Review Article

Aberrant methylations in cancer cells: Where do they come from?

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Cancer epigenetics is rapidly moving into a translational phase, and knowledge on how aberrant DNA methylation is induced is becoming important. Aging, chronic inflammation, and viral infections are known to promote methylation of non-core regions of promoter CpG islands (CGI). The non-core methylation and 'seeds of methylation', scattered methylation in a CGI, are considered to serve as triggers for dense methylation of a promoter CGI, which permanently represses expression of its downstream gene. Decreased gene transcription is an important factor that promotes induction of dense methylation. The presence of the CGI methylator phenotype (CIMP), in which methylation of multiple CGI was observed, is under dispute. Some gastric cancer cell lines have increased rates of *de novo* methylation, and neuroblastoma cases with CIMP show qualitatively different prognosis from those without. This strongly supports the presence of CIMP, but it seems to contain multiple entities. Limited knowledge is available for epimutagens, the chemicals that induce DNA demethylation or methylation. We have developed an assay system to detect demethylating agents, and an assay system for methylating agents is necessary. Efforts in the field on how aberrant methylation is induced will lead to new cancer prevention, diagnostics, and therapeutics. (*Cancer Sci* 2005; 96: 206–211)

DNA methylation of a CpG island (CGI) in the promoter region of a tumor-suppressor gene represses its transcription.[1] The first example was identified for the *RB* gene in sporadic retinoblastomas in 1993[2,3] followed by *VHL*,[4] *CDKN2A* (*p16*),[5,6] *CDH1* (*E-cadherin*),[7,8] and *hMLH1*.[9] Now, many tumor-suppressor genes are known to be inactivated by methylation of their promoter CGI in a wide variety of cancers.[1] Methylation of a promoter CGI excludes some methylation-sensitive transcription factors, such as CTCF, and recruits methyl-CpG binding proteins, such as MeCP2 and MBD1-MBD3.[10] These methyl-CpG binding proteins further recruit histone deacetylases, histone methyltransferases, and heterochromatin proteins.[11] It is believed that changes in chromatin structure will block the access of transcription complex to DNA, and repress transcription.

In parallel with the mechanistic studies on how DNA methylation leads to gene silencing, the search for genomic regions aberrantly methylated in cancers has also made a lot of progress.[12] In the late 1990s, before the human genome sequence was available, several genome-wide screening methods were developed, such as restriction landmark genomic scanning-methylation, methylation-sensitive-representational difference analysis (MS-RDA), methylation-sensitive-arbitrarily primed PCR, and methylated CpG island amplification-RDA.[13–17] These methods revealed that cancers harbor many aberrantly methylated genomic regions. Now, owing to completion of the sequencing of the human genome, it has become evident that, even if limited to CGI in promoter regions or putative promoter regions (5′ regions) of genes, most cancers have multiple aberrant methylations.[18–21] These aberrant methylations are considered to provide a good source of tumor markers,[22] and targets for chemotherapeutics.[23,24]

In contrast with these rapidly progressing areas of epigenetics, the etiology of aberrant DNA methylation needs more attention. What induces DNA methylation and how? As there are many excellent reviews on epigenetics and cancer,[1,3,10,22] here, we would like to focus on recent advancements on how aberrant DNA methylation is induced, based on our recent findings.[19–21,25–29]

Factors known to be associated with DNA methylation. Factors that are known to be associated with methylation of CGI include aging, chronic inflammation, and viral infections.[30–35]

It was first reported that a *Not*I restriction site in exon 1 of estrogen receptor (*ESR*) was methylated in normal colon mucosa in association with aging.[30] Further, the *N33* transcription start site and *MYOD* exon 1 were methylated in normal colon mucosa in association with aging, while *p16*, *THBS1*, *HIC-1* and *CALCA* were not.[31] These findings led to the establishment of a concept that some *regions* of CGI are methylated in association with aging in normal tissues, which has been confirmed by many subsequent studies using human samples and also in rats.[36,37] However, it is noteworthy that age-related methylation of tumor-suppressor genes applies mostly to exonic or far upstream regions within a promoter CGI, and that, even within the same promoter CGI, a small region covering the transcription start site is kept unmethylated.[33,36,38]

Chronic inflammation is also known to be associated with increased methylation. Normal-appearing colon mucosa of cases with ulcerative colitis are associated with increased methylation of *p16* exon 1, *MYOD* far upstream region, and *CSPG2* exon 1.[32,33] As in the case of age-related methylation, a region covering the *p16* transcription start site was spared from methylation.[33] Tobacco smoke, which contains various carcinogens and also induces inflammation, is also known to be associated with methylation of *p16* exon 1.[39] Along with the inflammation induced by viral infections as described below, chronic inflammation is considered as one of the factors that induce DNA methylation.

It is well established that viral DNA is methylated upon infection into mammalian cells.[40] It is becoming recognized that not only the viral DNA but also cellular DNA can be methylated as a consequence of viral infection.[41] Stomach cancers positive for Epstein-Barn (EB) virus are known to have more methylated CGI than those without.[34,35] As with age-related and inflammation-induced methylation, methylation of *p16* was present in its exon 1. In hepatocellular carcinomas, for which a precise comparison between virus-positive cancers and negative ones is very difficult, aberrant methylation was detected even in non-cancerous liver tissues showing chronic hepatitis or liver cirrhosis.[42] Detailed analysis of *MGMT* and *hMLH1* promoter CGI showed that weak

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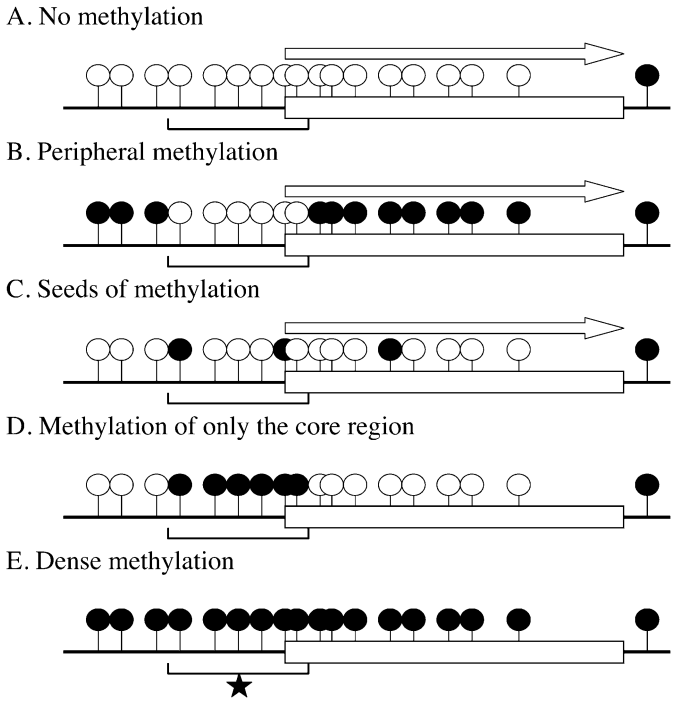

Fig. 1. Various types of methylation in a promoter CGI. When the core region (shown by brackets)* is methylated, transcription of the downstream gene is blocked (d, e). However, even if non-core regions are methylated, transcription is not blocked (b). 'Seeds of methylation' (c) is drawing attention as a precursor for dense methylation (e). Open lollipops show unmethylated CpG sites, and closed lollipops show methylated CpG sites.

methylation was present in far upstream regions.[43] Also, in the disease course of adult T-cell leukemia, endogenous genes, including *p16*, were shown to be methylated.[44] These points indicate that infection by EB virus, hepatitis viruses, and possibly other viruses triggers cellular machineries to methylate these viruses, which erroneously methylate endogenous genes.

As for *p16* and *hMLH1* genes, the above described factors do not induce methylation in critical regions for gene transcription (core regions), small regions covering their transcription start sites (Figs 1d, e),[12,45–47] but in other regions (non-core regions), such as exon 1 and far upstream regions (Fig. 1b). This shows that the core regions in promoter CGI are protected from *de novo* methylation. This leads to a hypothesis that the influence of the above factors can be more readily analyzed using non-core regions within promoter CGI or CGI outside promoter regions.

Dietary factors, such as folate deficiency and choline deficiency, are known to induce genomic *hypo*methylation, through induction of deficiency of methyl donors, such as S-adenosylmethionine.[48]

Molecular mechanisms for methylation induction. When a gene is silenced by methylation of its promoter CGI, the CGI is usually densely methylated (Fig. 1e), as is known for *CDH1*, *VHL*, *p16*, *hMLH1*, and many other genes.[19,25,38,45–47] Before this dense methylation is formed, two types of precursor methylation are considered to be present. One is methylation of non-core regions within a CGI (Fig. 1b), and the other is 'seeds of methylation', scattered methylation (Fig. 1c). The above-mentioned factors, aging, chronic inflammation, and viral infection, are involved in induction of non-core methylation and possibly 'seeds of methylation'.

Non-core methylation is frequently observed for various genes, including *p16*, and *hMLH1*, and does not block gene transcription.[45–47] Although a direct demonstration that non-core methylation leads to dense methylation with a significant frequency is not yet available, this model is widely believed. This is because methylation of core regions is almost always associated with methylation of non-core regions in cancer and normal cells, and because dense methylation in a cancer is often associated with methylation of non-core regions in its surrounding tissues. However, a quantitative correlation between the degree of non-core methylation and the chance of dense methylation needs to be carefully examined.

'Seeds of methylation' is emerging as an important precursor.[28,49,50] Stirzaker *et al*. introduced *GSTP1* with diminished promoter activity into LNCaP prostate cancer cells. When the construct was not methylated initially, little methylation was induced after 22 days. In contrast, when the construct was initially sparsely methylated with *Hpa*II methylase, a high degree of methylation all over the CGI was induced after 22 days. Also, we found that some gastric cancer cell lines tend to have scattered methylation, and that the scattered methylation leads to dense methylation with a low frequency.[28] These observations demonstrated that 'seeds of methylation' is an important precursor to dense methylation of a CGI.

Diminished transcription is also considered as a factor that promotes induction of dense methylation. Clark and colleagues demonstrated that even if 'seeds of methylation' are present, introduced *GSTP1* with active promoter was not methylated.[49,50] De Smet *et al*. demonstrated that impaired promoter activity or lack of cellular capacity for transcription promote re-methylation of demethylated *MAGE-A1*.[51] As circumstantial evidence, when we made genome-wide screenings for genes methylated in pancreatic and breast cancers, most of the genes whose 5′-CGI were methylated had low or no expression in normal counterpart cells.[20,21]

Antisense RNA to *HBA2* was shown to induce methylation of its promoter CGI.[52] Short interfering RNA targeted to *CDH1* and *EF1A* promoter CGI was shown to induce methylation of these CGI.[53,54] These mechanisms are very interesting since even transient expression of these RNA could lead to permanent inactivation of respective genes, and these CGI-specific mechanisms for methylation induction could be involved in physiological processes to induce tissue-specific methylation patterns. Also, a leukemia protein, PML-RAR fusion protein, has been shown to induce methylation of its target sequence by recruiting DNA methyltransferases.[55] A great deal of research is necessary on how sequence-specific methylation is induced in physiological processes, such as embryonic development.

In *Neurospora*, mutation of histone methyltransferase abolished DNA methylation, showing that histone methylation is indispensable for DNA methylation.[56] In mammalian cells, DNA methylation is known to induce histone modification such as histone deacetylation.[10] At the same time, recruitment of histone H3-Lys9 methyltransferase, SETDB1, along with heterochromatin protein 1 (HP1) to a euchromatic silenced gene-induced DNA methylation.[57] Also, temporal observation of DNA-demethylated cancer cells revealed that histone H3-Lys9 methylation preceded DNA re-methylation.[58] Although no reports have shown that histone modification is indispensable for DNA methylation in mammalian cells, histone methylation at H3-Lys9 seems to promote DNA methylation also in mammalian cells. The relationship among 'seeds of DNA methylation', induction of histone H3-Lys9 methylation, and dense DNA methylation needs to be clarified.

CpG island methylator phenotype (CIMP). Multiple CGI are methylated in some cancers. In 1999, Toyota *et al*. found that a subset of colon cancers had methylation of multiple CGI using 'Methylated in tumors (MINT)' clones they originally isolated.[59] They found a biological meaning in this subset, that is, this subset had a significantly higher incidence of *hMLH1* methylation than the others, and designated this phenotype as CIMP. CIMP was also observed in stomach and pancreatic cancers.[60,61] However,

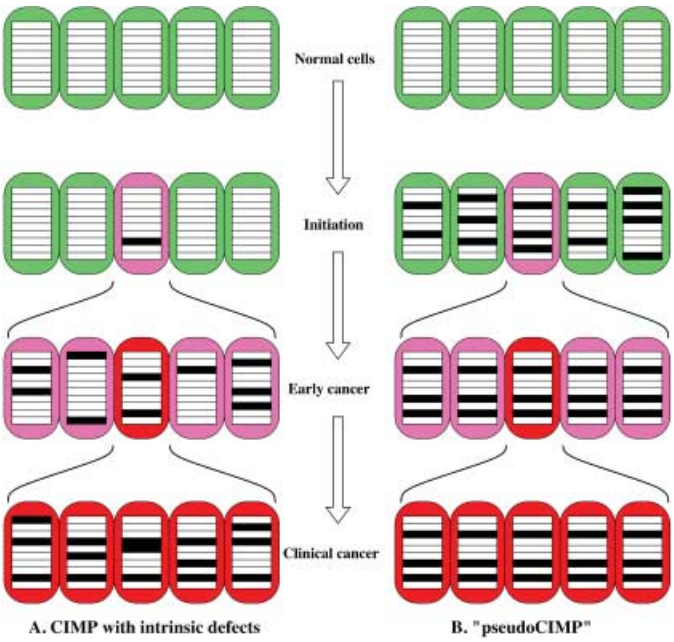

Fig. 2. CGI methylator phenotype (CIMP) and 'pseudoCIMP.' Open rectangles show unmethylated CGI, and closed rectangles show methylated CGI. In cancer cells (shown in pink or red) with intrinsic defects (a), aberrant methylation keeps occurring. After several clonal selections during multistep carcinogenesis, all cancer cells come to have methylation of multiple CGI. In contrast, if a cancer cell is derived from a precursor cell with methylation of multiple CGI (b), cancer cells derived from it will also display methylation of multiple CGI.

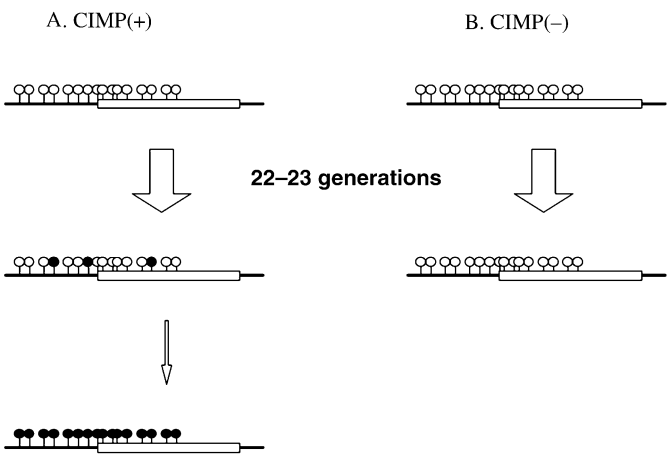

Fig. 3. Decreased fidelity in replicating methylation patterns and induction of dense methylation.[28] Two gastric cancer cell lines positive for CGI methylator phenotype (CIMP) displayed scattered methylation after 22–23 generations (a). This led to appearance of densely methylated DNA molecules, although the frequency was rare. In contrast, two different gastric cancer cell lines without CIMP did not display scattered methylation (b).

after a detailed analysis using 35 non-biased loci, most of which are derived from CGI, in 207 colorectal cancer samples, Yamashita *et al*. concluded that the number of cancers with specific numbers of methylated loci obeyed a normal distribution of random events, and qualitative distinction between CIMP(+) and CIMP(–) is impossible.[62]

We would like to note that several factors should be considered to solve this distortion. First, to honor the connotation of the word 'methylator', the rate of occurrence of methylation events in a defined number of cell divisions should be analyzed (Fig. 2a). If a cancer had originated from a precursor cell with many aberrantly methylated loci and high fidelity in its replicating methylated status, this is still observed as 'CIMP' (Fig. 2b). Second, the loci used are different. Different CGI show different susceptibility to methylation, and, even within a CGI, different regions show different susceptibility.[12,26,63] Third, CGI methylation that can affect cellular growth should be avoided, which was carefully done by Yamashita *et al*.[62]

To address the first point, whether or not some cancer cells have increased rates of occurrence of methylation events, we analyzed occurrence of *de novo* methylation in a defined generation of culture in four gastric cancer cell lines, two of which had multiple CGI methylated and the other two had few.[28] The former two cell lines showed increased rates of *de novo* methylation, which was measured as decreased fidelity in replicating CpG methylation patterns, and the increased rates resulted in the rare appearance of fully methylated molecules (Fig. 3). The latter two cell lines showed limited numbers of *de novo* methylation, and did not show appearance of fully methylated molecules at all. Interestingly, the increase of *de novo* methylation was observed in only limited CGI. This finding clearly demonstrated that at least some gastric cancer cell lines have intrinsic defects that manifest as increased *de novo* methylation that can lead to appearance of densely methylated CGI. The molecular mechanism for the increased rate of *de novo* methylation needs to be clarified.

Another piece of support for the presence of CIMP came from identification of a prognostic marker for neuroblastomas, one of the most common pediatric solid tumors. We made a genome-wide screening for differences in DNA methylation between neuroblastomas with a good prognosis and those with a poor prognosis by MS-RDA, and found that multiple CGI were methylated in the latter.[29] The multiple methylation was a very strong prognostic factor, surpassing the currently most reliable prognostic marker, N-*myc* amplification. This showed that methylation of multiple CGI, CIMP, was underlined by an important biological mechanism(s). Importantly, specific CGI were useful to sensitively detect CIMP in neuroblastomas; and MINT clones, which are good markers for CIMP in colon cancers, were not methylated in neuroblastomas with CIMP.

In contrast with the findings that support the presence of CIMP, we could not classify breast cancers into CIMP(+) and (–) groups using 13 CGI (Miyamoto *et al*. manuscript in preparation). Many different entities seem to be included in the current concept of 'CIMP', and careful description on how 'CIMP' was analyzed is requisite in future studies.

Chemicals that cause epigenetic alterations: Epimutagens. Some chemicals are reported to induce methylation or demethylation of CpG sites and CGI, in some cases, and are designated as epimutagens (Table 1).[64] Only a limited number of chemicals are known to induce methylation, including nickel, butyrate, and arsenic.[65–67] However, the meaning of these methylation changes, especially whether or not they can induce permanent changes in gene expression, needs to be carefully interpreted. It is possible that exposure to chemicals first induce gene expression changes, and then the altered gene expression induce DNA methylation (or demethylation) at limited number of CpG sites.

In contrast, the action of a group of demethylating agents, 5-aza-2′-deoxycytidine (5-aza-dC) and its derivatives,[24,68] is well documented. These chemicals are incorporated into DNA strands, and will trap DNA methyltransferase 1 (DNMT1). The trapped DNMT1 is degraded, and demethylation of cellular DNA is induced. (–)-Epigallocatechin-3-gallate (EGCG), a polyphenol in green tea, was recently shown to inhibit DNMT1 and induce demethylation of multiple CGI, and demethylation is proposed as one of its cancer-preventive mechanisms.[69] However, for

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Table 1. List of chemicals reported to alter methylation statuses, epimutagens

Chemical	Characteristics	Reference
Hypermethylation		
Butyrate	short-chain fatty acid	(66)
Nickel	metal	(65)
Arsenic	metal	(67)
Phenobarbital	tumor promoting agent	(70)
NNK	tobacco-specific carcinogen	(71)
Vinyl carbamate	urethane-derived carcinogen	(71)
Methylene chloride	occupational carcinogen	(71)
Hypomethylation		
5-aza-C, 5-aza-dC	cytidine analog	(24)
Zebularine	cytidine analog	(68)
Ethionine	methionine analog	(72)
Arsenic	metal	(73, 74)
Procaine	anesthetic agent	(75)
Procainamide	antiarrhythmic agent	(76, 77)
Hydralazine	antihypertensive agent	(77)
Valproic acid	antiepileptic agent	(78)
EGCG	major polyphenol from green tee	(69)
Trichloroacetic acid	corrosive agent	(79)

5-aza-C, 5-azacytidine; 5-aza-dC. 5-aza-2′-deoxycitidine.

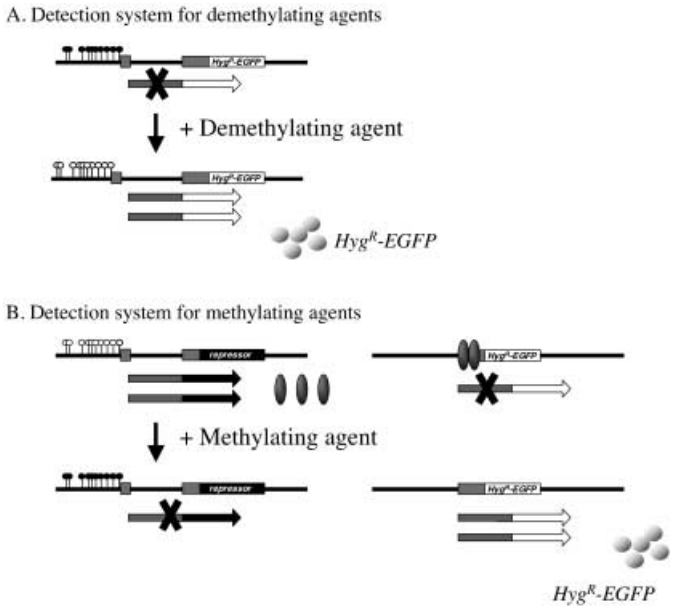

Fig. 4. Strategy to detect demethylating agents (a),[27] and methylating agents (b) using an endogenous promoter CGI. (a) In a human colon cancer cell line HCT116, the *Hyg^R-EGFP* marker gene was introduced into a downstream exon of the silenced *FLJ32130* gene by homologous recombination. As long as the promoter CGI was kept methylated, the recombinant *Hyg^R-EGFP* fusion gene was not expressed. However, by treatment with a known demethylating agent, 5-aza-dC, the promoter CGI was demethylated and the *Hyg^R-EGFP* expression was detected by its mRNA and fluorescence. (b) A repressor for transcription of a marker gene should be introduced into a downstream exon of a promoter CGI that can be readily methylated. The marker gene also must be introduced. As long as the CGI is kept unmethylated, the repressor is transcribed and the marker is not transcribed. However, if methylation is induced in the promoter CGI, the repressor is not expressed, and the marker will be transcribed.

other chemicals with 'demethylating activities', again, their significance needs careful interpretation.

In spite of the potential influence on human health, the number of known epimutagens is very small, possibly because there are no efficient assay systems for them. Taking this into account, we developed a detection system for demethylating agents using an endogenous promoter CGI (Fig. 4a).[27] We first searched for a CGI that is silenced in a cancer cell line, but can drive ample gene expression. Then, we inserted a *Hyg^R-EGFP* marker gene downstream of the CGI by homologous recombination. When the CGI was demethylated by 5-aza-dC, expression of the *Hyg^R-EGFP* marker gene was detected. To detect methylating agents (Fig. 4b), we need a promoter CGI that can be readily methylated, and a repression system of gene expression. The construction is also under way.

Future directions. No single mechanism will be able to explain how methylation of CGI is induced. It is mostly unknown which proteins regulate the frequency of *de novo* methylation and where it takes place. A particularly interesting issue is how 'seeds of methylation' lead to dense methylation of a CGI. A mechanism that unifies the methylation status of multiple CpG sites within a CGI is likely to exist.

Distinction between aberrant methylation and physiological methylation is very difficult, considering the presence of age-related methylation. In addition to the essentially confusing nature of methylation, inadequate description or analysis of methylation makes the situation worse. Does a study analyze the methylation status collectively of CGI or of a CpG site? Is the CGI located in the promoter region or not? Is methylation of the analyzed region critical for transcription repression? Precise analysis will open up new paths to cancer prevention, diagnostics, and treatment.

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