Methylation matters

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Abstract
DNA methylation is not just for basic scientists any more. There is a growing awareness in the medical field that having the correct pattern of genomic methylation is essential for healthy cells and organs. If methylation patterns are not properly established or maintained, disorders as diverse as mental retardation, immune deficiency, and sporadic or inherited cancers may follow. Through inappropriate silencing of growth regulating genes and simultaneous destabilisation of whole chromosomes, methylation defects help create a chaotic state from which cancer cells evolve. Methylation defects are present in cells before the onset of obvious malignancy and therefore cannot be explained simply as a consequence of a deregulated cancer cell. Researchers are now able to detect with exquisite sensitivity the cells harbouring methylation defects, sometimes months or years before the time when cancer is clinically detectable. Furthermore, aberrant methylation of specific genes has been directly linked with the tumour response to chemotherapy and patient survival. Advances in our ability to observe the methylation status of the entire cancer cell genome have led us to the unmistakable conclusion that methylation abnormalities are far more prevalent than expected. This methylomics approach permits the integration of an ever growing repertoire of methylation defects with the genetic alterations catalogued from tumours over the past two decades. Here we discuss the current knowledge of DNA methylation in normal cells and disease states, and how this relates directly to our current understanding of the mechanisms by which tumours arise.

Keywords: methylation; cancer

5'-methylcytosine, the fifth base
Methylation of cytosine is the only known endogenous modification of DNA in mammals and occurs by the enzymatic addition of a methyl group to the carbon-5 position of cytosine. The majority of 5'-methylcytosine in mammalian DNA is present in 5'-CpG-3' dinucleotides. Non-CpG sequences such as 5'-CpNpG-3' or non-symmetrical 5'-CpA-3' and 5'-CpT-3' may also exhibit methylation, but generally at a much lower frequency. In mouse embryonic stem cells, however, non-CpG methylation comprises 15-20% of total 5'-methylcytosine.

CpGs are not uniformly distributed in the human genome. In 98% of the genome, CpGs are present approximately once per 80 dinucleotides. In contrast, CpG islands, which comprise 1-2% of the genome, are approximately 200 base pairs (bp) to several kb in length and have a frequency of CpGs approximately five times greater than the genome as a whole. Based on the draft version of the human genome there are an estimated 29 000 CpG islands in the genome, roughly consistent with previous estimates, and CpG islands nearly always encompass gene promoters and/or exons. Approximately 50-60% of all genes contain a CpG island. With the noted exceptions of imprinted genes and several genes on the inactive X chromosome in females, CpGs within CpG islands are normally unmethylated while most CpGs outside CpG islands are methylated. It has been suggested that these patterns of methylation may serve to compartmentalise the genome into transcriptionally active and inactive zones.

DNA methylation is present in organisms from bacteria to humans. In bacteria, methylation is part of a defence mechanism to reduce the amount of gene transfer between species. Particular mutant strains of bacteria that lack detectable methylation nevertheless survive and proliferate. Early studies were unable to detect cytosine methylation in the fruit fly Drosophila melanogaster. Recent reports, however, show low level methylation of cytosine residues, particularly in early developmental stages. In contrast to bacteria, deletion of any one of three DNA methyltransferase genes from mice is lethal, suggesting that methylation has additional and indispensable functions in mammals.

Establishing DNA methylation patterns proceeds through defined phases during development of an organism. In general, germ cells of females are less methylated than those of males, and gamete methylation patterns are erased by a genome wide demethylation near the eight cell stage of blastocyst formation. During the implantation stage, methylation patterns are established following a wave of de novo methylation. In the adult, the amount and pattern of methylation are tissue and cell
Methylation machinery

Three DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B, have been identified in mammalian cells.\(^{16,17}\) Elimination of any of these genes from the germ line of mice is lethal.\(^{17,22}\) Mouse embryos having homozygous deletion of Dnmt1 or Dnmt3B die before birth, while Dnmt3A deletion leads to death approximately four weeks after birth.\(^{17,22}\) Mice that are heterozygous mutant for any one of the DNA methyltransferases appear normal and are fertile.\(^{17,22}\) Conditional deletion of Dnmt1 from mouse fibroblasts results in p53 dependent apoptosis and massive dysregulation of gene expression.\(^{23}\)

Initial methylation of DNA requires de novo methylase activity that is mostly present during early embryonic development.\(^{21}\) All three methyltransferases possess de novo activity,\(^{17,25}\) but appear to have certain distinct sequences targeted for methylation.\(^{1,17}\) The activity of Dnmt1 is far greater on hemimethylated DNA, and thus DNMT1 is termed a maintenance methylase. DNMT1 is ubiquitously expressed during development, and may therefore serve to maintain silencing, rather than initiating the event. Expression of the XIST (X inactive specific transcript) gene is also correlated with methylation status of its promoter, but XIST is unmethylated and expressed from the inactive X and methylated and silent on the active X.\(^{26}\) Dnmt1 deleted embryonic stem cells express the normally silenced XIST gene on the active X chromosome in males.\(^{27}\)

Methylation is also critical for the expression of imprinted genes. While the majority of genes are expressed from the maternal and the paternal alleles, a small number of “imprinted” genes are expressed in a parent of origin specific manner.\(^{22}\) Imprinting involves allele specific methylation in CpG islands associated with these genes, through mechanisms that are not fully understood.\(^{35,36}\) However, recent studies suggest the involvement of a protein with chromatin boundary function, CTCF, that binds to the unmethylated allele at the imprinting control region upstream of H19, but not to the methylated allele.\(^{37,38}\) Since methylation patterns are reproducibly established in imprinted genes and other genomic regions, sequence specificity for methyltransferases has been postulated. A first indication of how this might occur was described in a recent report of a protein complex consisting of DNMT1 together with RB, E2F1, and HDAC1. Theoretically, such a complex could specifically target genes that contain E2F1 binding sites.\(^{39}\)

Abnormal methylation in disease

The importance of DNA methylation patterns to human health is underlined by the recent identification of mutations in methylation type specific and there is evidence for aging related methylation changes of CpG islands in the promoter of genes, including the oestrogen receptor gene and MYOD1.\(^{20}\) Methylation patterns of certain genomic regions appear polymorphic between people and can be inherited, suggesting either the persistence of certain methylation at all stages of development, or the encryption of methylation pattern information.\(^{21}\)

Functions of methylation

Cytosine methylation has a number of functions, a few that are proven and others that are actively debated. Methylation within gene regulatory elements such as promoters, enhancers, insulators, and repressors generally suppresses their function. In normal cells, imprinted genes and genes on the inactive X chromosome are the most prominent examples of transcriptional repression by methylation. Methylation within gene deficient regions, such as in pericentromeric heterochromatin, appears crucial for maintaining the conformation and integrity of the chromosome.\(^{22}\) Methylation has also been proposed as a genome defence against surreptitious mobile genetic elements.\(^{35,36}\)

Two mechanisms by which methylation blocks transcription have been proposed.\(^{37,38}\) First, methylation inhibits binding of certain transcription factors to their CpG containing recognition sites.\(^{39,40}\) A second mechanism involves proteins or protein complexes, MeCP2 or MeCP1 respectively, that bind specifically to methylated CpGs and can indirectly inhibit the binding of transcription factors by limiting access to a regulatory element.\(^{40,41}\) The inhibitory effect is mediated by the ability of the methylated CpG binding proteins to recruit histone deacetylases (HDACs). For example, MeCP1 recruits HDAC1, HDAC2, and Rb related proteins 46 and 48,\(^{42}\) while MeCP2 binds to the Sin3-HDAC co-repressor complex.\(^{43}\) HDACs deacetylate lysine residues in the N-terminal tails of the histones to facilitate interactions between adjacent histones that in turn help form transcriptionally repressive chromatin structures. Other proteins with methyl binding domains (MBD) have been identified but their role in mediating the effects of DNA methylation remains to be determined.\(^{44}\)

During development, inactivation of one of the two X chromosomes in female cells occurs by a process dependent on methylation.\(^{45}\) CpG island containing promoters of the majority of genes on the inactive X chromosome, including housekeeping genes like HPRT, G6PD, and PGK1, are methylated and transcriptionally silent, presumably to ensure equivalent expression levels in male and female cells.\(^{46}\) For many of these genes, silencing precedes methylation\(^{10}\) and may therefore serve to maintain silencing, rather than initiating the event. Expression of the XIST (X inactive specific transcript) gene is also correlated with methylation status of its promoter, but XIST is unmethylated and expressed from the inactive X and methylated and silent on the active X.\(^{47,48}\) Dnmt1 deleted embryonic stem cells express the normally silenced XIST gene on the active X chromosome in males.\(^{51,52}\)

Abnormal methylation in disease

The importance of DNA methylation patterns to human health is underlined by the recent identification of mutations in methylation
related genes that are linked to human disease. Mutations in the methyltransferase gene DNMT3B are found in patients with ICF syndrome and mutations in the methylated CpG binding protein MeCP2 have been observed in patients with Rett syndrome.

ICF syndrome is a rare autosomal recessive disorder, characterised by the presence of variable immunodeficiency, instability of the pericentromeric heterochromatin in chromosomes 1, 9, and 16, and mild facial anomalies. The first observations indicating defects in the methylation machinery showed hypomethylation of satellite DNA in ICF patients. Additionally, chromosomal abnormalities such as those observed in ICF patients can also be induced in normal lymphocytes following treatment with the demethylating agents, 5-azacytidine and 5-azadeoxycytidine. Homozygosity mapping allowed localisation of the ICF syndrome candidate gene to chromosome 20q11-q13. The chromosomal location of DNMT3B. Recently, several groups reported mutations in DNMT3B in ICF patients consistent with the idea of a methylation defect.

Rett syndrome is an X linked, neurodevelopmental disorder characterised by mental retardation and autistic behaviour and occurs exclusively in females. Mutations in an X chromosome gene, MeCP2, which encodes a methylated DNA binding protein, occur in at least two thirds of sporadic Rett syndrome cases and 45% of familial cases. The majority of mutations occur either in the methylated CpG binding domain or in the transcriptional repression domain that recruits the Sin3-HDAC corepressor complex.

Other human diseases have been shown to be associated with imprinted regions and defects in imprinted genes or their epigenetic regulation. Examples include Beckwith-Wiedemann syndrome (BWS) on human chromosome 11p15 and the Prader-Willi syndrome (PWS) and Angelman syndrome (AS) both on chromosome 15q11-q13. PWS is characterised by mild to moderate mental retardation and patients are slow moving and overweight because of severe hyperphagia. Patients with AS show severe mental retardation and are thin, hyperactive, and show disorders of movement and uncontrolled laughter. The first hint of a possible imprinting effect in these syndromes came from the finding that the deleted fragments in both syndromes are from opposite parental origins. In PWS the deletion occurs in the paternal copy and in cases of AS the maternal copy is deleted. Additional evidence came from the finding of maternal disomy of chromosome 15 in PWS patients and paternal disomy of chromosome 15 in AS. These data suggest that the PWS gene(s) are transcribed from the paternal allele only and the AS gene(s) are expressed from the maternal allele. Several imprinted genes were identified in the critical region for PWS/AS, including paternally expressed SNRPN and maternally expressed UBE3A. Microdeletions in the SNRPN gene have been identified that alter DNA methylation patterns and lead to dysregulation of SNRPN and other genes in the imprinted gene cluster.

BWS is characterised by a number of growth abnormalities, including hemihypertrophy, macroGLOSSIA, visceralmegaly, and gigantism; however, the phenotypic expression is variable. Between 5 and 10% of BWS patients are prone to Wilms tumour, adenocortical carcinoma, hepatoblastoma, or embryonal rhabdomyosarcoma. Wilms tumours have been shown to exhibit preferential loss of maternal alleles at chromosome 11p. A cluster of at least 10 imprinted genes was identified in 11p15.5, including the paternally expressed IGF2 and the maternally expressed H19, and there is evidence for two independent imprinting control centres. The most common abnormality in BWS patients was LOI of IGF2 without any detectable chromosomal abnormalities. There is now overwhelming evidence implicating DNA methylation changes in BWS. Epigenetic changes include loss of imprinting in IGF2 and silencing of H19 by promoter methylation.

Defects in methylation may underlie or contribute to other disorders. Because of the heritable and reversible nature of methylation, intriguing theories have been proposed regarding the role that epigenetics (possibly aberrant methylation) might play in complex, non-Mendelian disorders such as schizophrenia and affective disorders.

The genomics of methylation imbalance in cancer
The underlying basis of cancer is a cumulative series of genetic and epigenetic alterations leading to deregulated cell growth. Particular alterations may provide a selective growth advantage to the tumour cell, whether by conferring resistance to therapies, increasing positive growth signals through the activation of oncogenes, or eliminating growth limiting signals through the inactivation of tumour suppressor genes. “Mutations” outside the nucleotide sequence occur frequently in human cancer and may contribute to the initiation and malignant progression of tumours. Although epigenetic mutations involving cytokine methylation were first observed in primary cancers nearly two decades ago, like most controversial ideas in science, it has taken a while to catch on.

An imbalance in cytokine methylation is prevalent in human sporadic cancers. Methyltransferase pattern defects include genome wide hypomethylation and localised aberrant hypermethylation of CpG islands. These imbalances can be present together in a single tumour, though the net effect is usually a decrease in total methylation levels. Whether genome hypomethylation and CpG island hypermethylation are linked by a common underlying mechanism or result from distinct abnormalities in the cancer cell is currently unknown. However, we do know that hypomethylation and hypermethylation occur at specific but distinct sites within the cancer cell.
Hypomethylation

The amount of 5'-methylcytosine in genomic DNA is measured directly by HPLC or indirectly as an inverse value of the capacity of a DNA sample to accept tritiated methyl groups from a universal methyl donor s-adenosylmethionine. These distinct methods have shown similar general trends of hypomethylation in tumours. The extent of genome wide hypomethylation in tumours parallels closely the degree of malignancy, though this is tumour type dependent. In breast, ovarian, cervical, and brain tumours, for example, hypomethylation increases progressively with increasing malignancy grade. Additionally, a study of 136 breast lesions has shown a significant correlation between the extent of hypomethylation and disease stage, tumour size, and degree of malignancy. Thus, hypomethylation may serve as a biological marker with prognostic value. Cells from non-malignant medical conditions such as gastritis and colitis also display a progressive hypomethylation, though lesser in degree relative to that in malignant cells. In contrast to escalating hypomethylation during tumour progression, the levels of hypomethylation in benign colon polyps and malignant colon adenocarcinoma are quantitatively similar. It is unlikely that hypomethylation reflects the dividing state of the premalignant or cancer cells, because normal tissues and cultured cells show no correlation between cell turnover or self renewal rates and overall levels of 5'-methylcytosine. These correlative data alone are consistent with either a contributory or reflective role of hypomethylation in tumour initiation and malignant progression.

What is the evidence that hypomethylation might contribute directly to malignancy, and what are the mechanisms by which this might occur? Several hypotheses have been proposed including hypomethylation mediated transcriptional activation of oncogenes, activation of latent retrotransposons, and chromosomal instability. Each of these hypotheses has received some support from the identification of genome sites subject to hypomethylation in cancer. Pioneering studies suggested that loss of methylation in tumours may involve all segments of the genome, including sequences of high, medium, and low copy number. Subsequent reports confirmed these findings in a more detailed fashion, providing additional rationale for an in depth investigation of each of the hypotheses. We now consider the data pertinent to each hypothesis.

ONCOGENE ACTIVATION

Holliday and Pugh proposed that if hypomethylation leads to inappropriate activation of genes important in neoplastic growth, then hypomethylation could provide a selective advantage for the tumour cell. Such cells could then clonally evolve and would appear as a prominent population in the tumour. Hypomethylation within the body of a number of genes has been found in primary cancers, including known oncogenes such as CMYC and HRAS. While oncogene overexpression in the absence of gene amplification is fairly common, to date there is no compelling mechanistic or correlative evidence that local hypomethylation causes overexpression. Hypomethylation in human cancers is causally related to transcriptional activation of a large group of genes of the MAGE, GAGE, CTA/LAGE, and SAGE families. These unrelated gene families are located on the X chromosome and their cellular function is unknown. MAGE genes, which are a prototype of this group, were first discovered as coding for tumour specific antigens recognised by cytolytic T lymphocytes and are currently being studied as potential anticancer vaccines. MAGE type genes are germline specific genes that are aberrantly activated in melanomas and many other tumour types. They are unmethylated in spermatogenic cells, but are methylated in all adult somatic tissues, including alleles on both the active and inactive X chromosomes.

Studies of MAGE promoters suggest that these genes use methylation as a primary mechanism for silencing in adult somatic tissues. The promoters of MAGE type genes have an intermediate density of CpGs and may constitute a unique class of promoters that fall somewhere between the constitutively unmethylated CpG island promoter and the conditionally methylated CpG poor promoter. MAGE promoter demethylation, possibly as a consequence of genome wide hypomethylation, leads to transcriptional activation of MAGE genes in cancer cells. The MAGE gene expression in tumour cells may stimulate the production of anti-MAGE T lymphocytes. Therefore, instead of providing a selective growth advantage, hypomethylation
Methylation matters

MOBILE DNA

Hypomethylation in cancer cells may lead to the transcriptional activation of mobile genetic elements called retrotransposons.\textsuperscript{103–106} This suggestion relates directly to a theory that a primary function of methylation is to defend the genome from the deleterious effects of these resident and invading parasites.\textsuperscript{66} The most abundant retrotransposons in the human genome are known as long interspersed nuclear elements (LINEs or L1s).\textsuperscript{119} Full length L1s have two open reading frames, one which encodes a nucleic acid binding protein and a second which encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilisation in genomes through an RNA intermediate.\textsuperscript{119} One hundred thousand L1s exist in the human genome, but most are inactive owing to truncations, rearrangements, and mutations. Only 30–60 may be competent for transposition.\textsuperscript{120} Additionally, many L1s are methylated and transcriptionally silent, though it is unknown if the non-methylated L1s and the intact L1s are both silenced in this manner. Loss of promoter methylation and transcriptional activation of L1 elements have been reported in a variety of sporadic cancer types.\textsuperscript{103–106}

If the full length, non-methylated transposable elements are transcribed (and then reverse transcribed), they might integrate in and disrupt important growth regulating genes. L1 mutational insertions in sporadic cancers have been found that disrupt the APC gene and CMYC gene in a sporadic tumour of the colon and breast, respectively, suggesting that certain L1s are active in human cancers.\textsuperscript{121} In the disrupted APC gene, the nucleotide sequences in and around the insertion site exhibited the signature of retrotranspose integration.\textsuperscript{121} Mutational insertion of non-autonomous retrotransposons such as Alu elements may also occur in the germline.\textsuperscript{119} Such Alu mediated “mutations” have been observed in BRCA1 and BRCA2 in families with hereditary predisposition to breast and ovarian cancer\textsuperscript{122} and in the MLH1 gene in families predisposed to colon cancer.\textsuperscript{121} Relative to other mutational mechanisms, transposon mediated mutational insertions are rare in well studied human cancer genes. A role of genome hypomethylation in permitting transposition in cancer cells is not resolved, but there is substantial evidence for the unleashing of transcription of large numbers of retrotranspose sequences in a methylation dependent manner.\textsuperscript{123–127}

The deleterious effect of retrotransposons in cancer may not require transposition. It has been suggested that because of the typically strong activity of the 5' LTRs or promoters of L1s, hypomethylation mediated transcriptional activation of L1s could also disrupt expression of nearby genes. While the promoters of most L1s have been deleted, other abundant retrotransposons such as human endogenous retroviruses (HERVs) retain the 5' LTR.\textsuperscript{119} HERVs are also demethylated and expressed in some cancers,\textsuperscript{128} but direct evidence for disrupted expression of genes near transcriptionally activated HERVs or L1s has not yet been reported in primary human cancers.

CHROMOSOME INSTABILITY

Hypomethylation of specific chromosomal domains has also been linked to chromosome instability.\textsuperscript{129} It has been proposed that the hypomethylation contributes to malignancy through disturbance of chromosomal domains and/or abnormal gene dosage effects from lost or gained chromosome fragments. In normal somatic cells, pericentromeric heterochromatin regions on chromosomes 1 and 16 are heavily methylated. In breast adenocarcinomas, ovarian epithelial tumours, and sporadic Wilms tumours, these regions are significantly hypomethylated and frequently unstable.\textsuperscript{124, 126–128}

Chromosome abnormalities associated with the hypomethylation of these regions include isochromosomes, unbalanced juxtacentromeric translocations, and whole arm deletions. Similar rearrangements involving chromosomes 1 and 16 are also induced in mitogen stimulated normal cells treated with either 5-azacytidine or 5-aza-2-deoxycytidine, but not with genotoxins which do not cause DNA hypomethylation.\textsuperscript{129, 130} Hypomethylation may be causally related to chromosome instability, though the apparent need for mitogen stimulation and cell division in this process suggests that the relationship is multifactorial.

An additional link between hypomethylation and chromosome instability has come from studies of ICF syndrome,\textsuperscript{132} a rare genetic disorder in humans that is caused by inherited mutations in the DNA methyltransferase DNMT3B.\textsuperscript{133, 136–137} In all somatic cells of ICF patients, the pericentromeric heterochromatin of chromosomes 1 and 16 is abnormally hypomethylated. Mitogen stimulation of lymphocytes from ICF patients results in a high frequency of abnormalities involving chromosomes 1 and 16, and to a lesser degree chromosome 9, which are similar in nature to the chromosomal abnormalities seen in sporadic cancers or in normal cells treated with demethylating agents.\textsuperscript{130, 133} It should be noted that ICF patients do not have an increased incidence of cancer.\textsuperscript{132–137}

A causal relationship between hypomethylation and chromosome instability is also supported directly by studies of mouse ES cells having homozygous deletion of the methyltransferase Dnmt1.\textsuperscript{138} The mutant ES cells are mostly euploid, but have a significantly increased mutation rate, primarily involving genomic deletion. Thus, data from sporadic human cancers, ICF patients, and mouse ES cells lacking Dnmt1 suggest that hypomethylation may predispose to chromosome abnormalities, possibly facilitated by additional growth stimulating factors or inappropriate cell division.

GOT FOLATE?

Several lines of evidence suggest that DNA hypomethylation and chromosome instability...
it was hypothesised that this particular MTHFR polymorphism may be a risk factor for maternal meiotic non-disjunction and Down syndrome in the children of young mothers.\footnote{85–87} Specific MTHFR polymorphisms are also associated with an increased risk of neural tube defects and vascular disease and may modify cancer risk.\footnote{140–142}

There is strong epidemiological evidence that sufficient dietary folate is important to reduce the risk of certain cancers.\footnote{143} Thus, a role of downstream genome hypomethylation on this cancer risk seems to be an important area for future studies. At present, reduced methyl donor via insufficient folate is the only known cellular mechanism leading to genome hypomethylation in cancer. A role for putative demethylating enzymes or dysfunction of methyltransferases in creating the hypomethylated state has been suggested but remains unproven.

**CpG island hypermethylation**

**THE CANDIDATE GENE APPROACH**

Beginning with its inception in the 1980s, the investigation of abnormal CpG island methylation has toppled the notion that the molecular underpinnings of sporadic cancers are purely genetic.\footnote{85–87} Methylation of CpG island promoters may inactivate both alleles of a proven cancer gene, or may act in concert with genetic mechanisms including point mutation or deletion (fig 1). Methylation of cancer suppressor genes is typically restricted to non-mutated alleles, and demethylating agents are capable of restoring gene activity and tumour suppressor function in cultured tumour cells. A great deal of excitement has come from the possibility that the dormant, but non-mutated genes could be chemically reactivated to restore functional tumour suppressor activity in cancer patients as an alternative to gene replacement therapy. Clinical trials to test this in haematopoietic and solid tumours will soon be under way.\footnote{144}

The candidate gene approach tests for aberrant methylation in established cancer genes, particularly in tumour samples and on specific alleles that do not harbour genetic alterations of the gene. This lucrative approach has uncovered methylation related gene silencing that can account for most types of malignant behaviour exhibited by human cancer cells (table 1). Genes involved in cell cycle regulation, DNA repair, drug resistance and detoxification, differentiation, apoptosis, angiogenesis, metastasis, and invasion are inappropriately silenced by methylation. Similar gene silencing events are recapitulated in chemically and genetically induced mouse models of human cancer.\footnote{145–146} In combination with functional studies of these cancer genes and mechanistic studies linking methylation with gene silencing, there is considerable evidence that CpG island methylation contributes directly to malignancy.\footnote{85–87}

Aberrant methylation may also influence the expression of imprinted genes in cancer cells. Methylation regulated expression of a number of imprinted genes is critical for embryonic
Methylation matters

Table 1  Aberrantly methylated genes in cancer

<table>
<thead>
<tr>
<th>Function</th>
<th>Genes</th>
<th>References (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Death associated protein kinase (DAP kinase, 9q34), Caspase 8 (CASP8, 2q33-34), Target of methylation induced silencing (TMS1, 16p11.2-12.1)</td>
<td>254–257, 258</td>
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<td>Angiogenesis</td>
<td>Thrombospondin-1 (THBS1, 15q15), Retinoblastoma (RB1, 13q14) p14ARF (9p21), Cyclin dependent kinase 2A (CDKN2A, 9p21), Cyclin dependent kinase 2B (CDKN2B, 9p21), p27KIP1 (12p13), p15INK4a (1p36)</td>
<td>183, 185, 187, 188</td>
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<tr>
<td>Cell cycle</td>
<td>14-3-3-ε (stratifin, SFN, 1p)</td>
<td>261–264</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Myogenic differentiation antigen-1 (MYOD, 11p15.4)</td>
<td>276</td>
</tr>
<tr>
<td>DNA repair</td>
<td>S雪花melanoma antigen (SNAI1, 3p21.3)</td>
<td>270</td>
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<td>Metastasis/invasion</td>
<td>E-cadherin (CDH1, 16q22.1)</td>
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<td>Glutathione S-transferase (GSTP1, 11q13)</td>
<td>301, 302</td>
</tr>
<tr>
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<td>Multi-drug resistance 1 (MDR1, 7q21.1)</td>
<td>303</td>
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<tr>
<td>Transcription/ transcription factors</td>
<td>Adenomatous polyposis of the colon (APC, 5q21-22), PTEN (10q23.3)</td>
<td>304, 305, 306</td>
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<td></td>
<td>Androgen receptor (AR, Xq11-12)</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>Androgen receptor 1 (ASOR, 6q25.1)</td>
<td>308–310</td>
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<tr>
<td></td>
<td>Ras association domain family member 1 (RASSF1A, 3p21.3)</td>
<td>304</td>
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<tr>
<td></td>
<td>Serine/threonine protein kinase 11 (STK11 or LKB1, 19p13.3)</td>
<td>313</td>
</tr>
<tr>
<td></td>
<td>Von Hippel-Lindau syndrome (VHL, 3p26-p25)</td>
<td>313, 314</td>
</tr>
<tr>
<td></td>
<td>Hypermethylated in cancer (HIC-1, 17p13.3)</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>Breast cancer, type 1 (BRCA1, 17q21)</td>
<td>315, 315–317</td>
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<td>CD44 antigen (CD44, 11pter-p13)</td>
<td>318</td>
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<tr>
<td></td>
<td>XO2 - oxysterol 2 (XO2, 1q25.2-25.3)</td>
<td>319</td>
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<tr>
<td></td>
<td>Calcium channel, voltage dependent, T type, alpha-1G subunit</td>
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<td></td>
<td>Calmodulin (CaM, 11p15.2-15.1)</td>
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<td>Fragile histidine triad gene (FHIT, 3p14.2)</td>
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<tr>
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<td>Telomerase reverse transcriptase (TERT, 5p15.33)</td>
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<td>Transmembrane protein containing epidermal growth factor and follistatin domains (TPEF, 2q33)</td>
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<td></td>
<td>Chondroitin sulphate proteoglycan 2 (CSPG2, 5q12-14)</td>
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<td>Androgen receptor (AR, Xq11-12)</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>Androgen receptor 1 (ASOR, 6q25.1)</td>
<td>308–310</td>
</tr>
<tr>
<td></td>
<td>Ras association domain family member 1 (RASSF1A, 3p21.3)</td>
<td>304</td>
</tr>
<tr>
<td></td>
<td>Serine/threonine protein kinase 11 (STK11 or LKB1, 19p13.3)</td>
<td>313</td>
</tr>
<tr>
<td></td>
<td>Von Hippel-Lindau syndrome (VHL, 3p26-p25)</td>
<td>313, 314</td>
</tr>
<tr>
<td></td>
<td>Hypermethylated in cancer (HIC-1, 17p13.3)</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>Breast cancer, type 1 (BRCA1, 17q21)</td>
<td>315, 315–317</td>
</tr>
<tr>
<td></td>
<td>CD44 antigen (CD44, 11pter-p13)</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>XO2 - oxysterol 2 (XO2, 1q25.2-25.3)</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>Calcium channel, voltage dependent, T type, alpha-1G subunit</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Calmodulin (CaM, 11p15.2-15.1)</td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>Fragile histidine triad gene (FHIT, 3p14.2)</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>Telomerase reverse transcriptase (TERT, 5p15.33)</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td>Transmembrane protein containing epidermal growth factor and follistatin domains (TPEF, 2q33)</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td>Chondroitin sulphate proteoglycan 2 (CSPG2, 5q12-14)</td>
<td>330</td>
</tr>
</tbody>
</table>

devlopment, but in the environment of a tumour cell, dysregulation of some imprinted genes may have oncogenic consequences. Complete loss of function of an imprinted gene could occur by deletion of the single transcriptionally active allele, as shown for the cyclin dependent kinase inhibitor p57KIP2 in lung cancers, H19 in Wilms tumours, and NOEV2, a member of the RAS superfamily, in breast and ovarian cancers. Uniparental disomy of the silent allele could also lead to complete inactivation of an imprinted gene that normally inhibits cell growth. Conversely, activation of a growth supporting gene such as IGF2 could occur by uniparental disomy of the active allele. In addition, loss of the imprinting signal and subsequent loss of imprinted gene expression (LOI) could result in biallelic expression of a growth promoting gene, as shown for IGF2 in Wilms tumours. In colorectal cancer, biallelic methylation of the CTCF binding site resulted in biallelic IGF2 expression, primarily in tumours that also showed methylation and silencing of MLH1 and p16.

Aberrant methylation of CpG islands has been observed in cells that are not overtly malignant. For example, cultured mammary epithelial cells having an extended life span are widely considered to be normal, yet they contain a densely methylated p16 promoter and lack p16 expression. The loss of p16 expression appears to be gradual, and proceeds coordinately with increasing promoter methylation. Aberrant CpG island methylation preceding malignancy is also observed in vivo. For example, frequent and widespread CpG island methylation is present in non-dysplastic tissue from patients with Barrett’s oesophagus and associated adenocarcinoma. In gastric cancer patients, the p16 and E-cadherin promoters are methylated in tumours and in normal gastric mucosa. Similarly, the promoter of the oestrogen receptor gene is aberrantly methylated in patients with inflammatory reflux oesophagitis. Thus, CpG island methylation is not simply a consequence of the malignant state. If it can be detected in normal appearing tissue before the onset of cancer, aberrant methylation may be a useful marker for early or precancer detection.

CANCER METHYLOMICS

Cancer genes may be inactivated by a variety of mechanisms, including point mutation, deletion, and methylation (fig 1). For particular genes, it is often one of the mechanisms that predominates in the inactivation. For example, the p16 tumour suppressor gene in brain and breast tumours is inactivated primarily by homozygous deletion. The p53 gene is most frequently affected by deletion of one allele and point mutation of the other allele in nearly all tumour types in which it is involved. These observations suggest that there may exist an entirely different set of important cancer genes that are inactivated primarily by aberrant methylation on one or both alleles. In theory, such genes would have remained undiscovered over the past two decades because of the exclusively genetic screening methods used.

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On the foundation set by discovery of aberrantly methylated genes, a number of methods to screen the genome for aberrantly methylated genes have been developed. These include PCR based methods, array hybridisation, and restriction landmark genome scanning (RLGS). Additional genome scanning methods involving mass spectrometry and non-radioactive oligo and CpG island array methods are also emerging. Suitable methods for addressing the hypotheses stated above should have a strong bias for 5' CpG islands and cover large numbers of genes. It should be noted that the current focus on CpG island promoters overlooks other less CpG rich promoters that also might be subjected to aberrant methylation and silencing.

Restriction landmark genome scanning (RLGS) is an approach that is uniquely suited for simultaneously assessing the methylation status of thousands of CpG islands (fig 2). RLGS separates radiolabelled NotI fragments in two dimensions and allows distinction of single copy CpG islands from multicopy CpG rich sequences. The methylation sensitivity of the endonuclease activity of NotI provides the basis for differential methylation analysis and NotI sites occur primarily in CpG islands and genes. RLGS has been used to identify novel imprinted genes, novel targets of DNA amplification, and methylation in human cancer and to identify deletion, methylation, and gene amplification in a mouse model of tumorigenesis. Additionally, the chromosome of origin of CpG islands displayed on the profiles has been determined. Such massively parallel analyses are critical for pattern recognition within and between tumour types and for estimating the overall influence of CpG island methylation on the cancer cell genome.

The total number of aberrantly methylated CpG islands in sporadic human tumours was estimated from RLGS profiles. The analysis covered 1184 CpG islands in each of 98 primary human tumours, for a total of 116 032 potential methylation events. An average of 600 methylated CpG islands per tumour was estimated, with a range of 0 to 4400. The total number of methylated sites is variable between and in some cases within different tumour types, suggesting there may be methylation subtypes within tumours having similar histology. Aberrant methylation of a proportion of these genes correlates with loss of gene expression.

The methylomics approach illuminates patterns of methylation that might yield clues to the underlying mechanism of aberrant methylation. For example, the observation that some CpG islands are preferentially methylated suggests that clonal selection and/or different susceptibilities of CpG islands may shape the patterns in tumours. The process may be stochastic, but the non-random outcome in the tumour suggests one or both of these mechanisms may be active. For methylation of proven cancer genes, an argument in support of clonal selection is straightforward since their tumour suppressing ability has been shown. An anatomical application of methylation data showed that aberrant methylation is usually found in a contiguous field in tissue from cancer patients, suggesting either a concerted methylation change or a clonal expansion of cells with aberrant hypermethylation.

Some genes are aberrantly methylated in a tumour type specific manner. Tumour
type and even histological subtype specificity is also observed in studies of the BRCA1 and other important cancer genes.\textsuperscript{177-178} These patterns, and resulting loss of gene activity in many cases, suggest that methylation of specific subsets of genes may contribute to the development of specific tumour types.

Homozgyous methylation of specific genes is quite frequent, even in low malignancy grade tumours.\textsuperscript{85, 172-176, 179-180} On statistical grounds the data suggest that methylation of one allele may predispose to methylation of the second allele of the same gene. Allelic transfer of methylation involving homologous gene pairing has been observed in plants and can result in suppressed expression of endogenous genes and transgenes.\textsuperscript{181} Pairing of one methylated and one unmethylated homologous chromosome segment during mitosis could lead to a transient hemimethylated state.\textsuperscript{87} If the maintenance methyltransferase DNMT1, which has a predilection for hemimethylated substrates and certain unusual DNA structures,\textsuperscript{182} is present at the precise time and location of homologous pairing, it may lead to homozgyous methylation of a particular gene. Depending on the rate of tumour cell specific and locus specific aberrant methylation, the exceptionally high frequency of homozygous methylation may be considered circumstantial support for an allelic transfer of methylation. The persistence of monoallelic methylation in many cases indicates that transallelic spreading of methylation is not an obligate event.

Central to understanding the impact and importance of CpG island methylation is the extent to which the methylation is capable of silencing the gene and the type of genes that are methylated. If methylation of a gene contributes to tumorigenesis, one would expect that: (1) the gene is expressed in the normal cells that give rise to the tumour, (2) the level or extent of methylation in the cancer cells is sufficient to silence or decrease expression of the gene in primary tumours, (3) and re-expression of the gene should have a measurable effect on the phenotype of the tumour cell. If methylation is the primary and sole mechanism of inactivation, it is expected that: (1) an unmethylated copy of the promoter would support transcription when transfected in cells having their endogenous promoter methylated, and (2) experimental demethylation by 5-aza-2-deoxycytidine should reactivate expression of the methylated gene. At the foundation of these expectations is the assumption that inappropriate gene silencing is the primary consequence of CpG island methylation. While this function is proven for many genes, it seems premature to suggest that all CpG island methylation events in cancer cells have a similar consequence or even arise through the same mechanism.

LOCATION, LOCATION, LOCATION

Aberrant CpG island methylation alone does not uniformly connote inappropriate gene silencing. Aberrant methylation that is not within the promoter may have no effect on gene expression or in some cases may promote expression.\textsuperscript{86} Alternatively, a lack of correlation could indicate that the single or few CpGs tested per island are not representative of the remainder of the island or that sparse methylation may be insufficient to silence the associated gene, particularly if the promoter activity is strong. Occasionally, aberrant methylation has been observed in genes that are transcriptionally inactive in the normal cell type from which the tumour originates, or which have been inactivated first by epigenetic mechanisms that do not involve methylation. Other explanations for non-random methylation, such as transcriptional effects on distant genes, or in dictating alternate promoter usage could also be involved. Alternatively, differing susceptibilities to aberrant methylation may contribute to the formation of these non-random patterns. These questions may be addressed in part by assessing the specificity of the DNA methyltransferases in cancer cells.\textsuperscript{183, 184} However, to account for the tumour type specificity of the methylation events, factors in addition to nucleotide sequence must be invoked. Potential factors that can influence methylation status and may differ between tissues include local chromatin conformation, gene activity, and exposure to exogenous agents. Clearly, the location and extent of the individual methylation events are important determinants of the effect of aberrant CpG island methylation in cancer.

METHYLOMICS AND GENOMICS

The prevalence and specificity of aberrant methylation raises important questions regarding the relative contribution of genetic and epigenetic mechanisms in the genesis of human tumours. For a comprehensive view of the underlying mechanisms of tumorigenesis, methylation patterns can be compared to genes and chromosome regions identified by traditional genomic analysis of tumours.

CpG island methylation may precede genetic instability in cancer cells. The MLH1 and 14-3-3\textsuperscript{\textalpha} genes, both important for genome integrity, are frequently silenced by aberrant methylation in cancer.\textsuperscript{85, 189-191} MLH1 encodes a DNA mismatch repair protein. Loss of MLH1 function in colon cancer is associated with a 100-fold greater mutation rate throughout the genome, which is particularly apparent at short repeated sequences, termed microsatellites.\textsuperscript{182, 183} MLH1 promoter methylation and gene silencing are significantly correlated with the microsatellite instability and experimental demethylation in tumour cell lines leads to re-expression of MLH1 and restoration of a DNA mismatch repair proficient phenotype.\textsuperscript{185} Additionally, in vitro studies of the MLH1 promotor indicate that methylation of a minimal region in the promotor, which is also methylated in the primary tumours, is sufficient to inhibit MLH1 transcription.\textsuperscript{184} MLH1 promoter methylation accounts for the majority of sporadic colon tumours exhibiting microsatellite instability,\textsuperscript{186} and has also been observed in sporadic endometrial cancer\textsuperscript{187} and in some hereditary colon and gastric tumours.\textsuperscript{181, 180} Methylation of a second gene indirectly involved in maintaining DNA integrity, the
The 14-3-3\(\gamma\) gene, is found in 91% of breast tumours and in other tumour types.\textsuperscript{185-188} The 14-3-3\(\gamma\) protein induces G2 arrest following DNA damage.\textsuperscript{189} Breast cancer cell lines that do not express 14-3-3\(\gamma\) accumulate a greater number of chromosomal breaks when exposed to \(\gamma\) irradiation.\textsuperscript{185} Thus, aberrant methylation and gene silencing may predispose to genetic instability, rather than being a reflection of it.

There are both random and recurrent components to genetic and methylation abnormalities. Nearly all chromosomal bands have been implicated in genetic loss within individual tumour types,\textsuperscript{197} while in an initial study considering 98 tumours from seven tumour types one or more aberrant methylation events were detected in 36% of the CpG islands tested.\textsuperscript{172} The “background” alterations may reflect an unstable genetic and/or methylation state of the tumour cell. The terms mutator phenotype\textsuperscript{192} and methylator phenotype\textsuperscript{196-200} are roughly equated with the former and latter states, respectively. Studies from colon tumours and cell lines have suggested an undefined linkage between the two phenotypes.\textsuperscript{199,201,202} In contrast, a direct test of methylation capacity and extent of existing methylation did not distinguish mutator from non-mutator colon cancer cell lines.\textsuperscript{203}

A proportion of the frequently methylated CpG islands are not located near regions of recurrent genetic loss in the same tumour type, suggesting that these targets are independent of recurrent genetic alterations. This is underlined by the fact that a significant proportion of low grade astrocytomas have relatively normal appearing genomes, while a methylocimic approach indicates that CpG island methylation is frequent and widespread.\textsuperscript{172} It will be of significant interest to determine the proportion of these silencing events that have a measurable role in tumorigenesis.

A number of aberrant methylation sites coincide with recurrent sites of deletion. The “two hit” mechanism combining deletion and methylation has not yet been addressed.\textsuperscript{184-188} Breast cancer cell lines that do not express 14-3-3\(\gamma\) accumulate a greater number of chromosomal breaks when exposed to \(\gamma\) irradiation.\textsuperscript{185} Thus, aberrant methylation and gene silencing may predispose to genetic instability, rather than being a reflection of it.

Is aberrant methylation of CpG islands in cancer cells a cause or consequence of gene inactivity? Possibly the most frequently posed question in the field, it may have arisen from studies of methylation associated X chromosome inactivation. Many genes on the inactive X chromosome are transcriptionally silenced before methylation, leading to the prevailing notion that methylation was not causal in the gene silencing, but perhaps required for maintenance of the inactive state.\textsuperscript{210} Recent studies of cells from the \textit{Dmm1} deleted mice suggest that methylation is necessary for proper X inactivation, potentially mediated through methylation of the \textit{XIST} gene promoter.\textsuperscript{211} Nevertheless, comparisons between X chromosome inactivation and aberrant CpG island methylation in cancer are problematic since the features of each are fundamentally different. X inactivation occurs during development of the organism, while aberrant CpG island methylation occurs in adult and paediatric tumour cells. X inactivation is a programmed cellular process and involves an entire chromosome, whereas aberrant CpG island occurs in deregulated cancer cells and can be localised to a CpG island without involvement of nearby CpG islands or genes. In this respect, aberrant
CpG island methylation is more similar to a local mutation than to more general defects involving deletion and chromosome copy number changes.

MECHANISMS OF ABERRANT CpG ISLAND METHYLATION

Two models by which CpG islands become methylated in cancer have been outlined. One proposed mechanism involves the loss of factors that normally protect the CpG island from methylation. Depending on the nature of the factor, aberrant methylation could be a cause or consequence of transcription inhibition. The protective factors would successfully compete with the methyltransferase for sites within the CpG island to prevent methylation. Protective factors might be structural proteins or transcription factors. For example, the recognition sites for SP1 transcription factor binding are found within most CpG islands and mutation of an SP1 site in a transgenic mouse leads to methylation of the transgene CpG island. However, in mice with homozygous deletion of the SP1 gene, CpG islands remain unmethylated. Certainly other transcription factors might serve a similar role, but the fact that even CpG islands from non-expressed genes remain unmethylated in normal cells implies that factors other than those associated with active transcription must be involved in protecting some CpG islands. In mouse fibroblasts, inhibition of poly ADP ribosylation leads to a decrease in the number of normally unmethylated CCGG sequences in the genome, suggestive of a pervasive loss of CpG island protection. This system may be a useful model for identification of the molecular mechanism(s) leading to aberrant CpG island methylation. Loss of protective factors in human tumour cells may allow spreading of methylation into the CpG island from flanking heavily methylated sequences that often contain Alu elements. In normal adult tissues, a well defined boundary exists between the methylated and unmethylated domains of the 5’ end of the GSTγ gene CpG island. The sharp demarcation and GSTγ expression are often lost in primary tumours. The nucleotide sequence at the boundary appears unique to the GSTγ gene.

A second model suggests that aberrant CpG island methylation is an active process and causes inappropriate gene silencing. In support of this model, experimental overexpression of murine Dnmt1 leads to transformation of NIH3T3 cells and in immortalised human fibroblasts, human Dnmt1 expression can result in massive methylation of CpG island associated promoters and gene silencing. Furthermore, inhibition of the methyltransferase using antisense to Dnmt1 reduces the tumorigenicity of murine adrenocortical tumour cells. Also in support of a causal role, inactivated tumour suppressor genes can be reactivated by demethylation and methylation appears to be dominant over chromatin mechanisms in the gene silencing. Early studies suggested that tumours have an increased activity and expression of the maintenance methyltransferase Dnmt1, but the level of this up regulation remains a contentious issue. Considering these and other data, it was quite surprising that aberrantly methylated CpG islands in a human colon cancer cell line remained methylated following homozygous deletion of the Dnmt1 gene. So although Dnmt1 overexpression can initiate aberrant CpG island methylation and facilitate transformation, it is not absolutely required for maintaining the aberrantly methylated state in these cells. Thus, debates of the exact initiating event for aberrant CpG island methylation are unsettled.

DNA METHYLATION AND MUTATIONAL HOTSPOTS

Spontaneous deamination of methylated cytosines can lead to C to T point mutations. Because a disproportionate number of point mutations in the p53 tumour suppressor gene (and other genes) are C to T mutations at CpGs, it has been speculated that deamination of the normally methylated CpGs in exons of the p53 gene is involved. An estimated 50% of all human tumours show a defect in p53, a situation that offers a unique opportunity to study mutation spectra in different neoplasias and to investigate the effects of endogenous and exogenous factors. Furthermore, mutation data for p53 are collected in a large database with currently over 10 000 entries. The body of the p53 gene contains 23 normally methylated CpG dinucleotides within the region encoding the DNA binding domain (codons 120 to 290). These CpGs represent only 8% of the total p53 gene sequence but 33% of the mutations in this region are found in the CpGs, suggesting a link between methylated sequences and mutational hot spots.

In addition to endogenous deamination, differing efficiencies of mismatch repair mechanisms of T/G versus U/G mismatches might contribute to the increased mutation rate of methylated CpGs relative to unmethylated CpG sites. Alternatively, involvement of exogenous factors was suggested by the identification of tumour type specific mutational hotspots. For example, mutation hotspots in codons 175, 248, and 273 are commonly found in breast, ovarian, and stomach cancers as well as in leukaemias and lymphomas. p53 codon 157 is a mutational hotspot in lung cancer patients with smoking history but not in other tumour types.

It was shown that BPDE, the activated metabolite of benz[a]pyrene, present at 20 ng to 40 ng per cigarette, forms adducts with DNA at the N2 position of guanine. Mapping the BPDE adducts in the p53 gene of BPDE treated HeLa cells and bronchial epithelial cells showed strong selective adduct formation in codons 157, 248, and 273, the mutational hotspots in smokers with lung cancer. Similar results were obtained for other polycyclic aromatic hydrocarbons present in combustion products of organic matter including cigarette smoke. Guanines
flanked by 5'-methylcytosines were the preferential targets for adduct formation. Considering a genome wide increase of methylation in CpG islands, it has been speculated that similar mechanisms result in increased mutation rates not only within coding regions of genes but also in promoter regions, leading to changes in gene regulation.

EARLY DETECTION, PREDICTION, AND CLASSIFICATION OF CANCER

One of the goals in cancer management is to identify the most effective therapy with the least toxicity for the patient. Successful treatment depends on an accurate, reliable, and reproducible classification of a tumour, using all available criteria including histopathology, cytogentic diagnosis, and histochemical stains. Molecular marker studies attempt to distinguish tumours that are similar in histology, but may have a widely variant clinical course. These studies are based on the assumption that the pattern of activation and inactivation of sets of genes will determine, or at least coincide with the biological and clinical behaviour of a tumour. Molecular biomarkers may be of use if they allow improved classification of tumour types and subtypes, can be used to predict future behaviour (for example, drug resistance or metastasis) of the tumour, or allow the early detection of tumour development or relapse.

There is now growing evidence that sites and patterns of aberrant DNA methylation may be useful molecular markers. Methylation can distinguish tumour types and subtypes. Hypermethylation of the major BRCA1 promoter was found exclusively in breast and ovarian cancer but not in colon cancer or leukemias. Similarly, hypermethylation of the VHL promoter was found only in clear cell renal carcinomas but not in a variety of other cancers. In AML and ALL, promoter methylation is a frequent mechanism for the inactivation of p15 while p16 remains active. In CML, inactivation was not found in either gene. However, in Hodgkin’s lymphomas, p16 is selectively inactivated by DNA methylation, while p15 remains unmethylated.

Methylation changes appear to precede apparent malignancy in many cases, and thus should be useful in improving early detection of potentially cancerous cells. For example, p16 promoter methylation is proposed as a biomarker for early detection of lung cancer and monitoring of prevention trials. Using sensitive PCR based methylation analysis, methylation in p16 and/or MGMT promoters were found in sputum of smokers up to three years before clinical diagnosis of squamous cell lung carcinoma. Other reports found early onset promoter methylation of MLH1 in endometrial cancers, p16 in prostate cancer, and hypermethylation on chromosome 16 in hepatocellular carcinomas. Whether methylation is causally related to the prognosis, or is a surrogate marker of the causative factor is unknown.

Yet other studies suggest that methylation markers may be used to predict response to chemotherapy or duration of patient survival.

Methylation of the CpG island within the WTI gene correlates with a chemoresistant phenotype in AML. Methylated DNA promoter methylation in lung cancer is also reportedly associated with poor survival. Using sensitivity PCR based methylation analysis, methylated DNA of the CpG-rich islands in the embryonic, extraembryonic and germ cell lineages during mouse embryo development are essential for de novo methylation and mammalian development.
Methylation matters

297


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Methylation matters


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Methylation matters

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302 Costello, Plass


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