosci 2006, 11:2672–2713
141. Israel B, Kenney S: Virally targeted therapies for EBV-associated malignancies. Oncogene 2003, 22:5122–5130
142. Verma M: Viral genes and methylation. Ann NY Acad Sci 2003, 983:170–180
143. Varier R, Kundu T: Chromatin modifications (acetylation/ deacetylation/ methylation) as new targets for HIV therapy. Curr Pharm Des 2006, 12:1975–1993
144. Tao Q, Robertson K: Stealth technology: how Epstein-Barr virus utilizes DNA methylation to cloak itself from immune detection. Clin Immunol 2003, 109:53–63
145. Minarovits J: Epigenotypes of latent herpesvirus genomes. Curr Top Microbiol Immunol 2006, 310:61–80
146. Li H, Leu Y, Chang Y: Epigenetic changes in virus-associated human cancers. Cell Res 2005, 15:262–271
147. Zheng Z, Baker C: Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci 2006, 11:2286–2302
148. Goel A, Li MS, Nagasaka T, Shin SK, Fuerst F, Ricciardiello L, Wasserman L, Boland CR: Association of JC virus T-antigen expression with the methylator phenotype in sporadic colorectal cancers. Gastroenterology 2006, 130:1950–1961
149. Etoh T, Kanai Y, Ushijima S, al. e. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 2004, 164:689–699
150. Bhende P, Seaman W, Delecluse H, Kenney S: The EBV lytic switch protein. Z, preferentially binds to and activates the methylated viral genome Nat Genet 2004, 36:1099–1104
151. Gologan A, Graham DY, Sepulveda AR: Molecular markers in helicobacter pylori-associated gastric carcinogenesis. Clin Lab Med 2005, 25:197–222
152. Issa JP: Age-related epigenetic changes and the immune system. Clin Immunol 2003, 109:103–108
153. Hsieh CJ, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R: Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. Cancer Res 1998, 58:3942–3945
154. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T: High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 2006, 12:989–995
155. Chan AO, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, Hui WM, Rashid A, Kwong YL: Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer. Gut 2003, 52:502–506
156. Yao Y, Tao H, Park DI, Sepulveda JL, Sepulveda AR: Demonstration and characterization of mutations induced by helicobacter pylori organisms in gastric epithelial cells. Helicobacter 2006, 11:272–286