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Review

Utility of DNA methylation markers for diagnosing cancer

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ABSTRACT

DNA methylation occurs at the CpG residues and serves as a powerful epigenetic mechanism that negatively regulates gene expression. This process is catalyzed by DNA methyltransferases and occurs within "CpG islands" found in the promoter regions of >70% of human genes. Given the important role of DNA methylation in regulating gene expression, un-programmed changes in methylation patterns are expected to either silence or activate transcription of tumor suppressor genes (via hypermethylation) or oncogenes (via demethylation), respectively, and by doing so promote a disease state. In light of the fact that a number of different cancers are frequently associated with hypermethylated tumor suppressor genes together with the observation that tumor derived genomic DNAs are present in various body fluids including serum/plasma, urine, sputum and bronchial lavage, methylated DNA has shown tremendous promise to serve as a robust biomarker for detecting cancer. Over the last several years protocols for capturing small amounts of DNA in circulation have been developed. Once captured, DNA methylation may be readily monitored by restriction enzyme digestion or bisulfite conversion followed by amplification of the desired genomic region with the polymerase chain reaction (PCR). New technologies which employ methyl-binding protein or antibodies that bind specifically to methylated-CpG residues have now enabled investigators to interrogate the status of entire "DNA methyome" of diseased tissue in an efficient and cost-effective manner. In this review, we describe the various tumor suppressor genes that are frequently hypermethylated in different cancers and how these and other methylated loci may be employed as clinically useful biomarkers for diagnosing cancer noninvasively using readily available body fluids.

1. Introduction

In humans DNA methylation occurs in the dinucleotide 5′-CpG-3′. It has been estimated that over 70% of genes harbor regions called "CpG islands" in which the frequency of CpG is considerably higher than expected. A vast majority of CpG islands are found in the upstream promoter regions of genes but in others they are located downstream of the transcription start point. Methylation at CpG dinucletode is catalyzed by three major DNA methyltransferases namely DNMT1, DNMT3a and DNMT3b which covalently attach a methyl group to the C5 position of cytosine residues. DNMT1 is responsible for maintaining genomic DNA methylation patterns and employs hemi-methylated-CpG dinucleotides, produced after DNA replication or repair, as substrate and fully methylates them. DNMT3a and 3b methylate previously unmodified CpG residues and hence are known as *de novo* methylases.

Since DNA methylation silences gene transcription such modifications must be carefully orchestrated during the course of development to ascertain that expression of certain sets of genes is spatially and temporally restricted to specific cell-types. DNA methylation patterns are tissue-specific and "frozen" once development is complete. Un-programmed changes in DNA methylation patterns brought about by gain or loss of function in any of the DNA methyltransferases or demethylases are likely to lead to developmental defects in the growing embryo or a disease state in individuals by altered expression of oncogenes and/or tumor suppressor genes. Nutrition also impacts genomic DNA methylation patterns. Since S-adenosylmethionine (SAM) serves as the source of methyl group which is recycled through the folate and cobalamin dependent pathways,[1] deficiency of dietary folate and vitamin B_{12} is expected to culminate in reduced global levels of DNA methylation.

The negative influence of DNA methylation on gene expression is mediated by methyl-CpG binding proteins (MBDs) that by

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recruiting histone modifying and chromatin remodeling enzymes facilitate the conversion of the loose 10 nm chromatin fiber into a more condensed 30 nm solenoid structure. Eight different MBDs have been identified to date which are MeCP2, MBDs 1, 2, 3, 4 along with Kaiso and its two related proteins ZBTB4 and ZBTB38. With the exception of MBD3, all MBDs bind to methylated DNA. Although the functions of various MBDs were considered overlapping, recent studies have shown that MeCP2 and MBD1 preferentially target specific gene promoters in prostate cancer derived PC3 cells suggesting that their functions may be mutually exclusive.[2] Interestingly, depletion of DNMT1 or DNMT3b has also been found to culminate in preferential loss of DNA methylation at certain genomic loci.[3,4]

DNA methylation patterns are dynamic during embryogenesis. Around the eight cell embryonic stage, genomic methylation patterns are erased but are then re-established during the implantation stage of the embryo[5]; subsequent embryonic development is associated with additional waves of DNA methylation and demethylation.[5,6] The biochemical signals responsible for promoting such dramatic fluctuations in DNA methylation patterns, be they local or global, remain unknown. In adults, distribution of genomic methylation marks is cell type specific and any un-programmed changes in these patterns have been linked to aging and disease.[7]

2. Role of DNA methylation in disease

Given the important role DNA methylation plays in regulating and restricting the expression of genes to certain cell-types, it is expected that aberrant changes in DNA methylome are likely to promote a disease state. Although infections as well as environmental factors are known to influence DNA methylation patterns, the mechanisms by which they impact the activity or targeting of DNA methyltransferases, MBDs and/or demethylases to different genomic regions remains unclear. There is now strong evidence suggesting that cardiovascular disease, hypertension, stroke, depression and type-2 diabetes originate early in development because of repeated environmental insults that influence the epigenome.[8] Similarly, neurological disorders such as schizophrenia, bipolar disorder and autism are associated with un-programmed changes in the epigenome.[9–11]

Aberrant DNA methylation has also been found to play an important role in cancer development and progression. Genome-wide hypomethylation that is restricted largely to gene-poor regions as well as gene-specific hypermethylation of CpG islands are the two commonest forms of epigenomic modifications that are frequently observed in cancer cells.[12,13] Hypomethylation of centromeric DNA promotes genomic instability and appears to increase as cancers progress from non-metastatic to metastatic state.[14–17] Cancer cell genomes invariably harbor one or more tumor suppressor genes that are hypermethylated. For instance, silencing of Rb by DNA hypermethylation in retinoblastomas was among the first reports which described how a gene may be inactivated epigenetically.[18] Subsequently, a number of other tumor suppressor genes such as VHL, p16, BRCA and hMLh1 have also been found to be hypermethylated in cancers.[19,20] Table 1 provides a list of all genes whose promoters have been found to be hypermethylated in various cancers.

3. Using methylated DNA in circulation as cancer biomarker

Clinical utility of even a reliable disease-specific biomarker is undermined if the tissue to be screened requires surgical removal. The ideal biomarker therefore is one which is found in readily available biological samples that can be obtained noninvasively. Due to the high cellular turnover, cancer patients carry elevated levels of free DNA (~200 ng/ml) in their blood.[25] Other body fluids such as urine,[26] bronchoalveolar lavage (BAL),[27] mammary aspiration fluids,[28] saliva,[29] sputum[27] and stools[30] that contact tumors are also potential sources of cancer cell DNA. These circulatory DNAs from blood or other body fluids can be captured easily and the status of DNA methylation at various gene promoters interrogated by various methods. According to one study 77% of prostate cancer patients showed GSTP1 hypermethylation in the DNA which was derived from urine as compared to a detection rate of 72% in plasma/serum samples.[31] Similarly, analysis of genomic DNA purified from the sputum of lung cancer patients found p16 to be most frequently methylated.[27,33] Studies using bronchoalveolaer lavage from early stage lung cancer patients have revealed frequent DNA methylation at p16, RARβ, DAPK and MGMT gene promoters.[34] Ductal lavage fluid and needle aspirates are also useful sources of tumor DNA which could be employed for detecting breast cancers.[28,35] Krassenstein et al. have found a number of genes which were abnormally methylated in genomic DNAs obtained from nipple aspirates of breast cancer patients.[35] Abnormally methylated DNAs are also found in the saliva from head and neck, and in stool samples from colon and rectal cancer patients.[29,30] Similarly, bladder cancer patients excrete urine that contains hypermethylated DAPK, RARβ, E-cadherin and p16 CpG islands; on their own these biomarkers were found to be unreliable but when combined together yielded an impressive detection rate of 91%.[32] Using serum samples from non-small cell lung cancer (NSLC) patients, Esteller et al found 15 out of 22 cancer patients to be positive for DNA methylation at p16, DAPK, GSTP1 or MGMT gene promoters,[21] however, detection rates of NSLCs varied from 6% to 76% when only p16 was used as a DNA methylation marker.[22–24]

DNA methylation markers that are specific for different cancers are obviously much sought after because if identified they will enable investigators to accurately predict different types of cancers. Until such cancer-specific DNA methylation markers are found, studies have shown that employing a panel of different DNA methylation biomarkers (i.e., several gene-specific CpG islands) is considerably more reliable for predicting cancer phenotypes as compared to using just one.

4. Methods for determining DNA methylation status of genes

Clinically ideal diagnostic tests are those which can be carried out on readily accessible body fluids (e.g., serum, urine, saliva, etc.). Such tests should be sensitive, specific, reproducible, cost-effective, and be of turn-key nature requiring minimal number of steps so that it could be carried out in a high-throughput manner. Body fluids of cancer patients serve an excellent source of tumor derived genomic DNA which can be used for interrogating the DNA methylation status of either a select panel of genes or the entire genome using a number of different methods. However since these samples invariably contain large amounts of background DNA from normal cells it is essential that the employed diagnostic test be sensitive enough to detect the few available copies of methylated DNA present in the sample.

Initially, DNA methylation sensitive and insensitive restriction endonucleases (e.g., HpaII and Msp1, respectively) were employed for detecting CpG methylation at specific genomic loci but since this approach requires large amounts of genomic DNA it is not deemed practical for clinical use. Over the past decade, a number of very sensitive as well as reliable methods have been developed which now allow investigators to detect locus-specific DNA methylation from very small amounts of genomic DNA. Among these, methylation-specific PCR (MSP) has been used most commonly.[36] In this assay, genomic DNA is treated with sodium bisulfite which deaminates unmodified cytosines (C) to uracil but

Table 1
Hypermethylated gene promoters implicated in different cancers.

Cancer	Hypermethylated genes	References
Colon	p14ARF (cell cycle), hMLH1 (DNA repair), p16, SFRP1, WRN	44–46
Breast	TMS1 (angiogenesis), E-cadherin (metastasis), ER (signal transduction), BRCA1 (transcription)	47,48
Lung	DAPK (apoptosis), RASSF1A (signal transduction), $p16^{INK4a}$	49,50
Glioma	THBS1 (angiogenesis), MGMT(DNA repair), EMP3	51,52
Leukemia	$p15^{INK4b}$, EXT1, ID4	53–55
Lymphomas	p73 (cell cycle), MGMT(DNA repair), $p16^{INK4a}$	56,57
Bladder	$p16^{INK4a}$, TPEF/HPP1, RASSF1A, RARβ (differentiation), DAPK	58
Kidney	VHL (transcription)	59
Prostate	GSTP1 (detoxification)	60
Esophageal	$p14^{ARF}$ (cell cycle), $p16^{INK4b}$	61
Stomach	$p14^{ARF}$ (cell cycle), hMLH1 (DNA repair)	62
Liver	GSTP1 (detoxification), $p16^{INK4a}$	63,64
Ovarian	BRCA1 (transcription)	65
Others	Apoptosis (CASP8), Cell cycle (RB), Differentiation (MYOD, PAX6), Metastasis (MASPIN, TIMP3), Signal transduction (APC, PTEN, AR)	66–74

spares methylated cytosines. This step converts all epigenetic information into DNA sequence information. The chemically converted DNA is subsequently subjected to polymerase chain reaction (PCR) using primer sets that are strategically designed to anneal to a specific region within a CpG island of interest and capable of differentiating methylated from unmethylated gene promoters. Although MSP is user-friendly and can detect DNA methylation with impressive sensitivity, it is neither quantitative nor can be performed in a high-throughput manner. Another disadvantage of MSP is that it allows screening of only a very small stretch (i.e. <40 bp) of DNA within a CpG island for methylated residues. These shortcomings render MSP ineffective in a clinical setting where the amount of methylated DNA sometimes needs to be correlated with disease severity for prognosis and where a number of genes need to be analyzed simultaneously. DNA sequencing of PCR products generated from primers flanking a CpG island of interest using bisulfite converted DNA as template offers the highest resolution in terms of providing detailed information about the methylated state of an entire CpG island, but its labor intensive protocol and expense precludes its use as a diagnostic tool. Alternatively, a specific CpG island may be PCR amplified using bisulfite converted DNA and the methylated-CpGs within it detected and scored via restriction enzyme analysis. This strategy forms the basis of another useful DNA methylation detection technique known as combined bisulpite restriction analysis (COBRA).[37–39] In COBRA, sequence changes in DNA that result as a consequence of bisulphite-conversion are analyzed through restriction enzyme digestion of the resulting PCR product.[39] The main limitation of this approach is that it is only useful for probing DNA methylation status of those CpGs that are harbored within a restriction enzyme site.

Methylight is another method capable of detecting methyated and unmmethylated DNA with exquisite sensitivity. This assay makes use of Taqman probes which anneal in between the two PCR primers and hence report DNA amplification in real-time. Since the probe contains a fluorophore at the 5′end and a quencher on either the 3′ end or in the middle, no fluorescence is emitted in the intact probe upon excitation. During the extension phase of PCR the probe is cleaved by the 5′→3′ exonuclease activity of Taq DNA polymerase which distances the fluorophore from the quencher and results in a fluorescent signal that is proportional to the amount of the generated PCR product. In this procedure, two different primer pairs are used to amplify bifulfite converted DNA: one which is specific for methylated DNA and the other specific for unmethylated DNA. Depending on the requirement, methylight assays may be performed either in a quantitative or semi-quantitative format, and report nucleic acid amplification in real-time without requiring gel electrophoresis.[40] Additionally these assays are user-friendly and amenable to automation.[41]

Table 2
Methods for detecting DNA methylation and their features.

Detection Method	Amount of DNA Required	Throughput	Cost
Enzymatic (MspI/HpaII)	Large	Low	Moderate
Methylation sensitive PCR	Low	Low-Medium	Low
Combined bisulfite restriction analysis (COBRA)	Low	Low-Medium	Low
Methylight	Low	Moderate	Low
DNA methylation microarrays	Moderate	Low	High

DNA methylation-specific microarrays are useful for interrogating the entire epigenome in an open-minded way.[42, 43] In this procedure, genomic DNA obtained from diseased tissue or body fluid is sheared and an antibody (or an MBD) with high affinity for 5-methylcytosine employed to selectively capture those genomic fragments that are methylated. After labeling, this population of DNAs is hybridized to a gene array in which all genomic CpG islands are represented. This powerful approach, albeit costly at present, is capable of identifying hundreds of specific genomic loci simultaneously that may be aberrantly methylated in different types of cancer. Additionally, this approach may also prove to be useful for monitoring cancers during or after treatment. Table 2 lists the DNA methylation detection methods discussed herein and their respective features.

5. Conclusion

Work over the past decade has shown that DNA methylation biomarkers can not only detect cancer at an early stage but may also be useful for monitoring disease progression during or after treatment. Since tumor derived DNAs are found in various body fluids of cancer patients, they can be obtained noninvasively and screened using various methods to probe presence or absence of DNA methylation at specific loci. Although a number of DNA methylation biomarkers have been identified to date none are reliable enough to be clinically useful. However, development of sophisticated new technology platforms and reagents should eventually lead to the identification of discrete sets of DNA methylation markers (i.e., "signatures") that are unique to different types of cancers. The clinical utility of DNA methylation markers for diagnosing cancers at an early stage therefore looks promising.

Conflict of interest statement
The authors declare that they have no conflict of interest.

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References

1. Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003;**3**:253–66.

2. Yaqinuddin A, Abbas F, Naqvi SZ, Bashir MU, Qazi R, Qureshi SA. Silencing of MBD1 and MeCP2 in prostate-cancer-derived PC3 cells produces differential gene expression profiles and cellular phenotypes. *Biosci Rep* 2008;**28**:319–26.
3. Yaqinuddin A, Qureshi SA, Qazi R, Abbas F. Down-regulation of DNMT3b in PC3 cells effects locus-specific DNA methylation, and represses cellular growth and migration. *Cancer Cell Int* 2008;**8**:13.
4. Yaqinuddin A, Qureshi SA, Qazi R, Farooq S, Abbas F. DNMT1 silencing affects locus specific DNA methylation and increases prostate cancer derived PC3 cell invasiveness. *J Urol* 2009;**182**:756–61.
5. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;**293**:1089–93.
6. Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* 1987;**99**:371–82.
7. Issa JP. CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol* 2000;**249**:101–18.
8. Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. *Rev Endocr Metab Disord* 2007;**8**:173–82.
9. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, Gao F, et al. Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *Hum Mol Genet* 2006;**15**:3132–45.
10. Deng C, Kaplan MJ, Yang J, Ray D, Zhang Z, McCune WJ, et al. Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. *Arthritis Rheum* 2001;**44**:397–407.
11. Samaco RC, Hogart A, LaSalle JM. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Hum Mol Genet* 2005;**14**:483–92.
12. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983;**301**:89–92.
13. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* 2005;**37**: 853–62.
14. Fraga MF, Herranz M, Espada J, Ballestar E, Paz MF, Ropero S, et al. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. *Cancer Res* 2004;**64**:5527–34.
15. Bestor TH. Transposons reanimated in mice. *Cell* 2005;**122**:322–5.
16. Cui J, Rohr LR, Swanson G, Speights VO, Maxwell T, Brothman AR. Hypermethylation of the caveolin-1 gene promoter in prostate cancer. *Prostate* 2001;**46**:249–56.
17. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003;**300**:455.
18. Greger V, Passarge E, Hopping W, Messmer E, Horsthemke B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum Genet* 1989;**83**:155–8.
19. Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, et al. Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res* 1995;**55**:4531–5.
20. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;**349**:2042–54.
21. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;**59**:67–70.
22. Kurakawa E, Shimamoto T, Utsumi K, Hirano T, Kato H, Ohyashiki K. Hypermethylation of p16(INK4a) and p15(INK4b) genes in non-small cell lung cancer. *Int J Oncol* 2001;**19**:277–81.
23. Liu Y, An Q, Li L, Zhang D, Huang J, Feng X, et al. Hypermethylation of p16INK4a in Chinese lung cancer patients: biological and clinical implications. *Carcinogenesis* 2003;**24**:1897–901.
24. Ng CS, Zhang J, Wan S, Lee TW, Arifi AA, Mok T, et al. Tumor p16M is a possible marker of advanced stage in non-small cell lung cancer. *J Surg Oncol* 2002;**79**:101–6.
25. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001;**61**:1659–65.
26. Goessl C, Muller M, Heicappell R, Krause H, Miller K. DNA-based detection of prostate cancer in blood, urine, and ejaculates. *Ann N Y Acad Sci* 2001;**945**:51–8.
27. Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin Chem* 2000;**46**:1078–84.
28. Belinsky SA, Palmisano WA, Gilliland FD, Crooks LA, Divine KK, Winters SA, et al. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. *Cancer Res* 2002;**62**:2370–7.
29. Ahrendt SA, Chow JT, Xu LH, Yang SC, Eisenberger CF, Esteller M, et al. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. *J Natl Cancer Inst* 1999;**91**:332–9.
30. Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet* 2001;**357**:1335–6.
31. Krassenstein R, Sauter E, Dulaimi E, Battagli C, Ehya H, Klein-Szanto A, et al. Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. *Clin Cancer Res* 2004;**10**:28–32.
32. Muller HM, Oberwalder M, Fiegl H, Morandell M, Goebel G, Zitt M, et al. Methylation changes in faecal DNA: a marker for colorectal cancer screening? *Lancet* 2004;**363**:1283–5.
33. Rosas SL, Koch W, da Costa Carvalho MG, Wu L, Califano J, Westra W, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer Res* 2001;**61**:939–42.
34. Chan MW, Chan LW, Tang NL, Tong JH, Lo KW, Lee TL, et al. Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. *Clin Cancer Res* 2002;**8**:464–70.
35. Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res* 2000;**60**:5954–8.
36. 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 U S A* 1996;**93**:9821–6.
37. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 1994;**22**:2990–7.
38. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* 1992;**89**:1827–31.
39. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* 1997;**25**:2532–4.
40. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, et al. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 2000;**28**. E32.
41. Trinh BN, Long TI, Laird PW. DNA methylation analysis by MethyLight technology. *Methods* 2001;**25**:456–62.
42. Schumacher A, Weinhausl A, Petronis A. Application of microarrays for DNA methylation profiling. *Methods Mol Biol* 2008;**439**:109–29.
43. Zilberman D, Henikoff S. Genome-wide analysis of DNA methylation patterns. *Development* 2007;**134**:3959–65.
44. Lee S, Hwang KS, Lee HJ, Kim JS, Kang GH. Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia. *Lab Invest* 2004;**84**: 884–93.
45. Malkhosyan SR, Yamamoto H, Piao Z, Perucho M. Late onset and high incidence of colon cancer of the mutator phenotype with hypermethylated hMLH1 gene in women. *Gastroenterology* 2000;**119**:598.
46. Xu XL, Yu J, Zhang HY, Sun MH, Gu J, Du X, et al. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World J Gastroenterol* 2004;**10**:3441–54.
47. Liu YN, Liu Y, Lee HJ, Hsu YH, Chen JH. Activated androgen receptor down-regulates E-cadherin gene expression and promotes tumor metastasis. *Mol Cell Biol* 2008;**28**:7096–108.
48. Mirza S, Sharma G, Prasad CP, Parshad R, Srivastava A, Gupta SD, et al. Promoter hypermethylation of TMS1, BRCA1, ERalpha and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. *Life Sci* 2007;**81**:280–7.
49. Licchesi JD, Westra WH, Hooker CM, Herman JG. Promoter hypermethylation of hallmark cancer genes in atypical adenomatous hyperplasia of the lung. *Clin Cancer Res* 2008;**14**:2570–8.
50. Yu ZH, Wang YC, Chen LB, Song Y, Liu C, Xia XY, et al. [Analysis of RASSF1A promoter hypermethylation in serum DNA of non-small cell lung cancer]. *Zhonghua Zhong Liu Za Zhi* 2008;**30**:284–7.
51. Alonso ME, Bello MJ, Gonzalez-Gomez P, Arjona D, de Campos JM, Gutierrez M, et al. Aberrant CpG island methylation of multiple genes in ependymal tumors. *J Neurooncol* 2004;**67**:159–65.
52. Uhlmann K, Rohde K, Zeller C, Szymas J, Vogel S, Marczinek K, et al. Distinct methylation profiles of glioma subtypes. *Int J Cancer* 2003;**106**:52–9.
53. Esteller M. Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. *Clin Immunol* 2003;**109**:80–8.
54. Ropero S, Setien F, Espada J, Fraga MF, Herranz M, Asp J, et al. Epigenetic loss of the familial tumor-suppressor gene exostosin-1 (EXT1) disrupts heparan sulfate synthesis in cancer cells. *Hum Mol Genet* 2004;**13**:2753–65.
55. Zhao Y, Yu L, Wang QS, Li HH, Bo J, Wang SH, et al. [Id4 gene methylation for detection of minimal residual disease in acute leukemia]. *Zhonghua Xue Ye Xue Za Zhi* 2006;**27**:298–301.
56. Gallardo F, Esteller M, Pujol RM, Costa C, Estrach T, Servitje O. Methylation status of the p15, p16 and MGMT promoter genes in primary cutaneous T-cell lymphomas. *Haematologica* 2004;**89**:1401–3.
57. van Doorn R, Zoutman WH, Dijkman R, de Menezes RX, Commandeur S, Mulder AA, et al. Epigenetic profiling of cutaneous T-cell lymphoma: promoter hypermethylation of multiple tumor suppressor genes including BCL7a, PTPRG, and p73. *J Clin Oncol* 2005;**23**:3886–96.
58. Jarmalaite S, Jankevicius F, Kurgonaite K, Suziedelis K, Mutanen P, Husgafvel-Pursiainen K. Promoter hypermethylation in tumour suppressor genes shows association with stage, grade and invasiveness of bladder cancer. *Oncology* 2008;**75**:145–51.
59. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 1994;**91**:9700–4.
60. Reibenwein J, Pils D, Horak P, Tomicek B, Goldner G, Worel N, et al. Promoter hypermethylation of GSTP1, AR, and 14-3-3sigma in serum of prostate cancer patients and its clinical relevance. *Prostate* 2007;**67**:427–32.
61. Smeds J, Berggren P, Ma X, Xu Z, Hemminki K, Kumar R. Genetic status of cell cycle regulators in squamous cell carcinoma of the oesophagus: the CDKN2A

(p16(INK4a) and p14(ARF)) and p53 genes are major targets for inactivation. *Carcinogenesis* 2002;**23**:645–55.
62. Kim TY, Jong HS, Jung Y, Kang GH, Bang YJ. *DNA hypermethylation in gastric cancer. Aliment Pharmacol Ther* 2004;**20**(Suppl. 1):131–42.
63. Chang H, Yi B, Li L, Zhang HY, Sun F, Dong SQ, et al. Methylation of tumor associated genes in tissue and plasma samples from liver disease patients. *Exp Mol Pathol* 2008;**85**:96–100.
64. Tchou JC, Lin X, Freije D, Isaacs WB, Brooks JD, Rashid A, et al. GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. *Int J Oncol* 2000;**16**:663–76.
65. Wilcox CB, Baysal BE, Gallion HH, Strange MA, DeLoia JA. High-resolution methylation analysis of the BRCA1 promoter in ovarian tumors. *Cancer Genet Cytogenet* 2005;**159**:114–22.
66. Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* 1999;**59**:798–802.
67. Banelli B, Gelvi I, Di Vinci A, Scaruffi P, Casciano I, Allemanni G, et al. Distinct CpG methylation profiles characterize different clinical groups of neuroblastic tumors. *Oncogene* 2005;**24**:5619–28.
68. Domann FE, Rice JC, Hendrix MJ, Futscher BW. Epigenetic silencing of maspin gene expression in human breast cancers. *Int J Cancer* 2000;**85**:805–10.
69. Jarrard DF, Kinoshita H, Shi Y, Sandefur C, Hoff D, Meisner LF, et al. Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. *Cancer Res* 1998;**58**:5310–4.
70. Jones PA, Wolkowicz MJ, Rideout 3rd WM, Gonzales FA, Marziasz CM, Coetzee GA, et al. De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc Natl Acad Sci U S A* 1990;**87**:6117–21.
71. Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am J Hum Genet* 1991;**48**:880–8.
72. Salem CE, Markl ID, Bender CM, Gonzales FA, Jones PA, Liang G. PAX6 methylation and ectopic expression in human tumor cells. *Int J Cancer* 2000;**87**:179–85.
73. Salvesen HB, MacDonald N, Ryan A, Jacobs IJ, Lynch ED, Akslen LA, et al. PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. *Int J Cancer* 2001;**91**:22–6.
74. Tsuchiya T, Tamura G, Sato K, Endoh Y, Sakata K, Jin Z, et al. Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. *Oncogene* 2000;**19**:3642–6.