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COMMENTARY

Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis

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Introduction

DNA methylation has at least two important roles in tumorigenesis. The target cytosines (C*) of (cytosine-5)-DNA methyltransferase (Mtase) are mutated to thymine (T) in ~30% of inherited diseases and cancer, and genome-wide alterations of DNA methylation patterns occur at early stages of tumor development. Insight into the normal function of DNA methylation will provide the knowledge to understand the origins of these aberrations and their importance for disease initiation and progression. Originally the aberrations seen in tumors were attributed to the higher spontaneous deamination rate of 5-methylcytosine (5-mC) as compared with C and to misregulation of the Mtase gene. Recently, it has become clear that the Mtase can actively participate in mutagenesis by enzymatically increasing both the rate of genetic and epigenetic alterations. Proteins that recognize and repair these alterations determine the frequency of their fixation as disease causing mutations. In addition, alterations in the metabolism of S-adenosylmethionine can disturb DNA methylation by depleting the cofactor S-adenosylmethionine or by increasing the level of metabolites acting as inhibitors of DNA methylation. This review concentrates on the normal role of DNA methylation in mammals and on aberrations of DNA methylation in inherited disease and cancer.

Function of DNA methylation

The only naturally occurring modification of DNA in higher eukaryotes is the methylation of the 5 position of cytosine (C) leading to the formation of 5-methylcytosine (5-mC). The reaction is catalyzed by the enzyme (cytosine-5)-DNA methyltransferase (Mtase), which has been isolated from prokaryotes and eukaryotes. Several aspects of the catalytic mechanism have been clarified mainly for prokaryotic Mtases (1). The crystal structure of the bacterial M.HhaI enzyme revealed an intermediate in which the target cytosine is flipped out of the DNA helix and covalently attached to the enzyme (2). The enzyme first binds to its target sequence, removes the target cytosine into its catalytic pocket and covalently attaches to the C6 position of cytosine (1). This generates a covalently bound enzyme-cytosine intermediate with the cytosine activated at C5, which can then accept the methyl group from the cofactor S-adenosylmethionine.

Most prokaryotic enzymes are '*de novo*' enzymes recognizing and methylating specific un- or hemi-methylated palindromic sequences of two to six base pairs (3). The eukaryotic enzymes on the other hand are believed to be mainly 'maintenance' enzymes that recognize and methylate hemi-methylated target sites generated by DNA replication. Since shortly after replication the parental strand is methylated whereas the newly synthesized is not, DNA methylation serves as a mechanism of strand discrimination for mismatch repair (4). Methylation occurs in mammals mainly at the dinucleotide CpG, although recent reports indicate that the sequence CpNpG can also be methylated albeit with a lower efficiency (5). About 70% of all CpG dinucleotides and ~3–6% of all cytosines in the genome are methylated in vertebrates. The eukaryotic enzymes contain a large domain at their amino terminus in addition to the catalytic domain and this is most likely involved in nuclear localization, targeting to the replication fork and in various aspects of enzyme regulation (6,7).

The 'maintenance' methyl-transferase also has a low *de novo* activity that can be stimulated by proteolytic cleavage of the amino terminal domain (8) and by specific target DNAs such as oligonucleotides containing looped and mismatched bases (9). It is still unclear whether this low *de novo* methylation activity is sufficient to account for the rapid genome-wide *de novo* DNA methylation that occurs during embryonic development or whether there is another methyl-transferase that has high *de novo* methylation activity. Homozygous deletion of the mouse Mtase gene leads to embryonic lethality, proving that DNA methylation is essential for embryonic development (10). Residual 5-mC in these mice suggests that there might be another gene in mice with Mtase activity (10).

Methylation of cytosine changes the structural characteristics of DNA in several ways. Methylation of DNA in the common B form facilitates a conformational change to the Z form, increases the helical pitch of DNA and alters the kinetics of cruciform extrusion (11,12). The methyl group of cytosine sterically extends into the hydrophilic major groove of B DNA and introduces hydrophobicity, two changes that may be responsible for the altered specificity of proteins interacting with DNA.

Regulation of gene expression by DNA methylation

Cytosine methylation can influence transcription directly by interfering with the binding of positively or negatively acting transcription factors or indirectly by the formation of inactive chromatin (13). Since 5-mC is structurally similar to T, the methylation of cytosine might lead to the generation of new consensus sequences for some transcription factors. Indeed, cytosine methylation has recently been found to convert a low affinity AP-1 binding site (CGAGTCA) into a high affinity site (mCGAGTCA), which is more similar to the consensus AP-1 binding site (TGAGTCA) (14). Several DNA binding proteins that contain a CpG in their recognition sequence are inhibited when the CpG is methylated (Table I).

Interestingly, the transcription factor Sp1 required to transcribe many housekeeping promoters can bind both methylated

***Abbreviations:** C, cytosines; Mtase, methyltransferase; T, thymine; 5-mC, 5-methylcytosine; LOH, loss of heterozygosity; LOI, loss of imprinting.

Table I. Transcription factors of vertebrates affected by cytosine methylation

Protein	DNA target[a]	Reference
AP-2	CCCGCCGGCG	(15)
AP-2	CTCCGGGG(C/T)TG	(16)
Ah receptor	TTGCGTG	(17)
CREB/ATF	TGACGTCA	(18)
CREB/ATF	CTGCGTCA	(19)
E2F	TTTTCGCGC	(20)
EBP-80	ATCTGCGCATATGCC	(21)
Ets	CGGAAG	(22)
MLTF	GGCCACGTGACC	(23)
MTF-1	TGCGCCCG	(24)
c-Myc, c-Myn	CACGTG	(25,26)
GABP	TTCCGGGC	(27)
NF-κB	GGGACTTTCCG	(28)
HiNF-P	CGGTTTTCAATCTGGTCCG	(29)
MSPF	ATGCGNNNNCGCCT	(30)

[a]Only the consensus sequence is shown and the CpG dinucleotide that affects DNA binding is underlined. The exact sequence requirements and the degree of binding inhibition by DNA methylation can obtained from the original publications.

and unmethylated target sequences and furthermore, prevents the methylation of adjacent sequences (31–34). Since Sp1 is only found in higher eukaryotes containing 5-mC in their DNA, Sp1 might have a specific role in regulating certain aspects of DNA methylation.

Promoters are generally inactive when methylated with the exception of the *H-2K* gene promoter (35). Methyl groups introduced at single sites into the herpes simplex thymidine kinase and the Epstein–Barr virus latency C promoters can interfere, with high efficiency, with their expression (36,37). For other promoters such as the human α- and γ-globin promoters and the mouse *MyoD1* promoter, the density of methylation rather than a specific CpG dinucleotide is responsible for interfering with transcription (38,39). The inhibition induced by partially methylating a promoter can be overridden with an enhancer whereas a fully methylated promoter remains silent (38,39). DNA methylation is generally believed to be a mechanism to determine either the active or inactive state of a gene rather than being responsible for the fine tuning of gene expression.

Chromatin structure and DNA methylation

The pattern of DNA methylation can influence the type and phasing of chromatin formed around a gene (40,41). A number of proteins have been described such as the MDBP1 protein, the MDBP2-histone H1 protein and the MeCP1/2 proteins that bind preferentially to methylated DNA (13). The MeCP1 protein requires several methylated CpGs for binding whereas the MeCP2 protein requires only one methylated CpG for binding (42). Antibodies against MeCP2 revealed that DNA covered by MeCP2 is mostly heterochromatin and localizes to G bands (43). Homozygous deletion of the mouse MeCP2 gene is lethal, suggesting an important role in embryonic development (44). MDBP2-histone H1 can bind to a single methylated CpG and interfere with transcription and the efficiency of binding and transcriptional inhibition is increased by phosphorylation (45).

The most striking example of gene inactivation by inactive chromatin formation and DNA methylation is seen at the inactive X chromosome in mammals. Most genes on the inactive X chromosome become inactivated during early embryonic development, irrespective of whether they contain a CpG island or not (46). X chromosome inactivation starts at the X chromosome inactivation center and DNA methylation and histone H4 acetylation is believed to reinforce and stabilize the silent state of the inactive X chromosome (47).

Activation of genes embedded in inactive chromatin requires disruption of the chromatin structure and demethylation. Recently, protein complexes have been discovered that are able to disrupt inactive chromatin (48). Demethylation is either the result of a passive process by interference with maintenance methylation after replication or of an active process mediated by enzymes with demethylase activity. Such activities have been characterized in differentiating cells with the ability to demethylate preferentially hemi-methylated (49) or also fully methylated DNA (50).

Since genes that are covered by inactive chromatin are mostly inaccessible to transcription factors, 5-mC mediated inactive chromatin formation may be involved in reducing the genome size and decreasing the number of target sequences that need to be recognized by transcription factors (51). At the same time, non-specific binding to related sequences is limited, which leads to transcriptional noise reduction (52). It has furthermore been proposed that in addition to genetic changes, species-specific epigenetic changes influence the evolution of higher eukaryotes (53,54). The heritability of DNA methylation patterns is expected to stabilize and propagate specific chromatin structures and the possibility of altering DNA methylation patterns may provide a way to increase the plasticity of the genomic information and to increase diversity in a population by generating epigenetic mutations that are subject to selection.

CpG islands

The genome is organized into isochores of G/C rich early replicating regions staining on chromosomal spreads as R bands and G/C poor late replicating regions staining as G bands (55). CpG islands are located mainly in the R bands and are defined as regions of at least 200 bp that are G/C-rich (60–70%) with a normal observed/expected ratio of the dinucleotide CpG (55,56). CpG islands are maintained in the unmethylated state in the germline and in normal tissue with the exception of imprinted genes, genes on the inactive X chromosome and repetitive elements such as LINES and SINES (Alus) (46,57–59). It is believed that CpG islands have evolved since they are not methylated in the germline and are therefore not subject to the higher mutation frequency of methylated cytosines as compared with unmethylated cytosines (60). There are an estimated 45 000 CpG islands per haploid genome in humans and it is believed that most of them are associated with genes (57,61). The promoters of many housekeeping genes contain unmethylated CpG islands that prevent the inactivation of gene expression by DNA methylation (57).

Since the Mtase is able to methylate CpG islands *in vitro*, the characteristics of the DNA structure at CpG islands and the high G/C content does not directly prevent their methylation (62). This is most likely achieved indirectly by means of specific proteins that interfere with DNA methylation and specific chromatin modifications. The chromatin located to CpG islands lacks histone H1, and the histones H2A and H2B covering CpG islands are hypoacetylated (63). Furthermore, CpG islands contain binding sites for the transcription factor Sp1, which binds both methylated and unmethylated target

sites, prevents DNA methylation in eukaryotic cells, and might be important to maintain housekeeping genes unmethylated and active (31–34,56).

When both normal and tumor cells are isolated from an organism and grown in culture, rapid changes in DNA methylation patterns can occur (64,65). These changes have been implicated in the limited division potential and aging of somatic cells *in vivo* and *in vitro* (66,67). Many genes are hypermethylated in cell culture, in particular autosomal CpG islands can become methylated which are normally not methylated *in vivo*. Methylation patterns and gene expression patterns observed in cell culture therefore do not necessarily reflect the *in vivo* state. The hypermethylation and inactivation of many genes in cell culture is believed to be a phenomenon of selection such that genes not absolutely required for the survival in culture are gradually switched off. Furthermore, cells in culture are selected for rapid division, such that slowly dividing cells are overgrown by rapidly dividing cells. Thus, genes that result in cell-cycle arrest and terminal differentiation such as *p16* and *MyoD1*, respectively, are inactivated in cultured cells by mechanisms such as deletion, mutation or hypermethylation (65,68).

Differentiation

DNA methylation patterns are mosaics in the various cell types found in different organs of an organism (69). Cell type and tissue specific mosaicism is not just the result of methylation errors that occurred during development since the methylation patterns found in organs of different individuals show a high degree of interindividual concordance (70). Tissue specific methylation patterns are most likely a result of an ordered demethylation and re-methylation process that occurs during development (53). It is, to date, unclear to what degree cell-type specific DNA methylation patterns are involved in establishing and stabilizing the gene expression pattern and therefore the identity of cell types (71,72).

A number of experiments, mainly performed *in vitro*, have shown that altering the DNA methylation patterns with demethylating drugs such as 5-azacytidine (5-Aza-CR) can change the differentiation state of cell lines. Mouse embryonic fibroblasts convert with high efficiency to myoblasts, adipocytes and chondrocytes when treated with 5-Aza-CR (73). Similarly, differentiation of Friend erythroleukemic cells can be induced by demethylating agents (74). It is believed that activation of cell lineage-determining regulatory genes leads to auto- and cross-regulation of further genes that synergistically activate and maintain a cell-type specific gene network active. It is conceivable that genes involved in determination and differentiation with a dominant role in defining the identity of cells should be tightly regulated such that any expression is prevented in cells of a different lineage. DNA methylation could be involved in establishing or maintaining the inactive state of determination genes and thus contribute to the stability of the differentiated state (39).

Parental imprinting and allelic exclusion

Parentally imprinted genes are differentially expressed depending on their parental origin and this is often reflected by the differential methylation of genes on the paternal and maternal chromosomes. About 15 imprinted regions have been identified in the mouse to date and it is believed that parental-origin specific methylation plays a role either in establishing or maintaining the imprint (75). Many imprinted genes contain directly repeated sequences that have been hypothesized to be involved in establishing or maintaining the imprint (76). The localization of a number of imprinted genes to the same chromosomal region on chromosome 11 suggests, furthermore, that the establishment of the imprint might involve entire chromosomal domains (77).

The inactivation of one allele leading to functional hemizygosity independent of the parental origin has been described as allelic exclusion in immunoglobulin genes where the V-D-J recombinase system arranges only one allele per cell (78). Interestingly, DNA methylation can interfere with V-D-J joining thus suggesting that it plays a role in allelic exclusion (79). Other examples of functional hemizygosity have been described mainly in cell culture, and it is unknown whether it has any developmental role and to what degree DNA methylation is involved (71,78,80,81). In addition to a developmentally regulated allelic exclusion such as described in lymphocytes, it appears possible that random methylation errors that occur during development and aging could reach a level that leads to the inactivation of some genes on one allele without affecting the function of the other allele (54). The frequent detection of partially methylated CpG sites in DNA isolated from tissues could originate from such DNA methylation errors (39,82). Mutation, deletion or hypermethylation of the remaining wild-type allele would lead to the complete loss of a gene function (54).

Inactivation of foreign and repeated DNA by cytosine methylation

One of the functions of restriction and modification systems in prokaryotes is to defend the organism against differentially modified, foreign DNA. Similarly, it has been proposed that DNA methylation in eukaryotes could be a host defense mechanism against foreign and repetitive DNA (51). Since no CpG-specific restriction enzymes have been identified in higher eukaryotes, this host defense mechanism presumably acts mainly via inactivation rather than degradation of foreign sequences. DNA from external sources such as virus DNA and transfected DNA, as well as from internal sources represented by transposable elements and repetitive sequences such as LINES and SINES (Alus), are subject to inactivation and *de novo* methylation (59,83,84). After integration, the adenovirus genome becomes gradually methylated by methylation spreading and for some virus DNA, cytosine methylation is involved in viral latency (83). Similarly, the high copy number of a transgene containing a CpG island is associated with extensive hypermethylation (85).

The mechanisms for selective recognition and methylation of foreign and repeated DNA in higher eukaryotes are not yet clear. The higher methylation activity of the mammalian methyl-transferase towards DNA-containing unusual structures such as mismatches and loops may be utilized to initiate methylation at foreign sequences (9). Direct repeats found in parentally imprinted regions have been hypothesized to be involved in generating and maintaining parental imprinting by serving as a preferred target for the methyl-transferase (76). Repeated sequences are believed to recombine with each other and the recombination intermediate has been suggested to be a preferential target for *de novo* DNA methylation (86). In the fungus *Ascobolus immersus* repeated sequences are efficiently recognized and inactivated by cytosine methylation by a process called methylation induced premeiotically (MIP) (87). Similarly, in the fungus *Neurospora crassa*, a process called repeat induced point mutations (RIP) recognizes repeated

sequences and either methylates the cytosines or mutates them to T (88). It has been speculated that in higher eukaryotes a similar process, that somehow recognizes repeated sequences and methylates and/or mutates them, might operate, possibly with a much lower efficiency (87–89).

DNA methylation and mutation

The target cytosines of the Mtases in prokaryotes and eukaryotes are mutated with increased frequency relative to other cytosines, suggesting that methylation could serve as a long-term inactivation mechanism by increasing the mutation rate at methylated foreign and repetitive DNA (59,87,89–91). A depletion of CpG dinucleotides is observed in pseudogenes suggesting that methylation also serves to increase the mutation rate of newly duplicated genes thus generating new gene variants (92,93). Furthermore, as can be seen in the high frequency of restriction fragment length polymorphisms (RFLP) at the CpG dinucleotide, the CpG dinucleotides are often mutated in different individuals, thus increasing the diversity of geno- and phenotypes in a population (60,94).

Similarly, alignment of Alu repetitive elements reveals that CpG dinucleotides are frequently mutated (89,95). Since Alu repetitive elements are believed to transpose when unmethylated and CpG rich, and to become methylated upon insertion, an insertion event can be used as a starting point for a molecular clock and the unidirectional C→T transition mutations at CpGs generating TpGs, and CpAs can be used as pacemaker. By comparing the sequence of an Alu repetitive element, which inserted into the *p53* gene of a common ancestor of humans and old world monkeys about 40 to 60 million years ago, an estimated *in vivo* rate constant ($k = 1.8\times10^{-8}$/years, $t_{1/2} = 82$ million years) of C→T germ-line transition mutations at CpGs was calculated (96).

When this rate constant is applied to the 10^7 CpGs of the haploid germ line genome, an estimated frequency of 21 C→T transition mutations at the CpG dinucleotide is expected per generation, which may contribute to the spontaneous mutational load leading to inherited disease (96). This compares to the 1.9 mutation events per generation and haploid genome calculated from the occurrence of factor IX mutations found in hemophilia B patients (97). Assuming that somatic cells have the same mutation rate as germ cells, a given CpG is expected to occur mutated ~10^7 times in the approximately 10^{13} to 10^{14} somatic cells of an adult human being. It is unknown whether somatic cells show the same mutation and repair rate as germ cells and to what degree somatic cells with mutated CpG dinucleotides survive. In contrast to germ cells, somatic cells are diploid and subject to processes such as terminal differentiation, developmental growth arrest and cell death, which would reduce the risk of generating cells with the same CpG dinucleotide mutated in both alleles. The frequency of transition mutations at the CpG dinucleotide in various mouse organs was found to be up to 9-fold higher than expected (98), suggesting that in somatic cells, DNA methylation errors at the CpG dinucleotide would lead not only to epigenetic mosaicism but also to genetic mosaicism (99,100). When the total number of CpGs that are targeted for methylation during the life of an organism are considered, DNA methylation errors appear to be an unavoidable burden to any organism containing 5-mC. The rate of accumulation and the rate of repair of methylation errors might vary depending on the species, the individual and possibly on environmental and nutritional factors (101). Interestingly, in mice the occurrence of the CpG dinucleotide in CpG islands is lower than in humans, suggesting that the cytosine deamination frequency is higher in mice resulting in the erosion of CpG islands (102).

Carcinogenesis

Ultimately, cancer is believed to be due to an accumulation of DNA aberrations and of their effects on gene function and expression. Analysis of DNA isolated from tumors has identified epigenetic and genetic alterations occurring at the target CpG of the Mtase as two of the most frequent and consistent changes observed in tumor cells (103–105). Whereas the role these genetic and epigenetic alterations can play in tumor development is well studied, less is known about their mechanistic origin.

Epigenetic changes induced by the (cytosine-5)-DNA methyltransferase

The mRNA level and enzyme activity of Mtase are higher in many tumor cells than in normal cells (106). Interestingly, the generally higher methyl-transferase activity does not lead to an overall increase but rather to a decrease of total genomic 5-mC content (107,108). Nevertheless, when the methylation status of various genes is measured in tumor cells, both hypermethylation as well as hypomethylation become apparent (109). It appears that the pattern of methylation of specific genes is altered in tumor cells and normally unmethylated CpG islands may become hypermethylated (110).

Hypermethylation and inactivation of one allele of a tumor suppressor gene by methylation errors introduced during development, aging or initiation by carcinogens (see also Table II) (71,111–113) and some chemotherapeutic agents (114) could lead to the formation of cells with increased tumorigenic potential, and mutation or hypermethylation of the second allele would lead to complete loss of function (Figure 1A). Genes involved in growth arrest and terminal differentiation such as the *MyoD1* gene (115) and tumor suppressor genes such as *pRb* (116,117), *bcr-abl* (118), *pVHL* (119), *pWT* (120), *estrogen receptor* (121), *$p57^{KIP2}$* (122), *MDGI* (123) and *p16* (124) are often found either mutated or hypermethylated and inactive in tumor cells. Abnormal hypermethylation of the regulatory region of genes could initiate and/or reinforce their inactivation either by directly interfering with the binding of transcription factors or by the generation of inactive chromatin.

On the other hand, growth inducing genes such as oncogenes can become overexpressed as a result of hypomethylation or gene amplification (Figure 1B) (109). In particular, demethylation and overexpression of the c-*fos*, c-*myc* and c-H-*ras* proto-oncogenes is known to be involved in hepatocarcinogenesis induced by conditions such as methyl-donor starvation (125). Other genes such as the *ornithine decarboxylase*, the *erb-A1* and the *bcl-2* proto-oncogenes can become hypomethylated in chronic lymphocytic leukemia (126,127). Various carcinogens, methyl-donor deficient diet, and some therapeutic drugs result in hypomethylation of genomic DNA and, therefore, might lead to genetic and to epigenetic alterations (see also Table II) (71,111,128).

Alteration of DNA methylation patterns observed in tumors does not have to be necessarily the direct result of *de novo* methylation or demethylation. Loss of heterozygosity (LOH) of partially methylated and imprinted genes and loss of imprinting (LOI) can lead to the formation of cells with either fully methylated or completely unmethylated gene sets. The

Table II. Inhibitors affecting (cytosine-5)-DNA methylation[a]

(I) Nucleotide analogs
5-azacytidine (5-Aza-CR) (73), 5-azadeoxycytidine (decitabine) (73)
arabinofuranosyl-5-azacytosine (177), 5-fluoro-2′-deoxycytidine (73), pyrimidone (178)
trifluoromethyldeoxycytidine (179), pseudoisocytidine (73), dihydro-5-azacytidine (180)
(II) AdoMet/AdoHcy analogs as competitive inhibitors
AdoHcy (181), sinefungin and analogs (160,175,182,183), 5′-deoxy-5′-S-isobutyladenosine (SIBA) (182),
5′-methylthio-5′-deoxyadenosine (MTA) (160,184,185)
(III) Drugs influencing the level of AdoMet
ethionine analogs
methionine (185–187), L-*cis*-AMB (188), cycloleucine (185)
antifolates
methotrexate (114,170)
(IV) Drugs influencing the level of AdoHcy, dc-AdoMet and MTA
inhibitors of AdoHcy hydrolase
3-deaza-adenosine (190), neplanocin A (188,191), 3-deazaneplanocin (188), 4'-thioadenosine (192),
3-deaza-aristeromycin (193)
inhibitors of ornithine decarboxylase
α-difluoromethylornithine (DFMO) (194)
inhibitors of spermine and spermidine synthetase
S-methyl-5′-methylthioadenosine (MTA), L-*cis*-AMB, AdoDATO, MGBG (188,195,196)
inhibitors of methylthioadenosine phosphorylase
difluoromethylthioadenosine (DFMTA) (184)
(V) Other inhibitors
methinin (197), spermine/spermidine (198), sodium butyrate (186,199), procainamide (200),
hydralazine (200), dimethylsulfoxide (186,187), free radical DNA adducts (201), UV-light (202),
8-hydroxy guanine (203), *N*-methyl-*N*-nitrosourea (204), novobiocine (205), phenobarbital (206)
benzo[*a*]pyrene (113,207), ethylmethansulfonate (113), ethylnitrosourea (112,113),
N-ethyl-*N*′-nitro-*N*-nitrosoguanidine (112,113), 9-aminoacridine (113), nitrogen mustard (113),
N-methyl-*N*′-nitro-*N*-nitrosoguanidine (113,208,209), diethylnitrosamine (210), chlordane (210),
N-acetoxy-*N*-2-acetylaminofluorene (113), aflatoxin B1 (112), nalidixic acid (205),
N-2-fluorenylacetamine (210), 3-methyl-4'-(dimethylamino)azobenzene (210),
1,3-bis(2-chlorethyl)-1-nitrosourea (113,114), cyclophosphamide (114), 6-mercaptopurine (211),
4-nitroquinoline-1-oxide (112), *N*-nitrosodiethylamine (112), hexamethylenebisacetamide (212),
retinoic acid (187), retinoic acid with cAMP (213), aromatic hydrocarbon carcinogens (207),
dibutyryl cAMP (214), antisense mRNA to the methyltransferase (215)
(VI) Increase of DNA methylation
AdoMet (169), Ni^{2+} (216), 5-methyl-CTP (217,218), butyric acid (219),
ethylation (220), ethyl methane sulfonate (221), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (214)
chemotherapeutic agents (114,205)
etoposide, nalidixic acid, doxorubicin, vincristine, vinblastine, cytosine arabinoside,
colchicine, cisplatinum, hydroxyurea, 1-β-D-arabinofuranosylcytosine,
5-fluorouracil, 5-fluorodeoxyuridine, 3′-azidodideoxythymidine, aphidicolin

[a]The table lists only inhibitors for which direct or indirect effects on DNA methylation have been observed.

partially methylated alleles arise either gradually by methylation errors occurring during development and aging or reflect the parental specific methylation, as described at the c-H-*ras* gene (80,81), the *pRb* gene (117) and at imprinted genes (100,129).

Loss of heterozygosity by deletion of one allele, by uniparental disomy or by somatic recombination has been implicated in many tumors (Figure 1C) (130). Either the active allele(s) is (are) retained leading to overexpression of a tumor inducing gene or the inactive allele(s) is (are) retained leading to a loss of a tumor suppressing gene. In most cases the retained allele(s) harbor a mutation that inactivates a tumor suppressor gene (130). DNA methylation can be considered as an allele specific epimutation that in the case of LOH might show the same characteristics as a sequence mutation (71).

An example for loss of heterozygosity at imprinted genes is described in Wilms' tumors and the Beckwith–Wiedemann syndrome, in which two active paternal alleles of the imprinted *IGF-II* gene are retained leading to overexpression of the *IGF-II* protein, whereas the two paternal alleles of *H19* are inactive (131,132). The *IGF-II* protein is an autocrine growth factor and might be required for the increased growth rate and autonomy of tumor cells and *H19* is a putative tumor suppressor gene (133). Another example for a disease caused by uniparental disomy at imprinted genes is described by the Prader–Willi syndrome and by the Angelman syndromes. Both syndromes are believed to occur by an embryonic loss of heterozygosity that retains either the paternal alleles (Angelman syndrome) (134) or the maternal alleles (Prader–Willi syndrome) (135).

As an additional mechanism, parentally imprinted genes might lose their functional haploidy by a loss of their imprint (LOI) without deletion or somatic recombination, which leads to gene dosage effects (Figure 1D). A case of such a relaxed imprinting has recently been described with the imprinted *IGF-II* gene in Wilms' tumor, with both an active paternal and maternal allele of *IGF-II*, which indicates that the mechanisms for establishing or maintaining the parental imprint might be impaired (136,137).

Changes in DNA methylation patterns can also render certain genomic regions unstable by altering the chromatin structure, leading to deletions, inversions and chromosomal losses (138,139). Although DNA does not recombine *in vitro* with a lower efficiency when methylated, the formation of inactive chromatin *in vivo* covering methylated repetitive elements could be involved in preventing their recombination (139,140). DNA methylation is also known to prevent site

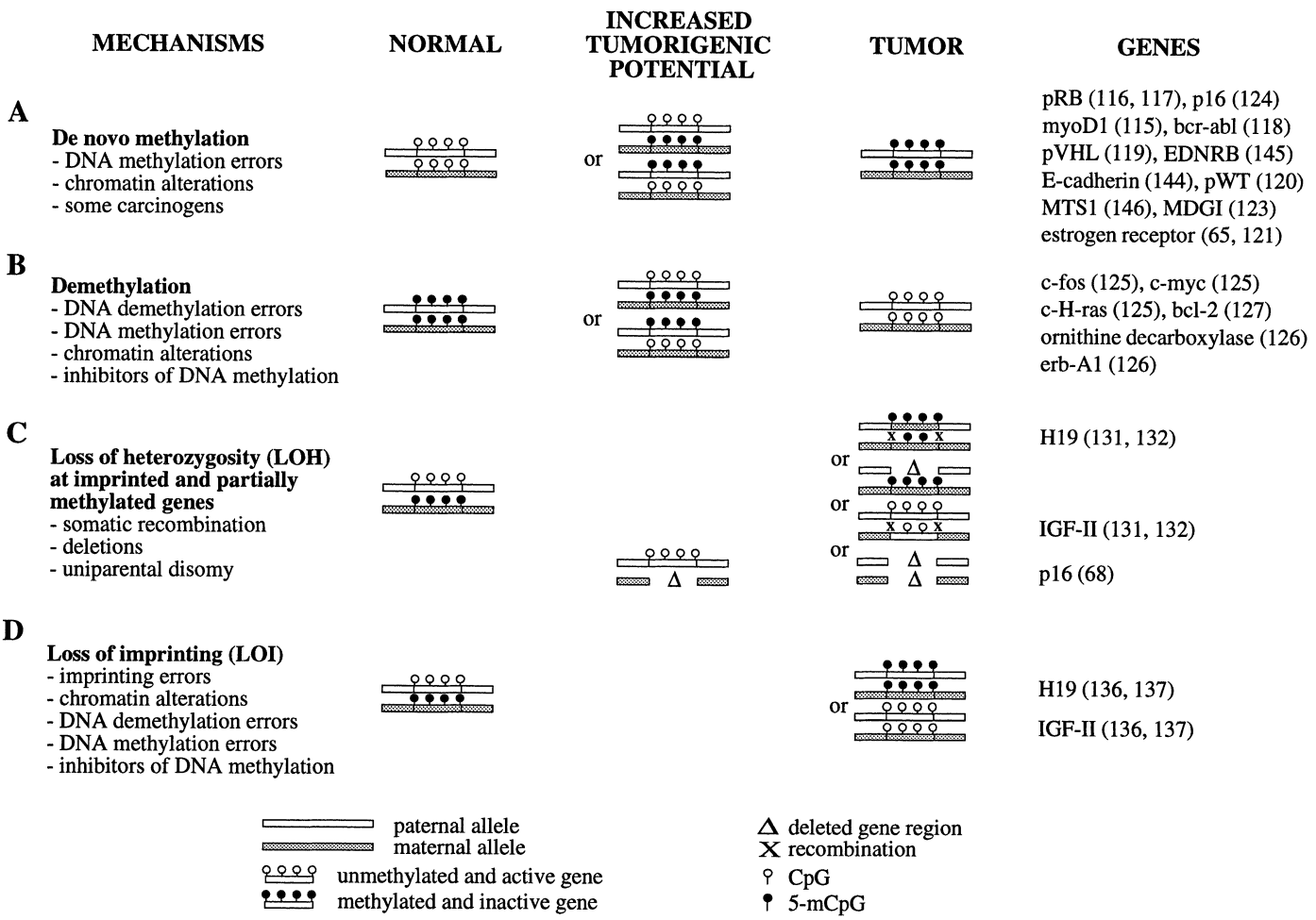

Fig. 1. Epigenetic changes as a result of DNA methylation errors. (**A**) DNA methylation errors can increase the tumorigenic potential by hypermethylation and inactivation of one allele of a negative growth regulatory gene. (A and **C**) Further methylation errors, deletions, mutations and somatic recombination at the second allele can lead to complete loss of a functional gene. (**B**) DNA methylation errors can also increase the tumorigenic potential by hypomethylation and activation of one or both alleles of a positive growth regulatory gene. (**D**) Loss of imprinting (LOI) can lead either to the loss of expression or to the overexpression of an imprinted gene with growth regulatory function.

specific V-D-J recombination occurring in B and T cells (79). Furthermore, abnormal DNA methylation could modulate certain aspects of the tumor phenotype such as antigen expression (141,142), invasion and metastatic behavior (141,143). The *E-cadherin* (144), the *EDNRB* (145), the *MTS1* (146) and the *estrogen receptor* (121) genes can be inactivated by hypermethylation leading to cells with altered surface antigens and increased metastatic potential.

The ultimate origins of alterations of DNA methylation patterns in tumor cells, although well documented, is still unclear. Many of the changes of DNA methylation patterns might occur randomly and are possibly not easily detected since they are without apparent biological effect on a cell. Therefore, it becomes crucial to distinguish specific DNA methylation changes required for the initiation and progression of tumors from non-specific changes, which might occur randomly. The probability of being methylated is specific for each CpG dinucleotide and the methylation state is estimated to be maintained with at least 99.9% efficiency (147). It is unknown whether naturally occurring methylation errors are sufficient to account for the genetic and epigenetic changes observed in tumorigenesis or whether specific changes occur in the DNA methylation machinery that somehow increase the error rate.

Studies addressing this question indicate that changes experimentally introduced to the DNA methylation machinery of normal cells can increase their tumorigenic potential. Inhibition of DNA methylation in APC^{Min} mice by treatment with 5-aza-CR reduces the risk for intestinal polyp formation (148). Experimentally induced overexpression of the Mtases from mouse and bacteria (M.HhaI) leads to hypermethylation and to an increased rate of transformation of NIH3T3 cells and to hypermethylation of CpG islands in fibroblasts, suggesting that the level of DNA methyl-transferase is important for the total methylation level of genomic DNA (149,150). On the contrary, high Mtase levels do not necessarily lead to hypermethylation, since cell lines with low methyl-transferase activity and unmethylated CpG islands mostly maintain their methylation pattern when fused to cells with high levels of methyl-transferase activity and methylated CpG islands (151).

Genetic changes at the target site of (cytosine-5)-DNA methyl-transferase

The target cytosines of Mtase in prokaryotes are mutated to thymine with an increased frequency when compared with non-target cytosines (90). In eukaryotes, the enhanced mutability of 5-mC contributes significantly to carcinogenesis and inherited disease (103,104). In humans, several genes such as *p53* (152)

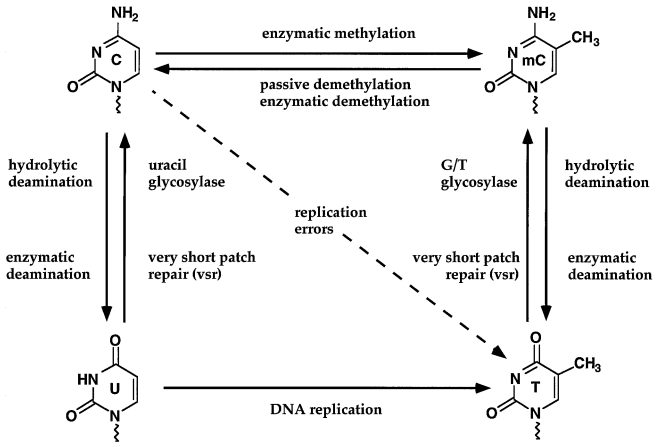

Fig. 2. Genetic changes as a result of DNA methylation. In addition to replication errors occurring at any base (diagonal), DNA methylation and possibly DNA demethylation increase the rate of C→T transition mutations (outside of chart). The efficiency of the repair machinery for these mutations determines the rate of their fixation (inside of chart).

and *p16* (153) are frequently mutated specifically at CpGs in tumor cells and ~30% of all inherited mutations are believed to occur by mutations at CpGs in the germ line (104,154). A search for the reason for this increased mutation frequency revealed that spontaneous hydrolytic deamination of 5-mC→T occurs with a higher frequency than C→U. In aqueous solution at 37°C, a 4- to 9-fold higher deamination frequency of 5-mC relative to C in single stranded DNA and in double stranded DNA an ~2-fold higher deamination frequency was measured for 5-mC when compared to C (155).

It has not been absolutely proven whether the observed high frequency of transition mutations in human cancer and inherited disease at the target cytosines of Mtase is simply the result of a normal spontaneous process of hydrolytic deamination of 5-mC or whether mutagens or other abnormalities exist that increase the rate of such mutations. It is not clear, furthermore, whether the mutations at CpGs seen in tumors were formed during organ development and aging and therefore pre-existed as cells with increased risk for tumorigenic potential, or whether these mutations occurred *de novo* during tumor initiation and progression. A 42-fold higher mutation frequency at 5-mC is observed at CpGs in human inherited disease and cancer, that is 40-fold higher than the expected frequency from the 2-fold higher spontaneous cytosine deamination rate measured for DNA in water (104,155) is observed. Several studies with bacterial enzymes have addressed this discrepancy between spontaneous and observed rate of cytosine deamination and have provided reasonable explanations for the high mutation frequency at 5-mC. A search for potential mutagens such as echinomycin or nitric oxide, that might lead to an increased mutation rate of 5-mC→T at the CpG dinucleotide in double-stranded DNA has so far been unsuccessful (156,157). Whereas DNA replication errors and spontaneous C→U deamination are believed to be the main mutagenic pathways occurring at unmethylated cytosines, the methylation reaction generates several additional pathways that increase the rate of transition mutation specifically at the cytosines targeted for methylation (Figure 2).

Under *in vitro* conditions of reduced AdoMet and AdoHcy concentrations, several bacterial Mtases actively increase the C→U deamination rate at the unmethylated target cytosine by up to 10^4-fold, by generating an unstable enzyme-dihydrocytidine intermediate, which in the presence of water deaminates rapidly to uracil leading to G/U mismatches (Figure 2) (158). These enzymes are also able to induce 5-mC→T mutations leading to G/T mismatches albeit at a much lower rate than C→U (Figure 2) (159). Mutations introduced into the AdoMet binding pocket of the bacterial Mtase M.HpaII increase the rate of enzyme-mediated C→U deaminations, suggesting that the Mtases can become mutagenic when structural alterations are introduced, which interfere with optimal AdoMet binding (189). We recently discovered that two analogs of AdoMet, sinefungin and 5′-amino-5′-deoxyadenosine can compete with AdoMet binding and increase the rate of enzyme-mediated C→U deamination by donating a proton and facilitating the formation of the unstable enzyme-dihydrocytidine intermediate (160). It is conceivable that further compounds with affinity to the AdoMet binding pocket of the Mtase could serve as proton donors and increase the rate of enzyme-mediated cytosine deamination.

Generally, the *in vivo* frequency of mutation is determined by the frequency of occurrence and the efficiency of repair. Specific repair systems such as the very short patch repair system (vsr) in bacteria (161) and the G/T glycosylase in mammals (162) have evolved that recognize and repair the G/U and G/T mismatches generated at the target cytosines of Mtase. It is believed that an increased generation of G/U and G/T mismatches at CpGs in genomic DNA would lead also to an increased frequency of mutation, since the repair mechanisms directed against these mismatches are not absolutely efficient. G/T mismatches are more difficult to recognize than G/U mismatches, since thymine is a normal constituent of DNA whereas uracil is not. The *in vitro* repair efficiency of the eukaryotic G/T glycosylase is much lower than that of uracil glycosylase (163). The sequence context in which a mutation occurs might also be important for the efficiency of mismatch repair (101). The bacterial uracil glycosylase repairs uracil in looped and damaged DNA inefficiently (164). Furthermore, the bacterial Mtases strongly bind G/U mismatches and block the repair by uracil glycosylase, suggesting that the accessibility of mismatches to the repair machinery can determine the frequency of their repair (165,166).

The metabolism of the cofactor AdoMet and its involvement in carcinogenesis

A large number of studies indicate that aberrations of the metabolism of the cofactor AdoMet may also play an important role in carcinogenesis (167). Aberrations of the AdoMet metabolism may not only affect DNA methylation since AdoMet is a universal methyl donor for a large number of other methyl-transfer reactions besides DNA methylation as well as the sole aminopropyl-donor for the synthesis of spermine and spermidine. Interference with DNA methylation can be achieved at several steps of the AdoMet metabolism (Figure 3 and Table II).

Insufficient supply of methionine, folate and choline leads to hypomethylation of liver DNA, increased expression of the c-H-*ras*, c-*jun* and c-*myc* genes and to the generation of liver tumors in rats (125,168–170). Feeding such methyl-donor deficient rats with AdoMet leads to the re-methylation of genomic liver DNA and to the reversion of the tumorigenic state, suggesting that the level of AdoMet can influence the activity of the eukaryotic Mtase (169). In humans, methyl-donor deficiency is correlated with an increased risk for liver and colon tumors (171). It is not clear to what degree the

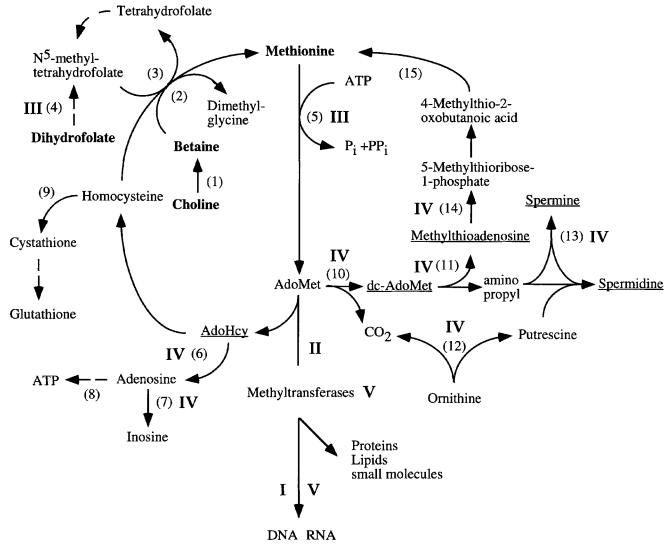

Fig. 3. The metabolism of S-adenosylmethionine and the targets affecting DNA methylation. Interference with the enzymes (1)–(15) involved in the metabolism of AdoMet can affect DNA methylation either by limiting AdoMet synthesis or by the accumulation of metabolites with inhibitory activity (underlined). Enzymes are: (1) choline dehydrogenase, (2) betaine-homocysteine methyltransferase, (3) methionine synthase, (4) dihydrofolate reductase, (5) AdoMet synthase, (6) AdoHcy hydrolase, (7) adenosine deaminase, (8) adenosine kinase, (9) cystathione β-synthase, (10) AdoMet decarboxylase, (11) spermidine synthase, (12) ornithine decarboxylase, (13) spermine synthase, (14) methylthioadenosine phosphorylase (MTAP). (15) transaminases. **I–V**: The targets of inhibitors affecting DNA methylation as listed in Table II. **Bold**: insufficient supply of choline, folate, vitamin B12 and methionine can result in hypomethylation of genomic DNA. Underlined: Accumulation of AdoHcy, dc-AdoMet, MTA, spermine and spermidine can result in hypomethylation of genomic DNA.

observed phenotypes of methyl-donor deficient animals can be attributed to changes in genomic DNA methylation patterns. Methyl-donor deficient animals are also known to be more susceptible to a number of carcinogens and to show other symptoms not directly related to DNA methylation (172).

An insufficient supply of AdoMet during methyl-donor deficiency could lead, in addition to hypomethylation, to the induction of enzyme-mediated C→U deamination, as is observed with the bacterial enzymes and in methyl-donor deficient rats (158,173). Whereas conditions favoring the enzyme mediated cytosine deamination pathway have not been found in human colon tumor cells (174), it remains possible that chronic depletion of methyl-donors, possibly combined with a higher level of DNA methyl-transferase, represents a cancer-predisposing condition to normal cells leading to hypomethylation and to enzyme-mediated cytosine deaminations.

Inhibitors of DNA methylation

The role of DNA methylation in cancer suggests a potential target for chemotherapeutic approaches that can either directly or indirectly affect DNA methylation (175,176). Agents that interfere with the metabolism of AdoMet and drugs that bind to DNA are expected to lead to alterations of DNA methylation patterns (Figure 3). In addition, several carcinogens can affect DNA methylation either by modifying the target DNA or by inactivating the Mtase protein (71,111–113). In dealing with inhibitors of transmethylation reactions, it should be noted that they inhibit not only DNA methylation, but can have various side-effects on such factors as DNA synthesis, other methyl-transferases and on spermine and spermidine synthesis. The synthesis of a specific inhibitor of Mtase would help to correlate the aberrations seen in tumors with changes in DNA methylation. Inhibitors of DNA methyl-transferase can be divided, based on their mechanism, into five groups (Figure 3 and Table II).

The nucleotide analogs (group I) are incorporated into DNA and inactivate the Mtase by forming a mechanism-based covalent enzyme–DNA adduct. The most studied inhibitor of DNA methyl-transferase, the nucleotide analog 5-azacytidine, has been used to treat solid tumors and myelogenous leukemia (222). Since 5-azacytidine is unstable and requires continuous infusion in the clinical setting, more stable analogs such as arabinofuranosyl-5-azacytosine (177), pseudo-isocytidine (73), 5-fluorocytidine (73), pyrimidone (178) and dihydro-5-azacytidine (180) have been used to inhibit DNA methylation.

Another approach is taken by the synthesis of analogs of AdoMet and AdoHcy (group II) such as sinefungin and derivatives (160,175,182,183), that act as competitive inhibitors of AdoMet and inhibit not only DNA methyl-transferase but also other AdoMet requiring enzymes such as AdoMet decarboxylase and AdoHcy hydrolase. Some of these analogs have potential use as antifungal, antiparasitic, anticancer and antibacterial agents (183).

The third group (III) inhibits the synthesis of AdoMet by interfering with methionine metabolism such as the antifolate methotrexate, which inhibits dihydrofolate reductase (114,170), and ethionine (185,186) and L-*cis*-AMB (188), which both inhibit AdoMet synthase.

A fourth group (IV) acts by inhibiting the degradation of AdoHcy or the synthesis of spermine and spermidine, leading to the accumulation of the inhibitory AdoHcy, decarboxylated AdoMet (dc-AdoMet), 5′-methylthio-5′-deoxyadenosine (MTA) and inhibition of DNA methylation by feedback inhibition. Inhibitors of AdoHcy hydrolase (223) lead to the accumulation of AdoHcy; inhibitors of ornithine decarboxylase (194,196) and spermidine and spermine synthase (195,196) lead to the accumulation of dc-AdoMet; and inhibitors of methyl-thioadenosine phosphorylase (184,185) lead to the accumulation of MTA.

A fifth group (V) inhibits DNA methylation by various mechanisms such as peptides (197), butyric acid (186,199), spermine and spermidine (198), some carcinogens (71,111–113), or alkylating agents that modify the target DNA (113) or inactivate the Mtase protein (113). Another approach targets the Mtase mRNA by antisense mRNA (215).

Only a few pathways (VI) are known that can increase the DNA methylation, such as the hypermethylation induced by AdoMet (169), carcinogenic nickel (216) and some chemotherapeutic agents (Table II).

As discussed in this review, genetic and epigenetic alterations at the target CpG of Mtase are among the most frequently found aberrations in human cancer, inherited disease and aging. It is therefore interesting to evaluate whether these alterations could be prevented or reversed by specific treatments with agents affecting DNA methylation. Sufficient dietary supply with methyl-donors is known to have preventive characteristics against liver and colon tumors in rats (224). Treatment of APC^{Min} mice with 5-aza-CR lowers the risk for intestinal polyp formation, presumably by reducing the level of Mtase (148). Inhibitors of Mtase could prevent the hypermethylation seen with a number of chemotherapeutic agents and therefore prevent the formation of drug-resistant tumor cells containing hypermethylated drug-activating genes (114). Reversion of the

tumorigenic state could possibly be achieved by the activation of hypermethylated wild-type tumor suppressor genes such as *pRb* (116) and *p16* (124) by inhibitors of DNA methylation, or by the silencing of hypomethylated oncogenes by agents that stimulate *de novo* methylation such as AdoMet (169,224). It appears possible, furthermore, that inhibitors of DNA methylation could induce apoptosis (225) or activate antigens in tumor cells thus rendering them accessible to immunoglobulin mediated therapy (226–228).

The high mutability of the target C of Mtase is traditionally believed to be an endogenous mutagenic process, not accessible to preventive measures, such as those described for antioxidants, which reduce mutations induced by oxidative damage (229). Nevertheless, an insufficient supply of dietary methyl-donors leads to hypomethylation and can increase the risk for liver and colon tumors in rats, suggesting that conditions might exist that increase the risk for such mutations (169,173). Since enzyme-mediated C→U deaminations do not occur in the presence of AdoMet and AdoHcy, it is possible that a sufficient supply of AdoMet and AdoHcy could prevent enzyme-mediated C→T transition mutations. In particular, chemotherapeutic treatments with agents affecting the AdoMet and AdoHcy pool could be supplemented by the administration of AdoHcy or analogs to prevent the potential accumulation of enzyme-mediated C→T transition mutations. When analogs of AdoMet, AdoHcy, MTA and adenosine are designed as inhibitors of methylation and deamination reactions, their potential enzyme-mediated mutagenic effects have to be considered. Two analogs, sinefungin and 5′-amino-5′-deoxyadenosine, dramatically increased the *in vitro* rate of C→U deaminations mediated by bacterial methyl-transferases even in the presence of high concentrations of AdoMet and AdoHcy (160). Although it remains to be shown whether this or similar pathways can lead to an increase of the *in vivo* C→T mutation frequency in eukaryotic cells, it suggests that the use of analogs of AdoMet, AdoHcy, MTA and adenosine could have some unwanted side effects.

Perspectives

The regulation of developmental gene expression, parental imprinting, inactivation of repeated and foreign DNA and the generation of genetic and epigenetic diversity have been described as being the normal functions of DNA methylation. Genetic and epigenetic aberrations at the target CpG of the Mtase are among the most frequently observed events in human cancer and inherited disease. The effects of these aberrations on individual genes and cells have been well studied, but their ultimate origins are only starting to be understood. The identification of the pathways leading to aberrations of methylation patterns and deaminations at the target cytosine of Mtase in tumors and inherited disease could ultimately lead to clues of how to reverse and prevent them. Specific alterations of DNA methylation and AdoMet metabolism seen in tumor cells could furthermore serve as targets for chemotherapeutic intervention and possibly as diagnostic markers for assessing disease progression and therapy.

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