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Aberrant DNA methylation in cancer: potential clinical interventions

G. Strathdee and R. Brown

DNA methylation, the addition of a methyl group to the carbon-5 position of cytosine residues, is the only common covalent modification of human DNA and occurs almost exclusively at cytosines that are followed immediately by a guanine (so-called CpG dinucleotides). The bulk of the genome displays a clear depletion of CpG dinucleotides, and those that are present are nearly always methylated. By contrast, small stretches of DNA, known as CpG islands, are comparatively rich in CpG nucleotides and are nearly always free of methylation. These CpG islands are frequently located within the promoter regions of human genes, and methylation within the islands has been shown to be associated with transcriptional inactivation of the corresponding gene. Alterations in DNA methylation might be pivotal in the development of most cancers. In recent years, it has become apparent that the pattern of DNA methylation observed in cancer generally shows a dramatic shift compared with that of normal tissue. Although cancers often exhibit clear reductions throughout their genomes in the levels of DNA methylation, this goes hand-in-hand with increased methylation at the CpG islands. Such changes in methylation have a central role in tumourigenesis; in particular, methylation of CpG islands has been shown to be important in transcriptional repression of numerous genes that function to prevent tumour growth or development. Studies of DNA methylation in cancer have thus opened up new opportunities for diagnosis, prognosis and ultimately treatment of human tumours.

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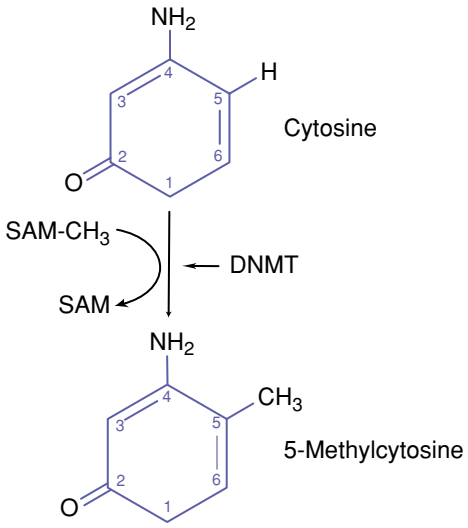
DNA methylation is the only commonly occurring modification of human DNA (Fig. 1) and results from the activity of a family of DNA methyltransferase (DNMT) enzymes that catalyse the addition of a methyl group to cytosine residues at CpG dinucleotides (Ref. 1). Alterations in DNA methylation are regarded as epigenetic, and not genetic, changes, as although they affect the structure of DNA they do not materially affect the genetic code. The human genome exhibits a clear depletion of CpG dinucleotides, presumably due to the high rate of deamination of 5-methylcytosine to thymine (Ref. 1). However, the genome also contains small stretches, up to a few kilobases in length, that are comparatively rich in CpG dinucleotides, and these stretches are known as CpG islands. Unlike the bulk of DNA, where the CpG dinucleotides are highly methylated, the CpG dinucleotides in these islands are usually methylation-free in adult tissue, and this pattern of DNA methylation is stably inherited from one cell generation to the next (Ref. 1). The genome consists of ~30 000 CpG islands and 50–60% of these are associated with genes, usually within the promoter region (Ref. 2). The potential functional importance of CpG islands was revealed by studies demonstrating that methylation of CpG islands within gene promoters is associated with transcriptional repression of the genes (Ref. 1).

DNA methylation is clearly important during development. Homozygous loss of any of the three known mammalian DNMTs (DNMT1, 3a and 3b) has been shown to be lethal in mice and their action is probably vital for establishing the correct pattern of gene expression (Refs 3, 4). However, the requirement for DNA methylation in adult tissue might be much lower (Ref. 5) and its primary role might be to maintain the bulk, non-coding, portion of the genome in a transcriptionally inactive state, effectively increasing the specificity of transcription factors for their target sites within genes (Ref. 6). DNA methylation at CpG islands as a mechanism for control of specific gene expression in adult tissues appears to be mainly restricted to two small classes of genes. First, inactivation of the X chromosome in females is associated with widespread methylation of CpG islands on the inactivated X chromosome (Ref. 7). Second, in imprinted genes, where either only the paternally or only the maternally inherited allele of the gene is expressed, inactivation of the non-expressed allele is associated with methylation within its promoter (Ref. 8). However, in normal tissue, for the majority of genes associated with CpG islands, the island remains methylation-free regardless of whether the gene is expressed or not.



Mechanism of DNA methylation
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Figure 1. Mechanism of DNA methylation. 5-Methylcytosine is produced by the action of the DNA methyltransferases (DNMT 1, 3a or 3b), which catalyse the transfer of a methyl group (CH_3) from *S*-adenosylmethionine (SAM) to the carbon-5 position of cytosine (**fig001gsb**).

This situation is in sharp contrast to that observed in human cancer cell lines, where increased methylation of CpG islands is much more common. Pioneering work by Bird and co-workers in the early 1990s demonstrated that inactivation of genes in cancer cell lines was frequently associated with methylation of CpG islands (Ref. 9). Furthermore, almost all human cancer types appear to show a loss of the normal control of DNA methylation. Analysis of DNA methylation at a genome-wide level has revealed that tumours often exhibit overall decreased levels of DNA methylation (Ref. 2). In addition, analysis of individual CpG islands has shown that these often exhibit increased methylation in cancer. Indeed, recent methods that allow large-scale analysis of CpG islands indicate that cancers probably exhibit aberrant increased methylation

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of hundreds, or even thousands, of CpG islands within a single tumour. This review article discusses the potential roles of both increased methylation at CpG islands and overall decreases in the genome-wide level of methylation in the development of cancer, and possible mechanisms by which such methylation abnormalities arise. In addition, the potential of DNA methylation as a target for novel therapeutic and diagnostic approaches is discussed.

How does DNA methylation result in transcriptional repression?

To date, three members of the DNMT family have been described in mammalian cells. The first DNMT to be identified was DNMT1 (Ref. 10). This enzyme is believed to function primarily to maintain the DNA methylation pattern after the synthesis of new DNA during cell division, because it exhibits much higher activity on hemimethylated DNA than on unmethylated DNA (Ref. 11). Subsequently two more enzymes – DNMT3a and 3b – were also cloned (Ref. 12). Unlike DNMT1, these show no preference for hemimethylated DNA, and, based on inactivation of the *DNMT3a* and *3b* genes in mice, are believed to function principally as de novo methyltransferases (Ref. 4).

The first mechanism suggested for the suppression of transcription by DNA methylation proposed direct inhibition of transcription factor binding, and, indeed, the binding of a number of important transcription factors has been shown to be sensitive to methylation within their recognition sites (Ref. 13). However, some transcription factors are insensitive to methylation (Ref. 13), and many more do not have CpG sites within their recognition sequences. In recent years though, a more generally applicable mechanism by which DNA methylation can maintain transcriptional repression has begun to be elucidated (Fig. 2). DNA methylation leads to the binding of a recently discovered family of proteins known as methyl-binding domain (MBD) proteins (Ref. 14). The members of this protein family all share a common MBD, which allows them to bind specifically to DNA containing methylated CpG sites (Ref. 14). At least three of the five known members of this family (MeCP2, MBD2 and MBD3) have been shown to be associated with large protein complexes (Refs 15, 16) containing histone deacetylase (HDAC1 and HDAC2) and chromatin-remodelling (Sin3a and mi-2) activities. The action of these histone deacetylase and chromatin-remodelling activities is thought to result in the production of compacted chromatin that is refractory to transcription (Ref. 17). The functional role of the other members of these protein complexes (see Fig. 2) remains to be elucidated. In addition to the complexes referred to above, the MBD proteins might associate with several other complexes involved in transcriptional repression. A recent report by Kokura et al. (Ref. 18) demonstrated that MeCP2 interacts with at least two other proteins – c-ski and N-CoR – known to be involved in transcriptional repression.

How does DNA methylation contribute to carcinogenesis?

Hypermethylation of CpG islands

As discussed above, cancer cells exhibit two apparently opposing changes in their pattern of DNA methylation – an overall decrease in DNA methylation associated with increased methylation of CpG islands – and both might play important roles in the tumourigenic process (Fig. 3). Of the two, the increased methylation at CpG islands has been by far the most studied and has a much clearer role in carcinogenesis.

Analysis of candidate genes

Increased methylation of a CpG island in a human tumour was first reported in 1986 (Ref. 19). However, it was not until recently, particularly following the advent of polymerase chain reaction (PCR)-based techniques for methylation analysis (Ref. 20), that analysis of CpG island methylation in tumours has become widespread. In the past few years, many genes known to play important roles in tumour development have been assessed for methylation changes, and an ever-growing list of genes have been shown to be susceptible to CpG island methylation in cancer (examples are given in Table 1; for a more complete list, see Ref. 2). Indeed, such changes might be as important as the more widely studied genetic mutations and chromosomal aberrations that characterise tumour cells.

The importance of hypermethylation of CpG islands during tumour development has been particularly highlighted by a number of examples in which epigenetic, and not genetic, inactivation is the primary mechanism for loss of gene expression during tumourigenesis. Perhaps the most striking example is the *GSTP1*

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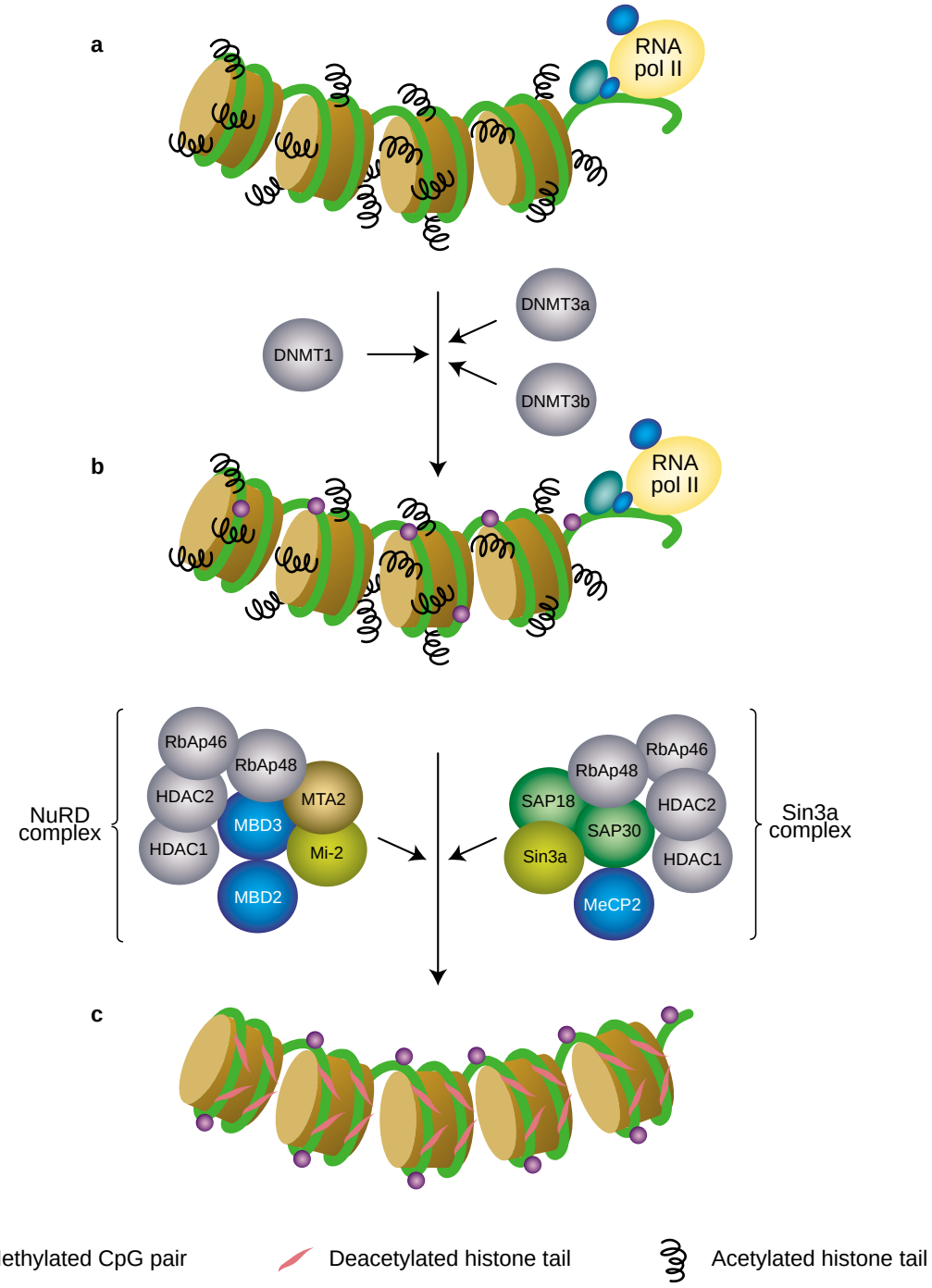

Proposed mechanism by which DNA methylation leads to transcriptional repression

Figure 2. Proposed mechanism by which DNA methylation leads to transcriptional repression (*see next page for legend*) **(fig002gsb).**

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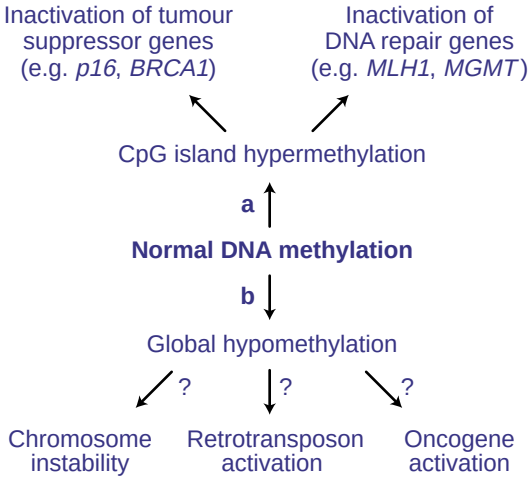
Figure 2. Proposed mechanism by which DNA methylation leads to transcriptional repression. (a) Transcriptionally active chromatin is predominantly unmethylated and has high levels of acetylated histone tails (short black squiggles). (b) Methylation at CpG dinucleotides can be carried out by one of the three known human DNA methyltransferases (DNMT1, 3a and 3b), resulting in DNA with high levels of CpG methylation (purple circles), but still containing predominantly acetylated histone tails. DNA in this form would still be expected to be transcriptionally competent. (c) Methylated DNA is targeted by methyl-binding domain (MBD) proteins such as MBD2 and MeCP2, which are found associated with large protein complexes such as the NuRD complex (MBD2) and the Sin3a complex (MeCP2). Histone deacetylase (HDAC1 and 2) and chromatin-remodelling activities (Mi-2 and Sin3a) within these complexes result in alterations in chromatin structure, producing chromatin that is refractory to transcriptional activation (pink streaks represent deacetylated histone tails). The functional roles of other components in these complexes are not yet known. Abbreviations: MTA2, metastasis-associated protein 2; RbAp46/48, retinoblastoma-associated protein 46/48; RNA pol II, RNA polymerase II; SAP18/30, Sin3-associated polypeptides 18/30 (**fig002gsb).**

gene, which encodes glutathione *S*-transferase π and is involved in detoxification of potentially DNA-damaging electrophiles. In prostate cancer, loss of expression of this protein is seen in the vast majority (>90%) of tumours. In almost all cases this loss of expression correlates with hypermethylation of the *GSTP1* promoter (Refs 21, 22). Indeed, promoter hypermethylation and loss of *GSTP1* expression is probably one of the earliest events in the development of prostate cancer as it is even seen in the majority of pre-cancerous prostatic intraepithelial neoplasia (PIN) lesions (Ref. 23). Loss of *GSTP1* expression is likely to result in an increase of oxidative DNA damage, as a result of the loss of the detoxifying activity of GSTP1, and this might be the key initiating event in the majority of prostate tumours.

A similar example is the mismatch repair (MMR) gene *MLH1*, first identified as one of the two MMR genes frequently mutated in the cancer predisposition syndrome hereditary non-polyposis colorectal carcinoma (Ref. 24). The characteristic phenotype of such MMR-deficient tumours is microsatellite instability and this has also been observed in many sporadic tumours (Ref. 25), but mutations in MMR genes were rarely seen (Ref. 26). Recently though, Kane et al. (Ref. 27) were able to demonstrate that, in colon cancer cell lines, loss of *MLH1* expression was instead the result of promoter hypermethylation. Subsequently, numerous studies have confirmed that the clear majority of sporadic tumours with the microsatellite instability phenotype also exhibit *MLH1* promoter hypermethylation (Refs 28, 29, 30, 31).

Another key observation demonstrating the importance of DNA methylation in tumour development is the presence of DNA methylation abnormalities even in pre-malignant hyperplasias. In addition to hypermethylation of *GSTP1* in PIN, in other tissues increased CpG island methylation of key tumour suppressor genes has been detected in samples taken from areas



Possible roles of increased CpG island and decreased global DNA methylation in tumour development

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Figure 3. Possible roles of increased CpG island and decreased global DNA methylation in tumour development. (a) Increased CpG island methylation can result in the inactivation of many well-characterised tumour suppressor genes (*BRCA1*, breast cancer 1 gene) as well as inactivation of DNA repair genes, resulting in increased levels of genetic damage. (b) The role of reduced global DNA methylation is still unclear; however, this might lead to reduced chromosome stability, to activation of retrotransposon elements (resulting in insertional mutagenesis) or to activation of oncogenes **(fig003gsb).**

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Table 1. Examples of genes exhibiting hypermethylation in cancer (tab001gsb)

Gene[a]	Effect of loss of function in tumour development	Tumour types
Rb	Loss of cell-cycle control	Retinoblastoma
MLH1	Increased mutation rate, drug resistance	Colon, ovarian, endometrial, gastric
BRCA1	Genomic instability	Breast, ovarian
E-CAD	Increased cell motility	Breast, gastric, lung, prostate, colon, leukaemia
APC	Aberrant signal transduction	Breast, lung, colon, gastric, oesophageal, pancreatic, hepatocellular
p16	Loss of cell-cycle control	Most tumour types
VHL	Altered protein degradation	Clear-cell renal cell carcinoma
p73	Loss of cell-cycle control	Leukaemia, lymphoma, ovarian
RASSF1A	Aberrant signal transduction	Lung, breast, ovarian, kidney, nasopharangeal
p15	Loss of cell-cycle control	Leukaemia, lymphoma, gastric, squamous cell carcinoma, hepatocellular
GSTP1	Increased DNA damage	Prostate
DAPK	Reduced apoptosis	Lymphoma, lung
MGMT	Increased mutation rate	Colon, lung, brain, oesophageal, gastric

[a] For a more complete list, see Ref. 2.
Abbreviations: APC, adenomatous polyposis coli; BRCA1, breast cancer 1; DAPK, death-associated protein kinase; E-cad, epithelial cadherin; GSTP1, glutathione *S*-transferase π1; MLH1, MutL homologue 1; MGMT, *O*(6)-methylguanine-DNA methyltransferase; p15, $p15^{INK4b}$; p16, $p16^{INK4}$; p73, p73; Rb, retinoblastoma; RASSF1a, Ras association domain family 1A; VHL, von Hippel–Lindau.

of hyperproliferation known to precede the development of frank tumour (Refs 32, 33, 34, 35, 36, 37). This clearly suggests that CpG island methylation is important in the early stages of tumour development and might be a key initiating event.

Although the mechanism that leads to aberrant methylation of CpG islands is still not clear, evidence does suggest that some tumours contain specific defects that cause them to be susceptible to such abnormal DNA methylation. Toyota and colleagues (Ref. 38) demonstrated that, in colon cancer, CpG island hypermethylation is not randomly distributed between tumours: whereas some appeared to be comparatively resistant to CpG island methylation, others exhibited methylation at a high percentage of the loci studied.

Large-scale methylation analysis of CpG islands

To date, nearly all of the studies of CpG island methylation have used a candidate gene approach to identify genes targeted by methylation. Although this has already identified a long list of silenced genes with methylated CpG islands, ascertaining the true extent of CpG island methylation in cancer will require larger-scale screening of unselected CpG islands. Two recently developed techniques are now being used to begin such investigations.

First, differential methylation hybridisation (DMH), a technique devised by Huang and co-workers (Ref. 39), combines digestion using methylation-sensitive restriction enzymes with microarray-based technologies to allow the screening of several thousand CpG islands

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simultaneously. This technique has already been used to uncover extensive methylation of CpG islands in breast cancer, and the authors also demonstrated that the extent of methylation correlated with tumour grade.

Second, Costello et al. (Ref. 40) reported the analysis of >1000 CpG islands in multiple tumour types using a technique, based on two-dimensional gel electrophoresis, known as restriction landmark genomic scanning (RLGS). This analysis identified patterns of methylation shared between different tumour types as well as type-specific methylation events. On the basis of their analysis, and assuming a total of 30 000 CpG islands in the human genome (Ref. 2), the authors estimated that the maximum number of CpG islands aberrantly methylated in a single tumour in their tumour set was about 3000 and the average number was about 400.

In addition to the identification of tumour-specific patterns of methylation, the RLGS technique has been used to identify novel targets of DNA methylation in several different tumour types (Refs 41, 42, 43, 44). In the case of medulloblastomas, the methylation status of several of these loci correlated with patient prognosis (Ref. 44). However, unlike in the candidate gene approach, which focuses primarily on genes for which a role in tumour development has already been demonstrated, novel methylation targets identified by large-scale analysis will require much further investigation before their significance to tumourigenesis can be fully appreciated. This is especially the case given the large numbers of CpG islands found to be targeted in a single tumour; this suggests that many of the aberrantly methylated loci will not play significant roles in tumour development.

Genome-wide hypomethylation

Hypomethylation of DNA in human tumours was first identified even earlier than CpG island hypermethylation (Ref. 45). However, whereas the role of CpG island methylation in tumourigenesis has become increasingly established, the role, if any, of DNA hypomethylation in the carcinogenic process remains to be elucidated. Nevertheless, extensive demethylation of DNA outside of CpG islands has now been observed in many tumour types (Refs 46, 47, 48), and several potential mechanisms by which this might be involved in tumour development have been put forward, as described below.

Chromosome stability

Chromosome aberrations are common in cancer, and it has been suggested that DNA methylation could be involved in the control of chromosome stability. Lengauer et al. (Ref. 49) demonstrated that colon cancer cell lines varied markedly in their ability to methylate and silence exogenously introduced retroviral sequences and that failure in this correlated with extensive aneuploidy of the cell lines. The authors suggested that this assay might be detecting a continuing methylation defect that could be the basis of the chromosome instability observed in these cell lines. In support of this it has been shown that patients with the autosomal recessive ICF (for immunodeficiency, centromere instability and facial anomalies) syndrome, caused by mutation of one of the DNMTs (*DNMT3b*) (Ref. 50), exhibit demethylation and instability of the pericentric heterochromatic regions on chromosomes 1, 9 and 16 (Ref. 51). Furthermore, hypomethylation and instability of these regions on chromosomes 1 and 16 have also been observed in ovarian, breast and Wilms' tumours (Refs 52, 53, 54). However, a clear association between the extent of genomic hypomethylation and chromosome instability has yet to be described, and patients with ICF syndrome have not been reported to show increases in tumour frequency.

Retrotransposon activation

The human genome contains numerous mobile genetic elements, termed retrotransposons (Ref. 55), and expression from these elements is usually suppressed by DNA methylation. However, hypomethylation and consequent re-activation of expression from the elements has been detected in human cancer (Ref. 56). This could potentially lead to movement of the retrotransposons and re-integration at new sites in the genome, leading to insertional mutagenesis. However, although mutation due to insertion of mobile genetic elements has been observed in cancer (Refs 57, 58, 59), such mutations are not frequent, arguing against a major role for such insertional mutagenesis in tumour development.

Oncogene activation

Perhaps the most obvious potential role for DNA hypomethylation would be in the activation of genes, particularly oncogenes. Hypomethylation within the H-*ras* (Ref. 60) and c-*myc* (Ref. 61) oncogenes has indeed been observed; however,

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this is not associated with increased expression. Several genes mapping to the X-chromosome, in particular the *MAGE* gene family (Ref. 62), undergo demethylation within their promoter regions and activation in a tumour-specific manner. However, no role has been identified for these genes in tumour development. Nevertheless, these results do at least demonstrate that activation of specific genes can occur following DNA hypomethylation.

What causes the cancer-specific methylation changes?

Increased expression of DNMTs

Although the importance of CpG island methylation in cancer is now becoming apparent, the mechanisms that lead to this phenomenon in tumours are still unknown. The most widely investigated potential cause is the increased expression in tumours of one or more of the DNMT enzymes.

Vertino et al. (Ref. 63) demonstrated that overexpression of *DNMT1* in immortalised human fibroblasts resulted in increased methylation at a number of CpG islands. A subsequent report, focusing on the E-cadherin loci, suggested that increased DNMT1 activity resulted in the spreading of methylation from methylation centres, often associated with repetitive elements, lying both 5' and 3' of the E-cadherin CpG island (Ref. 64). This eventually led to complete methylation of the CpG island and loss of expression. Initially, a number of authors identified similar upregulation of *DNMT1* in vivo, in several different tumour types (Refs 65, 66, 67, 68). However, subsequent studies determined that when levels of *DNMT1* were compared with markers of proliferation the apparent increased expression was lost (Refs 69, 70, 71), suggesting that the increased expression of *DNMT1* is most probably a result of increased proliferation of the tumour cells, as opposed to genuine upregulation of *DNMT1*. Nevertheless, the field remains contentious, and other recent reports indicate that upregulation can still be observed after normalisation to proliferation markers (Refs 72, 73). DNMT3a and 3b could be thought to represent better candidates for causing the increased CpG island methylation observed in cancer cells, because of their de novo methylation capability (Ref. 4). However, similarly conflicting studies indicating both increased expression (Refs 72, 73) or the absence of increased expression (Refs 70, 71) of *DNMT3a* and *3b* have been reported. In addition, studies by Rhee et al. (Ref. 74) cast further doubt on the role of DNMT1. The authors genetically inactivated both copies of the *DNMT1* gene in the HCT116 colon cancer cell line. Surprisingly, this had very little effect on the level of DNA methylation and failed to result in demethylation or activation of genes such as *p16* that are hypermethylated in this cell line.

In summary, it is still unclear whether upregulation of DNMTs plays some role in the abnormal methylation seen in tumour cells. However, as the increased methylation of CpG islands is frequently observed within the context of an overall decrease in genome-wide methylation, it is apparent that other factors must also play a role in the abnormal methylation pattern seen in cancer.

A role for $p21^{WAF1}$?

The opposing changes of increased CpG island methylation but decreased overall methylation in cancer suggest that the normal control of targeting of the DNMTs might have been lost. A potential role for the cyclin-dependent kinase inhibitor $p21^{WAF1}$ in the control of at least DNMT1 activity was suggested by a study by Chuang and colleagues (Ref. 75) in which the authors determined that both DNMT1 and $p21^{WAF1}$ bind to the same region of proliferating cell nuclear antigen (PCNA). The binding of DNMT1 to PCNA is thought to be important in targeting DNMT1 to replication complexes during late S phase of the cell cycle, and in vitro assays have demonstrated that oligopeptides derived from $p21^{WAF1}$ were highly efficient at inhibiting the DNMT1–PCNA complex (Ref. 75). A recent report by De Marzo et al. (Ref. 76) found that although $p21^{WAF1}$ and DNMT1 expression was mutually exclusive in normal colonic epithelia, in adenomatous polyps DNMT1-expressing cells were also found to express $p21^{WAF1}$. Furthermore, $p21^{WAF1}$ is overexpressed in the early stages of breast, lung, ovarian and hepatocellular carcinomas (Refs 77, 78, 79, 80).

A potential speculative model based on these observations would be that $p21^{WAF1}$ expression early during tumour development displaces DNMT1 from replication complexes, resulting in reduced genome-wide methylation. This could directly lead to mistargeting of free DNMT1 to CpG islands or, alternatively, the genome-

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wide hypomethylation could lead to increased expression of DNMTs, which subsequently aberrantly methylate CpG islands.

Involvement of factors downstream of methylation

It has been suggested that the relationship between DNA methylation and chromatin modification could be viewed as a feedback loop. Thus, in addition to DNA methylation inducing the formation of inactive chromatin states (see Fig. 2), inactive chromatin states could lead to increased DNA methylation. This was proposed by Ng and Bird (Ref. 81), partly on the basis of two studies in non-human models that showed inhibitors of histone deacetylation lead to alterations in DNA methylation (Refs 82, 83). This therefore suggests that factors that alter chromatin structure could also alter DNA methylation patterns. In support of such a model, it has recently been demonstrated that mutations in the *ATRX* gene, which are responsible for the ATRX (X-linked α-thalassaemia and mental retardation) syndrome in humans, also cause diverse changes in the pattern of global methylation (Ref. 84). The ATRX protein demonstrates clear homology to chromatin-remodelling proteins, in particular Mi-2 (see Fig. 2) and probably functions as a transcriptional regulator through modification of chromatin structure. Of particular interest is the fact that the alterations in methylation induced by mutation of *ATRX* include both increased and decreased methylation of specific sequences (Ref. 84) – a clear similarity to the altered methylation patterns observed in cancer. These results suggest that alterations in the protein complexes that act downstream of methylation to induce transcriptional silencing could result not only in altered transcription but also in changes to the DNA methylation pattern itself.

Clinical implications/applications

DNA methylation patterns are likely to become of increasing importance in the management of cancer patients in the near future. Already, several clinical trials using agents targeted against DNMTs have been completed or are under way

Table 2. Clinical trials with inhibitors of DNA methylation (tab002gsb)

Phase	DNMT inhibitor	Trial design	Target tumour	Progress	Reference
I	Anti-DNMT1	Single agent antisense	Multiple	Completed: some anti-tumour activity	www.methylgene.com
II	Anti-DNMT1	Single agent antisense	Renal cell, squamous cell carcinoma	On-going	www.methylgene.com
I	Decitabine	In combination with carboplatin	Multiple	On-going	www.crc.org.uk
I	Decitabine	In combination with phenyl butyrate	Multiple	On-going	Ref. 98
II[a]	Decitabine	Single agent	Chronic myelogenous leukaemia	26% response rate	www.supergen.com
II[a]	Decitabine	Single agent	Myelodysplastic syndrome	49% response rate	www.supergen.com
III[a]	Decitabine	Single agent	Myelodysplastic syndrome	On-going	www.supergen.com

[a] Therapeutic effect may be due to cytotoxicity at high doses of Decitabine, as opposed to demethylation.
Abbreviation: DNMT, DNA methyltransferase.

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(Table 2). In addition, DNA methylation shows great promise as a marker for the early detection of cancer, and DNA methylation patterns might be of use in determining patient prognosis.

Therapeutics targeting DNA methylation

Methylation probably causes the inactivation of numerous genes that are important in the development of most or all tumour types; thus, inhibition of DNA methylation and consequent re-activation of these genes is an attractive avenue for the development of novel therapeutics. This strategy is particularly appealing because, in normal cells, these genes are not normally regulated by DNA methylation and therefore the toxicity of inhibitors of DNA methylation to non-cancer tissue could potentially be well below that seen with conventional cytotoxic anti-cancer agents [although reductions in global levels of methylation might have some deleterious effects (Fig. 3)]. However, as yet, few effective inhibitors of DNMTs are known. The two closely related drugs 5-azacytidine and 2'deoxy-5-azacytidine (also known as Decitabine) have long been used experimentally to inhibit DNA methylation in tissue culture and have been shown to re-activate numerous methylation-silenced genes (Refs 85, 86). In addition, Decitabine has also been shown to induce cell differentiation (Ref. 87) and has been used to treat a number of haematopoietic disorders (Ref. 88). However, its use in the activation of genes silenced by methylation in cancer is likely to be limited by its toxicity (Ref. 89). Nevertheless, in combination with other drugs, Decitabine might be of use in the treatment of malignancy. For example, in mouse xenograft models it has been demonstrated that treatment with relatively low doses of Decitabine can result in re-activation of a methylation-silenced gene, *MLH1*, in the xenografts (Ref. 90). The MLH1 protein, part of the human DNA MMR system, has been shown to be important in determining sensitivity to a number of important chemotherapeutic agents (Ref. 91), and, indeed, the treated xenografts exhibited clear increases in sensitivity to drugs such as carboplatin, temozolomide and epirubicin. On the basis of these results, a Phase I clinical trial of the combination of Decitabine and carboplatin is scheduled to begin shortly.

As described above, histone deacetylase activity is important in the transcriptional repression of methylated sequences. Cameron and colleagues (Ref. 92) demonstrated that combining treatment with Decitabine and treatment with an inhibitor of histone deacetylase, trichostatin A, caused a synergistic re-activation of expression of the *MLH1* and *TIMP3* genes in the colorectal cancer cell line RKO. Several other reports have now confirmed these original observations (Refs 93, 94, 95), although there appears to be some locus or cell-type specificity because other investigators have failed to observe such synergism (Refs 96, 97). A Phase I clinical trial aimed at assessing the clinical potential of this synergistic interaction has been initiated using the combination of Decitabine and another histone deacetylase inhibitor, phenyl butyrate (Ref. 98).

Another approach being used to inhibit DNA methylation is the use of antisense oligonucleotides. Antisense oligonucleotides directed against DNMT1 mRNA have been shown to reduce DNMT1 protein levels and induce demethylation and expression of the *p16* tumour suppressor gene in human tumour cells (Ref. 99), and also inhibit tumour growth in mouse models (Ref. 100). This DNMT1 antisense molecule has also been used in Phase I clinical trials [Davis, A.J. et al. (OCI/Princess Margaret Hospital, Toronto, Canada), pers. commun.; Stewart, D. et al. (Ottawa Regional Cancer Centre, Canada), pers. commun.] and has shown some anti-tumour activity. Phase II clinical trials of this agent in renal cell carcinoma and squamous cell carcinoma of the head and neck will begin in the near future (for further details see http://www.methylgene.com).

Like many other novel therapeutics currently being developed, inhibitors of DNA methylation are unlikely to function through being directly cytotoxic. An important consequence of this is that, unlike conventional cytotoxic agents, it might be best to use such drugs at concentrations other than the maximum tolerated dose. For this reason, an important element of clinical trials involving methylation inhibitors will be the use of molecular endpoints to specifically monitor changes in DNA methylation. Clearly, the most satisfactory approach would be to monitor levels of methylation of important tumour suppressor genes in tumour DNA. However, such an approach would be possible in a limited number of tumour types, such as leukaemia, where repeated sampling of the tumour was feasible. Another approach is to determine total

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genomic levels of 5-methylcytosine in a surrogate tissue, such as lymphocytes. It has previously been shown, using a high-performance liquid chromatography (HPLC)-based method, that reductions in total genomic levels of 5-methylcytosine in lymphocytes of xenograft-bearing mice closely mirrors reduced methylation of the *MLH1* promoter following Decitabine treatment (Ref. 90). Similarly, genes known to be methylated in normal tissue, such as imprinted genes (Ref. 8) or the *MAGE* gene family (Ref. 62), can also be assessed for reductions in methylation in surrogate tissues. Levels of foetal haemoglobin are known to be increased by treatment with inhibitors of DNA methylation and this is currently used as a treatment for sickle cell anaemia (Ref. 101); this therefore represents another potential molecular endpoint to monitor the effect of DNA methylation inhibitors.

As discussed above, Decitabine has been used in several clinical trials for haematopoietic disorders. However, this generally involves treating patients with high doses of Decitabine that are likely to be directly cytotoxic (Ref. 89), and the importance of the demethylating activity of Decitabine in such treatments is unclear (Ref. 102).

Classification and tumour prognosis

Another potential use of DNA methylation is in the classification of tumours depending on their methylation status. Such classification might be of use in determining patient prognosis or potential response to therapy. Indeed, a number of DNA methylation studies have already identified links between methylation and patient outcome. For example, a study of methylation of the DNA repair gene *MGMT* identified a clear link between methylation of the *MGMT* promoter and increased overall and disease-free survival (Ref. 103), probably due to increased responsiveness to alkylating agents in MGMT-deficient tumours. Similarly, Tang et al. (Ref. 104) determined that increased methylation of the *DAP* (*death-associated protein*) *kinase* gene was strongly associated with decreased survival in patients with non-small-cell lung carcinoma, and found that *DAP kinase* gene methylation was probably the strongest independent prognostic factor in these patients. In addition, the development of methods for the large-scale analysis of CpG island methylation referred to above raises the prospect of dramatically increasing our ability to classify tumours based on DNA methylation. These methods have already been able to identify differences between methylation patterns in different tumours (Ref. 40) and correlations between methylation and tumour grade (Ref. 39). Further studies using such techniques for large-scale CpG island analysis will be necessary to determine whether methylation-based classification of tumours will be useful in predicting patient outcome or response to particular therapeutic regimes.

DNA methylation in the early detection of cancer

Increased methylation at CpG islands is probably one of the most attractive markers for the development of techniques for the early detection of cancer. Three key features of DNA methylation make it possibly the best tumour marker for this purpose. First, for many CpG islands methylated at high rates in cancer, the corresponding CpG islands in normal tissue are rarely or never found to exhibit methylation (Ref. 1). Second, unlike genetic mutations, which can occur in numerous positions throughout genes, changes in DNA methylation associated with loss of transcription always occur within a specific region of the gene. Third, the development of PCR-based techniques, in particular methylation-specific PCR (Ref. 20), to identify aberrant methylation allows methylation abnormalities to be detected in readily obtainable samples in which small amounts of tumour-derived DNA are present, such as serum (Refs 105, 106, 107) and saliva (Refs 32, 108, 109). Tumour-derived DNA extracted from the serum of patients has already been used to identify successfully tumour-specific methylation changes in several tumour types [for example colon, head and neck, and lung cancer (Refs 98, 99, 105)]. In addition, methylation abnormalities have been detected in various tumour types by using tumour-specific DNA isolated from particular samples, such as semen for prostate cancer (Ref. 110), ductal lavage fluid for breast cancer (Ref. 111) and saliva for head and neck and for lung cancer (Refs 32, 108, 109).

A potential problem with this approach is that although many of the genes aberrantly methylated in cancer are not normally methylated in normal cells, recent reports suggest many might be susceptible to age-related methylation (Ref. 112). Such studies have shown that for genes such as the those encoding the oestrogen receptor

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or IGF2, promoter methylation can be detected within normal tissue and this methylation increases with increasing age. However, it might be that not all genes are susceptible to such age-related methylation (Ref. 113), and one of the early studies using saliva that also examined healthy, non-high-risk patients detected no methylation in this group (Ref. 109). Nevertheless, methylation abnormalities have been detected in saliva from apparently healthy individuals thought to be at high risk for lung and for head and neck cancer (Refs 32, 109), suggesting that this type of analysis could be used as a screening method in high-risk patients. Further studies will be required to determine if the abnormalities detected are evidence of pre-neoplastic changes that identify patients likely to develop frank tumours or are age-related methylation changes that do not represent a high risk of tumour development.

Outstanding research questions and future prospects

The past few years have seen an upsurge of interest in the role of DNA methylation, and in particular its role in cancer. In this period, the number of known DNMT enzymes has increased from one to three, the number of genes known to be targeted by methylation has increased exponentially and the mechanisms by which DNA methylation leads to transcriptional inactivation have been greatly elucidated. However, despite all of these advances, little more is known about the mechanisms that underlie the methylation changes observed in cancer. The identification of these mechanisms clearly represents the major basic research challenge in this field in the near future. Clinically, the role of DNA methylation in the management of cancer patients is likely to increase greatly. Already, a number of approaches aimed at reversing tumour-specific methylation are being investigated in the clinic and, as the mechanisms underlying the control of methylation are elucidated further, novel targets, offering the potential for therapeutic intervention, are likely to be identified.

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Further reading, resources and contacts

The CpG Island Methylation in Aging and Cancer website provides general information on methylation in cancer, as well as useful lists of both genes affected and genes unaffected by methylation in cancer.

http://www3.mdanderson.org/leukemia/methylation/

The MethDB website (database of methylation) includes information on ^{m}CpG content as well as patterns and profiles of methylation at specific sites.

http://www.methdb.de/

The ClinicalTrials.gov website provides information about ongoing clinical trials.

http://clinicaltrials.gov

The DNA Methylation Society website includes news and reviews about DNA methylation (subscription required for full access).

http://dnamethsoc.server101.com/

Features associated with this article

Figures

Figure 1. Mechanism of DNA methylation (fig001gsb).
Figure 2. Proposed mechanism by which DNA methylation leads to transcriptional repression (fig002gsb).
Figure 3. Possible roles of increased CpG island and decreased global DNA methylation in tumour development (fig003gsb).

Tables

Table 1. Examples of genes exhibiting hypermethylation in cancer (tab001gsb).
Table 2. Clinical trials with inhibitors of DNA methylation (tab002gsb.)

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