"mutational fingerprint" [54, 55]. The presence of methylation fingerprints was first suggested by analysis of cancer tissues, and was recently shown by analysis of non-cancerous tissues as well [56].

14.4.1 Target Gene Specificity of DNA Methylation in Cancers

The presence of target gene specificity in DNA methylation induction was initially indicated by the presence of methylation of specific genes in cancer cells [57–59]. A pioneering study of 1,184 CGIs using restriction landmark genomic scanning, an early-stage genome-wide scanning technique for differences in DNA methylation, revealed that certain CGIs were more frequently methylated in specific tumor types [57]. Analyses of promoter CGIs of mostly tumor-suppressor genes suggested that some CGIs were methylated at high incidences in specific tumor types [58]. Methylated DNA immunoprecipitation (MeDIP)-microarray analysis of colon cancer tissue revealed that most methylated genes were located within defined genomic clusters [59]. Comparison of 77 HCCs associated with either HBV or HCV revealed that some genes are preferentially methylated in HCCs, depending on the specific hepatitis virus [6].

14.4.2 Strategy to Analyze Methylation Fingerprints in Non-cancerous Tissues

Analysis of methylation in non-cancerous tissues should be conducted using a method with high sensitivity, such as methylation-specific PCR (MSP), because aberrant methylation of a gene is expected to be present only in a minor fraction of cells in a polyclonal tissue. Since methods with high sensitivity can analyze only selected genes, this requires pre-specification of genes to be studied. By a genome-wide methylation analysis of cancer tissues, we can obtain candidate genes methylated also in non-cancerous tissues. Using these pre-specified genes, we can perform a high-sensitivity methylation analysis in non-cancerous tissues [9, 60]. Recent methylome analyses yielded many promoter CGIs methylated in various cancers, including gastric, colon, lung, and breast cancers [26, 61–63]. High-sensitivity methylation analysis of these CGIs in non-cancerous tissues of patients with documented exposure to specific agents will lead to establishment of methylation fingerprints of the agents.

14.4.3 Methylation Fingerprint in Gastric Mucosae Exposed to *H. pylori* Infection

To clarify the presence of target gene specificity in methylation induction by *H. pylori*, methylation analysis of a set of candidate CGIs in gastric mucosae was

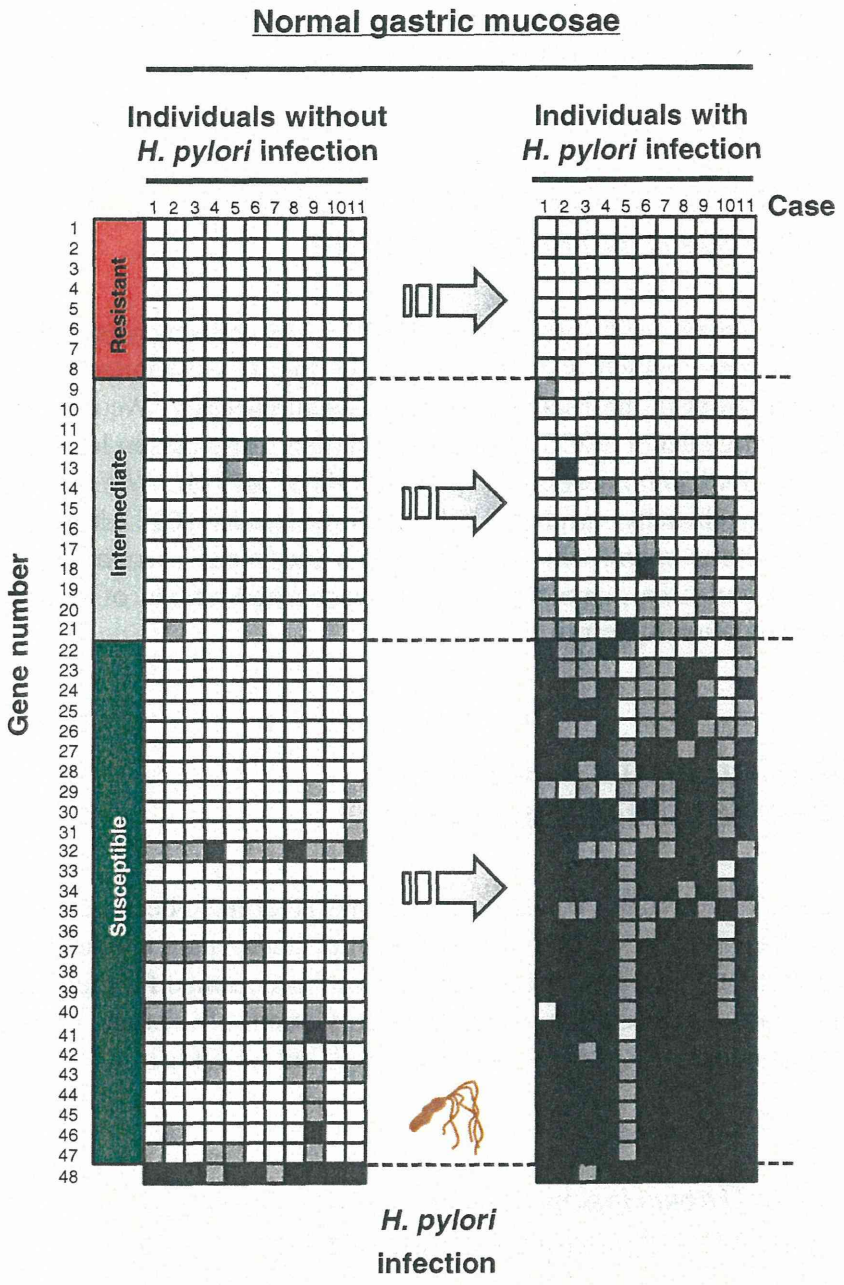

Fig. 14.3 The presence of a methylation fingerprint of *H. pylori* infection. Methylation of promoter CGIs of 48 genes was analyzed in gastric mucosae of individuals without and with *H. pylori* infection using a high-sensitivity method, MSP. Although genes 1–8 were not methylated at all, genes 22–47 were consistently methylated in individuals with *H. pylori* infection (Modified from Nakajima et al. [9])

conducted by searching for promoter CGIs methylated in gastric cancers [26]. MSP of 48 promoter CGIs in gastric mucosae with and without *H. pylori* infection suggested that specific genes were consistently methylated in gastric mucosae with *H. pylori* infection while others were not, forming a methylation fingerprint (Fig. 14.3) [9]. The susceptible genes had lower transcription levels in normal

gastric mucosae than had the resistant genes. Up-regulation of DNMTs was not observed in gastric mucosae with *H. pylori* infection.

14.4.4 Methylation Fingerprint in Esophageal Mucosae of Long-Term Smokers

Esophageal squamous cell carcinomas (ESCCs) are cancer types infamous for repeated occurrence, which is considered to be due to accumulation of genetic/epigenetic alterations in the background esophageal mucosae. We isolated 13 promoter CGIs methylated in ESCCs, and analyzed their methylation levels in esophageal mucosae. Methylation levels of 5 genes (*HOXA9*, *MT1M*, *NEFH*, *RSPO4*, and, *UCHL1*) were significantly correlated with smoking duration [60]. Although smoking is not an infectious agent, this finding in non-cancerous esophageal mucosae supports the theory that a specific agent induces methylation of specific genes, leaving a methylation fingerprint.

14.5 Mechanisms for Formation of a Methylation Fingerprint

A methylation fingerprint in non-cancerous tissues is formed because some specific genes are susceptible and others are resistant to methylation induction. Factors involved in such susceptibility and resistance have been studied for the past decade, and include low transcription levels, trimethylation of histone H3 lysine 27 (H3K27me3), and binding of RNA polymerase II (Pol II) (Fig. 14.4).

14.5.1 Low Transcription Levels and DNA Methylation Susceptibility

A low transcription level of a gene was proposed to be involved in methylation induction in the early 2000s [64–67]. Song et al. demonstrated that disruption of promoter activity (thus low transcription levels) of a transfected gene leads to aberrant DNA methylation of promoter CGIs in a cancer cell line [64]. de Smet et al. demonstrated that a gene demethylated by a DNA demethylating agent, 5-aza-2′-deoxycitidine, tends to be re-methylated when it is not transcribed [65]. The majority of genes methylated in cancer tend to have low transcription levels in normal cells [59, 66–68]. Among genes methylated in non-cancerous tissues, genes susceptible to methylation induction had lower transcription levels than resistant genes [9].

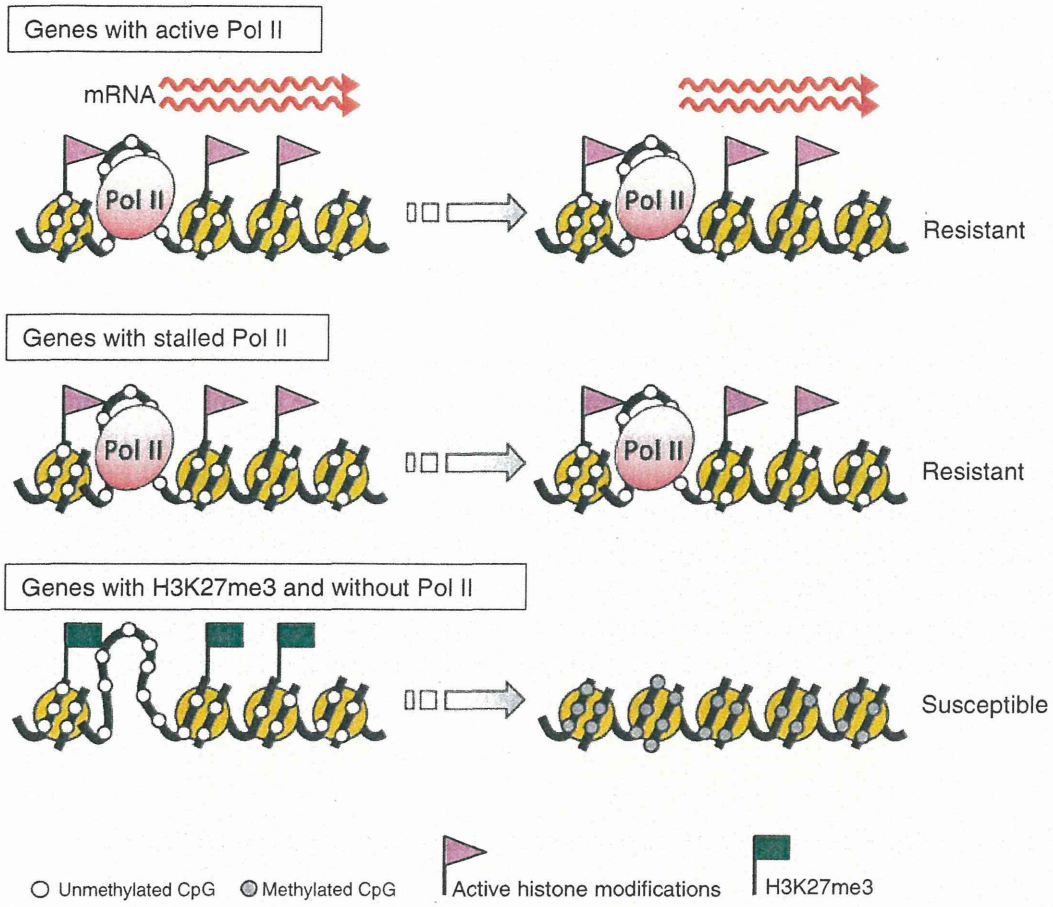

Fig. 14.4 Players involved in induction of DNA methylation in specific genes. Genes with active Pol II, 68% of which have active histone modifications (H3Ac), are resistant to DNA methylation induction. Genes with stalled Pol II, 19% of which also have active histone modifications (H3Ac), are also resistant. On the other hand, genes without Pol II, 90% of which are associated with H3K27me3 modification, are susceptible to methylation induction

14.5.2 Trimethylation of Histone H3 Lysine 27 and DNA Methylation Susceptibility

Histone modifications and DNA methylation depend upon each other. Using a limited number of genes, genes methylated in cancers were shown to be pre-marked by H3K27me3 in embryonic stem cells [69–71] and normal corresponding tissue [71]. This finding was also confirmed by genome-wide analyses [68, 72, 73]. H3K27me3 is recognized by a polycomb repressive complex (PRC) [74–76]. EZH2, a component of PRC2, and CBX7, a component of PRC1, are known to interact with DNA methyltransferases (DNMTs) [77, 78], and H3K27me3 may function as a recruiting signal for DNMTs. Another representative repressive histone modification, trimethylation of histone H3 lysine 9 (H3K9me3), was not associated with DNA methylation susceptibility [68]. Conversely, histone modifications

of active chromatin, such as acetylation of histone H3 (H3Ac) and trimethylation of histone H3 lysine 4 (H3K4me3), are weakly associated with resistance to DNA methylation [68].

14.5.3 Stalled RNA Polymerase II and DNA Methylation Resistance

Even among the genes with low transcription levels, some genes are still resistant to methylation induction. We have demonstrated that binding of Pol II to promoter CGIs (stalled Pol II) is associated with resistance to methylation induction [68]. Multivariate analysis of transcription levels, H3K27me3, H3Ac, and Pol II binding suggested that Pol II binding had a stronger effect on DNA methylation resistance than active histone modifications. Taken together with the fact that transcribed genes are resistant, binding of Pol II, active or stalled, is associated with resistance to methylation induction during carcinogenesis. Molecular mechanisms of how Pol II binding confers resistance to methylation induction remain to be clarified, but protection of CGIs from accession by DNMTs is one possible mechanism.

14.5.4 Role of Genomic Position Relative to Repetitive Elements

In addition, genomic architecture is also involved in gene-specificity of methylation induction. Compared with methylation-resistant genes, methylation-prone genes are located further apart from SINE and LINE retrotransposons [79]. However, since genome architecture does not change according to inducers of aberrant methylation, its role in the formation of a methylation fingerprint of an inducer is limited.

14.5.5 A Model for Formation of a Methylation Fingerprint of an Agent

As a mechanism of how a specific agent induces methylation of specific genes, we speculate that the agent first induces changes in transcription, H3K27me3, and binding of Pol II in its target genes, and that the changes then lead to methylation induction of the genes that acquired a susceptible epigenetic status (Fig. 14.5). Since the first changes in epigenetic status are consistently induced depending upon an agent, DNA methylation is expected to be induced in specific genes, forming a methylation fingerprint. This model is currently being validated using animal models.

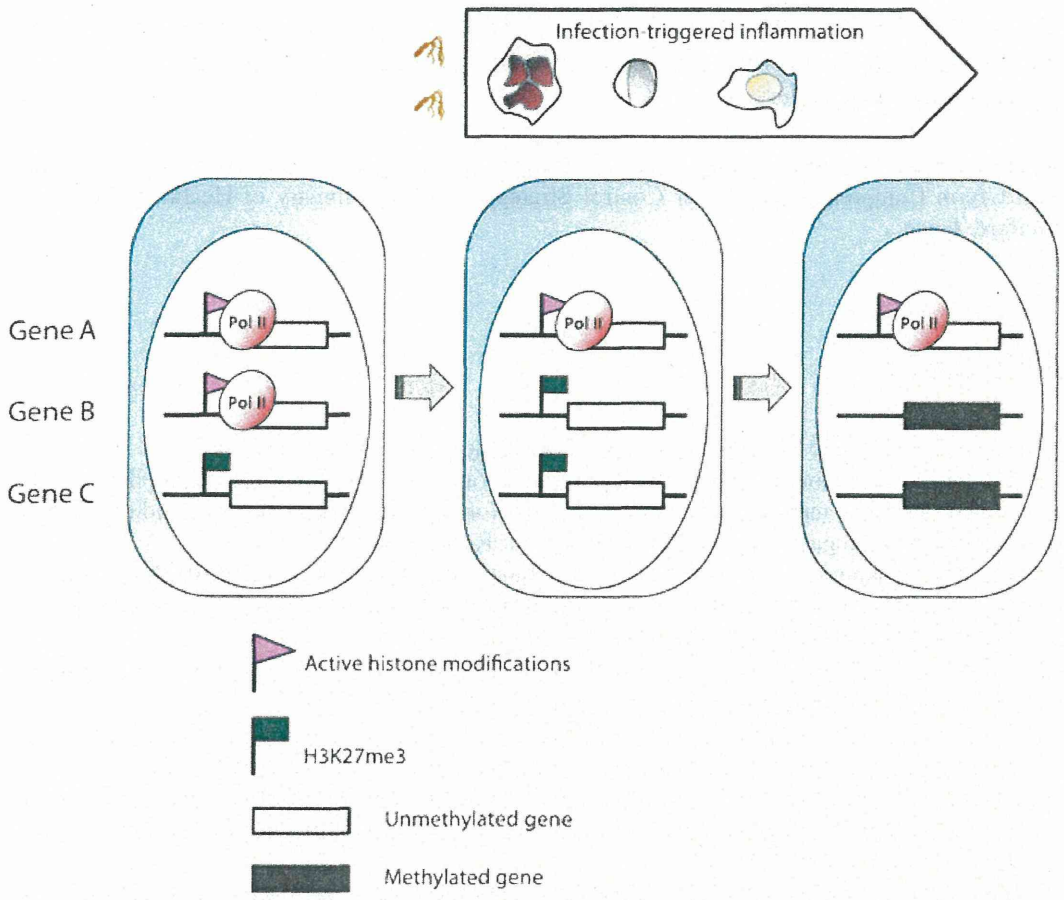

Fig. 14.5 A model for formation of a methylation fingerprint by an infectious agent. An agent is expected to induce changes in epigenetic statuses, including H3K27me3 and binding of Pol II. A gene that acquires a susceptible epigenetic status by exposure to an agent, such as *H. pylori* infection, is expected to become methylated (Gene B). Since the changes in epigenetic statuses are consistently induced by an agent, specific genes are expected to be methylated by the agent. A gene with Pol II (Gene A) and one with H3K27me3 (Gene C) regardless of the exposure to an agent are expected to be resistant and susceptible, respectively, to methylation induction. This model needs to be validated

14.6 Epilogue

Aberrant DNA methylation is induced by various infectious agents, and inflammation is an important element in the induction. Specific genes are methylated by specific inducers, forming methylation fingerprints. The potential application of methylation fingerprints in molecular epidemiology is to identify past exposure to infectious agents. An antibody to an agent usually gradually decreases after elimination of the agent, but a methylation fingerprint is expected to remain forever. So far, the number of known methylation fingerprints of specific agents is limited. However, a strategy to identify the fingerprint of an agent is already established if the agent induces cancer.

Once methylation fingerprints of various infectious agents become available, their use is expected to add new information to the involvement of infectious agents in various human disorders.

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CHAPTER 3

DNA Methylation Alterations in Human Cancers

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CHAPTER OUTLINE

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3.1 INTRODUCTION: BIOLOGICAL ROLES OF DNA METHYLATION

Epigenetic processes, i.e. alterations to biological information without changes in the DNA sequences that are mitotically and/or meiotically heritable, go beyond DNA-stored information and are essential for packaging and interpretation of the genome [1]. The modulation of epigenetic profiles contributes significantly to embryonic development, differentiation, and transition from a stem cell to a lineage-committed cell, and underlies responses to environmental signals such as hormones, nutrients and inflammation [2]. DNA methylation is a key element of epigenetic mechanisms that include histone-modification, positioning of histone variants, nucleosome remodeling, and non-coding RNA. DNA methylation is a covalent

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chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring of CpG dinucleotides. CpG sites are concentrated either in repetitive sequences or CpG islands in promoter regions.

The C-terminal catalytic domain of DNA (cytosine-5-)-methyltransferases (DNMTs) transfers methyl groups from S-adenosyl-L-methionine (AdoMet) to cytosines. Dietary folate, vitamins B6 and B12, methionine and choline can critically affect the synthesis of AdoMet [3]. The C-terminal catalytic domain of DNMTs is composed of five conserved amino acid motifs, namely I, IV, VI, IX and X [4,5]. Motifs I and X are filed together to form the binding site for AdoMet. Motif IV contains the prolylcysteinyl dipeptide that provides the thiolate at the active site. Motif VI contains the glutamyl residue that protonates the 3 position of the target cytosine. Motif IX forms the target recognition domain. The N-terminal regulatory domain of DNMT1 contains a PCNA (proliferating cell nuclear antigen)-binding domain, a cysteine-rich ATRX (alpha thalassemia/mental retardation syndrome X-linked) zinc finger DNA-binding motif, and a polybromo homology domain targeting DNMT1 to the replication foci. The preference of DNMT1 for hemimethylated over unmethylated substrates in vitro and its targeting of replication foci are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand. Thus, DNMT1 has been recognized as the "maintenance" DNMT, whereas DNMT3A and DNMT3B show de novo DNA methylation activity in vitro [6]. However, since de novo methylation of CpG islands has actually been observed in human fibroblasts overexpressing DNMT1, DNMT1 is capable of de novo DNA methylation activity in vivo as well as having a maintenance function [3]. DNA methylation profiles in vivo may be determined on the basis of cooperation between DNMT1 and the DNMT3 family. DNMT3L lacks conserved motifs of the catalytic domain and cooperates with the DNMT3 family to establish an imprinting pattern [7].

DNA methylation plays critical roles in the maintenance of chromatin integrity and regulation of gene expression [8]: (a) repetitive and parasitic sequences, such as retrotransposons and endogenous retroviral elements, are usually repressed due to DNA methylation and (b) methylation of CpGs islands can directly impede the binding of transcription factors to their target sites, thus prohibiting the transcription of specific genes. Moreover, methylation of CpG islands normally promotes a highly condensed heterochromatin structure, where active transcription does not occur. Weber et al. reported that approximately 70% of human genes are linked to promoter CpG islands and about 4% of CpG island promoters are methylated in somatic cells [9]. Methylation of CpG islands naturally takes place during X chromosome inactivation and imprinting, though the majority of CpG islands remain unmethylated during development and differentiation. Extensive changes in DNA methylation during the processes of differentiation are known to take place at CpG island shores, regions of comparatively low CpG density close to CpG islands [10].

On the other hand, DNA demethylation is a process involving removal of a methyl group from a nucleotide in DNA. Although passive demethylation occurs in the absence of methylation of newly synthesized DNA strands by "maintenance" DNMT during replication rounds, active removal of cytosine methylation has long remained a mystery. Recently, it has been proved that 5-methylcytosine can be converted to 5-hydroxymethylcytosine, an intermediate form potentially involved in active demethylation, by the 2-oxoglutarate and Fe (II)-dependent oxygenases TET1, TET2 and TET3 [11,12]. MLL (myeloid/lymphoid or mixed-lineage leukemia)–TET1 translocations have been found in patients with acute lymphoblastic leukemia [13] and deletions or mutations at the TET2 locus have been reported in myelodysplastic syndrome and acute myeloid leukemia [14], indicating that impairment of the conversion of 5-methylcytosine to 5-hydroxymethylcytosine may also participate in tumorigenesis [15]. Further investigation will be needed to elucidate the significance of conversion to 5-hydroxymethylcytosine.

3.2 DNA METHYLATION ALTERATIONS IN HUMAN CANCERS

Heterozygosity of the *Dnmt1* gene, in conjunction with treatment using the DNMT inhibitor 5-aza-deoxycytidine, reduces the average number of intestinal adenomas in ApcMin mice [16]. On the other hand, genomic hypomethylation in p53+/− mice due to the introduction of a hypomorphic allele of Dnmt 1 induces sarcomas at an earlier age in comparison with littermates possessing normal levels of DNMT1 activity [17,18]. Increased loss of heterozygosity (LOH) accompanied by activation of endogenous retroviral elements has been observed in Dnmt1 hypomorphic mice [19]. These observations of genetically engineered animals clearly demonstrate a causal relationship between alterations of DNA methylation and human cancers.

In fact, human cancer cells obtained from clinical tissue specimens frequently show genome-wide DNA hypomethylation and region-specific DNA hypermethylation [20]. DNA hypomethylation induces a higher probability of translocation of parasitic sequences to other genomic regions, and chromosomal rearrangement resulting in chromosomal instability [21]. Furthermore, aberrant DNA hypomethylation can also induce activation of oncogenes and loss of imprinting. However, a more widely recognized epigenetic change in human cancers is DNA hypermethylation at the CpG islands of promoters that silences specific genes, including tumor-suppressor genes [20] such as CDKN2A (cyclin-dependent kinase inhibitor 2A), *CDKN2B* (cyclin-dependent kinase inhibitor 2B), *TP73* (tumor protein p73), *MLH1* (mutL homolog 1), *Apc* (adenomatosis polyposis coli), *BRCA1* (breast cancer 1), *MGMT* (O-6-methylguanine-DNA methyltransferase), *VHL* (von Hippel-Lindau tumor-suppressor), *GSTP1* (glutathione S-transferase pi 1), *CDH1* (cadherin 1) and *DAPK1* (death-associated protein kinase 1). DNA hypermethylation of tumor-suppressor genes frequently becomes the second hit for driver events in accordance with the two-hit theory [22]. Moreover, some tumor-suppressor genes, such as *TIMP3* (tissue inhibitor of metalloproteinase 3), *SFRP1* (secreted frizzled-related protein 1), *SFRP2*, *SFRP4*, *SFRP5* and *RASSF1* (Ras association (RalGDS/AF-6) domain family member 1), are seldom mutated, or their mutations have never been reported in human cancers [23]. Therefore, intensive screening of genes that are methylated in human cancers may be a strategy for identification of tumor-related genes that have potential as therapeutic targets. In some instances, genes can be silenced simultaneously due to a process of long-range epigenetic silencing, and the spreading of silencing seems to affect neighboring unmethylated genes through repressive chromatin [24].

MiRNAs are the best-known class of short non-coding RNAs, which are typically around 21 nucleotides in length, imperfectly aligned with the 3′UTR of target mRNAs, and induce their translational repression. Observations of silencing due to DNA hypermethylation have expanded to tumor-suppressive microRNAs (25), such as miR-34a and 34b/c, miR-124, miR-137, miR-152, miR-193a, miR-200, miR-203, miR-205, miR-218 and miR-345. In addition to their tumor-suppressor function, miRNAs can also serve as oncogenes to promote cancer growth. B-cell integration cluster (BIC)/miR-155 is the first miRNA shown to have such tumor-promoting activity. miR-10b is another oncogene highly associated with cancer metastasis. Transcription of miR-10b is regulated by the transcription factor Twist, and the downstream targets of miR-10b include homeobox D10. Other miRNAs with oncogene function include miR-17 clusters, miR-21, and miR-373 and miR-520c as metastasis-promoting miRNAs [26].

3.3 ABERRANT DNA METHYLATION IN PRECANCEROUS CONDITIONS ASSOCIATED WITH CHRONIC INFLAMMATION, PERSISTENT VIRAL INFECTION AND SMOKING

DNA methylation alterations are frequently observed even in precancerous conditions and early-stage cancers, suggesting that epigenetic alterations may precede the classical transforming events, such as mutations of tumor-suppressor genes, amplification of oncogenes and

chromosomal instability. Environmental factors influence health, and epigenetic profiles are known to be responses to environmental signals. Thus, aberrant DNA methylation participates especially in precancerous conditions associated with chronic inflammation, persistent viral infection and smoking [27,28]. For example, in the 1990s, although LOH on chromosome 16 was frequently detected by classical Southern blotting in hepatocellular carcinomas (HCCs) associated with metastasis, the molecular events occurring in non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, were unknown. When we examined the DNA methylation status on chromosome 16 using Southern blotting with a DNA methylation-sensitive restriction enzyme, DNA methylation alterations at multiple loci were frequently revealed even in chronic hepatitis or liver cirrhosis, compared with normal liver tissue, indicating that DNA methylation alterations are a very early event during multistage hepatocarcinogenesis [29]. This was one of the earliest reports of DNA methylation alterations at the precancerous stage.

We then examined whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis. Bisulfite modification, which converts unmethylated cytosine residues to uracil, leaving methylated cytosine residues unchanged, was applied to microdissected specimens obtained from lobules, pseudo-lobules or regenerative nodules in non-cancerous liver tissue from patients with HCCs. Although no degree of DNA methylation of any of the examined C-type CpG islands, which are generally methylated in a cancer-specific but not age-dependent manner, was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation of such islands was frequently found even in microdissected specimens of non-cancerous liver tissue showing no remarkable histological changes obtained from patients with HCCs in which LOH was never detected by PCR using multiple microsatellite markers. Thus it was directly confirmed that aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis [30].

DNA hypermethylation around the promoter region of the CDH1 gene at 16q22.1 [31], which encodes a Ca^{2+}-dependent cell–cell adhesion molecule [32], has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis [33]. Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation [33]. Reduction of E-cadherin expression due to DNA hypermethylation around the promoter region may participate even in the very early stage of hepatocarcinogenesis through loss of intercellular adhesiveness and destruction of tissue morphology.

In addition to the chronic hepatitis and liver cirrhosis stages resulting from infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) [30,34,35], DNA methylation alterations are frequently found at the precancerous stage in various organs, especially in association with chronic inflammation and/or persistent infection with viruses. Epstein–Barr virus (EBV) infection in stomach cancers is significantly associated with marked accumulation of DNA hypermethylation of C-type CpG islands [36,37], and viral latent membrane protein 2A up-regulates DNMT1 in cultured cancer cells [38]. *Helicobacter pylori* infection, another etiologic factor that is believed to be involved in stomach carcinogenesis, has also been reported to strongly promote regional DNA hypermethylation [39]. Cervical intraepithelial neoplasia (CIN) is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with human papillomavirus (HPV) infection. DNMT1 protein expression is increased even in low-grade CINs relative to normal squamous epithelia, and is further increased in higher-grade CINs and squamous cell carcinomas of the uterine cervix [40]. HPV-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the methyltransferase activity of DNMT1 in vitro [41].

DNA hypermethylation at the HIC1 (hypermethylated in cancer 1) locus has been observed in non-cancerous lung tissues, which may contain progenitor cells for cancers, obtained from

patients with non-small-cell lung cancers, and in the corresponding non-small-cell lung cancers [42]. HIC1 is a growth-regulatory and tumor-repressor gene [43] that was first identified in the commonly methylated chromosomal region in human cancer cells [44]. The incidence of DNA hypermethylation at this locus was significantly associated with poorer differentiation of lung adenocarcinomas. The incidence of DNA hypermethylation in samples of both non-cancerous lung tissue and non-small-cell lung cancer from patients who were current smokers was significantly higher than in patients who had never smoked [42]. The incidence of DNA hypermethylation in non-cancerous lung tissue from patients with non-small-cell lung cancers was significantly correlated with the extent of pulmonary anthracosis, as an index of the cumulative effects of smoking [27]. Cigarette smoking seems to be another background factor associated with alterations of DNA methylation during multistage carcinogenesis.

3.4 ABNORMAL EXPRESSION OF DNMTS IN HUMAN CANCERS

At least a proportion of DNA methylation alterations in human cancers may be attributable to abnormalities of DNMTs. In fact, altered expression of DNMTs has been reported in human cancers. For example, the levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs [45,46]. The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein tumor involvement. Moreover, DNMT1 overexpression in tumors is inversely correlated with the recurrence-free and overall survival rates of patients with HCCs [47].

Ductal adenocarcinomas of the pancreas frequently develop after chronic damage due to pancreatitis. At least a proportion of peripheral pancreatic ductal epithelia with an inflammatory background may be at the precancerous stage. The incidence of DNMT1 protein expression increases with progression from peripheral pancreatic ductal epithelia with an inflammatory background, to another precancerous lesion, pancreatic intraductal neoplasia (PanIN), to well-differentiated ductal adenocarcinoma, and finally to poorly differentiated ductal adenocarcinoma, in comparison with normal peripheral pancreatic duct epithelia [48]. DNMT1 overexpression in ductal adenocarcinomas of the pancreas is significantly correlated with the extent of invasion to surrounding tissue, an advanced stage, and poorer patient outcome [48]. The average number of methylated CpG islands of examined tumor-suppressor genes in microdissected specimens of peripheral pancreatic ductal epithelia with an inflammatory background, PanIN and ductal adenocarcinoma was significantly correlated with the level of DNMT1 protein expression demonstrated immunohistochemically in precisely microdissected areas [49].

When the human DNMT3A and DNMT3B genes were first cloned, the expression levels of DNMT1, 3A and 3B were reported in ten paired samples of normal and cancerous tissue obtained from various organs. Robertson et al. observed $\geq$2-fold overexpression of DNMT3A in five of ten samples, DNMT1 in six of ten samples, and DNMT3B in eight of ten samples, and DNMT3B clearly showing the largest fold increases among the three enzymes [50]. On the other hand, the cancer phenotype associated with accumulation of DNA methylation on C-type CpG islands is defined as the CpG-island methylator phenotype (CIMP) [51], and such accumulation is generally associated with frequent silencing of tumor-related genes due to DNA hypermethylation only, or a two-hit mechanism involving DNA hypermethylation and LOH in human cancers of various organs [52]. Expression levels of DNMT1 mRNA and protein are significantly correlated with poorer differentiation and CIMP in stomach cancers, but no such association has been observed for the expression of DNMT2, DNMT3A or DNMT3B [53]. EBV infection in stomach cancers is significantly associated with marked accumulation of DNA methylation on C-type CpG islands and overexpression of DNMT1 protein, although

Helicobacter pylori infection, another etiologic factor strongly promoting regional DNA hypermethylation, was not correlated with DNMT1 expression levels.

Urothelial carcinomas (UCs) of the urinary bladder are clinically remarkable because of their multicentricity and tendency to recur due to a "field effect". Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered precancerous, because they may have been exposed to carcinogens in the urine. Our immunohistochemical examinations have clearly revealed that the incidence of nuclear DNMT1 immunoreactivity is already higher in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs, where the PCNA labeling index had not yet increased, compared to that in normal urothelia from patients without UCs, indicating that DNMT1 overexpression was not a secondary result of increased cell proliferative activity, but in fact preceded such activity [54]. The incidence of nuclear DNMT1 immunoreactivity showed a progressive increase in dysplastic urothelia, and during transition to UCs, being significantly correlated with accumulation of DNA methylation on C-type CpG islands [55].

With respect to the mechanisms regulating the expression levels of DNMTs [56], the members of the miR-29 family, including miR-29a, miR-29b and mir-29c, have been shown to directly target DNMT3A and DNMT3B [57]. Enforced expression of miR-29s in lung cancer cell lines restores the normal patterns of DNA methylation, induces re-expression of methylation-silenced tumor-suppressor genes, and inhibits tumorigenicity in vitro and in vivo [57]. Enforced expression of miR-29b in acute myeloid leukemia cells resulted in markedly reduced expression of DNMT1, DNMT3A, and DNMT3B at both the RNA and protein levels [58]. Although down-regulation of DNMT3A and DNMT3B was the result of direct interaction of miR-29b with the 3′UTRs of these genes, miR-29b down-regulates DNMT1 indirectly by targeting Sp1, a transactivator of the DNMT1 gene [58]. miR-148 has been observed to bind to the coding region, outside the usual 3′UTR, of DNMT3B and to induce splicing alteration of DNMT3B in human cancer cells [59]. DNMT1 may also be directly regulated by miR-148 [60] and miR-126 [61]. Down-regulated miR-152 induces aberrant DNA methylation in HCC cells by targeting DNMT1 [62]. In addition to miRNAs, Hu-antigen R (HuR) proteins bind to target mRNAs and modify their levels of expression by altering their stability. HuR proteins target the 3′UTR of DNMT3B in human colon cancer cells, resulting in DNA hypermethylation of its target genes [63].

3.5 MUTATIONS, POLYMORPHISM AND SPLICING ALTERATIONS OF DNMTS AND HUMAN CANCERS

Even though our previous screening indicated that mutations of DNMT1 are not the major event during carcinogenesis in the liver and stomach [64], recent massively parallel DNA sequencing has identified somatic mutations including missense mutations, frameshifts, splice-site mutations and large deletions, which were predicted to affect DNMT3A translation in acute myeloid leukemia cells [65]. The overall survival of patients showing DNMT3A mutations was significantly shorter than that of patients without such mutations. Mutations of the DNMT3A gene, which reduce its enzymatic activity and alter the DNA methylation profiles, have also been reported in acute monocytic leukemia [66]. These observations add to the evidence for participation of aberrant DNMT activity in the pathogenesis of malignancies.

DNMT3A gene polymorphism can affect transcriptional levels of DNMT3A and susceptibility to cancers. The effect of a single nucleotide polymorphism, A/G, in the DNMT3A promoter region on transcriptional activity has been evaluated using a luciferase assay. Carriage of the A allele conferred significantly higher promoter activity in comparison with the G allele, and AA homozygotes had a six-fold increased risk of gastric cancer [67]. Similarly, a marked association between DNMT3B6 promoter C/T polymorphism and overall survival of patients

with head and neck squamous cell carcinoma has been reported [68]: the homozygotes (CC-genotype and TT-genotype) survived significantly longer than the heterozygotes (CT-type). Such polymorphism may affect the gene expression profiles through distinct DNA methylation patterns.

Pericentromeric satellite regions are considered to be one of the specific targets of DNMT3B, since Dnmt3B−/− mice lack DNA methylation in such regions and die in utero [6]. DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. In fact, germline mutations of the DNMT3B gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions [69]. In HCCs [70] and UCs [71], DNA hypomethylation of these regions is correlated with copy number alterations on chromosomes 1 and 9, respectively, where satellite regions are rich. The major splice variant of DNMT3B in normal liver tissue samples is DNMT3B3, which possesses the conserved catalytic domains. DNMT activity of human DNMT3B3 has been confirmed in vitro [72]. On the other hand, DNMT3B4 lacks the conserved catalytic domains, although it retains the N-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3B4 expression. The levels of DNMT3B4 mRNA expression and the ratio of DNMT3B4 mRNA to DNMT3B3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, are significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions [73]. DNA demethylation on satellite 2 has been observed in DNMT3B4-transfected human epithelial 293 cells [73]. Since DNMT3B4 lacking DNMT activity competes with DNMT3B3 for targeting to pericentromeric satellite regions, DNMT3B4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

As another molecular mechanism involved in site-specific DNA methylation alterations, interaction between DNMT3A and c-myc has been reported. This interaction promotes the site-specific methylation of CpG dinucleotides localized in c-myc boxes in the promoter regions of the CDKN2a, CCND1 and TIMP2 genes [74]. The invalidation of c-myc reveals that c-myc allows recruitment of DNMT 3A on the c-myc box of c-myc-regulated genes. Monitoring transcription factor arrays have identified transcription factors interacting with DNMT3A and DNMT3B (such as CREB and FOS), those interacting with DNMT 3A (such as AP2alpha and p53) and those interacting with DNMT 3B (such as SP1 and SP4) [74]. Thus, direct interaction between DNMT 3A and/or DNMT 3B and transcription factors provides a rational molecular explanation for the mechanism of targeted DNA methylation.

3.6 SIGNAL PATHWAYS AFFECTING DNA METHYLATION STATUS DURING TUMORIGENESIS

Molecular links between the major signaling pathways involved in tumorigenesis and epigenetic events have been reported [75]. For example, correlations between the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and epigenetic events in tumorigenesis and progression have been attracting attention. It has been reported that PTEN methylation becomes progressively higher from benign thyroid adenoma to follicular thyroid cancer and to aggressive anaplastic thyroid cancer, which harbors activating genetic alterations in the PI3K/AKT pathway that correspond to a progressively higher prevalence [76]. An association of PTEN methylation with PIK3CA alterations and ras mutations has been reported in thyroid tumors [76]. Aberrant methylation and hence silencing of the PTEN gene, which coexists with activating genetic alterations of the PI3K/AKT pathway, may enhance the signaling of this pathway and contribute to tumor progression.

With regard to BRAF-MEK signaling, BRAF is highly expressed in neurons. Expression of MAP2, a neuron-specific microtubule-associated protein that binds and stabilizes dendritic microtubules, is expressed in cutaneous primary melanomas and inversely associated with melanoma progression. Ectopic expression of MAP2 in metastatic melanoma cells inhibits cell growth by inducing mitotic spindle defects and apoptosis [77]. Levels of MAP2 promoter activity in melanoma cell lines are correlated with activating mutation in BRAF: hyperactivation of BRAF-MEK signaling activates MAP2 expression in melanoma cells through promoter demethylation or down-regulation of the neuronal transcription repressor HES1 [77]. Thus, BRAF oncogene levels can regulate the neuronal differentiation and tumor progression of melanoma. Genome-wide DNA methylation analysis after shRNA knockdown of BRAF V600E in thyroid cancer cells has revealed numerous methylation targets including hyper- or hypo-methylated genes with metabolic and cellular functions [78]. Among such genes, the HMGB2 gene plays a role in thyroid cancer cell proliferation, and the FDG1 gene in cell invasion [78]. A prominent epigenetic mechanism through which BRAF V600E can promote tumorigenesis is alteration of the expression of numerous important genes through DNA methylation alterations.

The Ras signaling pathway also regulates DNA methylation status. Forced expression of a cDNA encoding human GAP120 (hGAP), a down-modulator of Ras activity, or delta 9-Jun, a transdominant negative mutant of Jun, in adrenocortical tumor Y1 cells causes transformed cells to revert to their original morphology, resulting in a reduced level of DNA methylation through a reduction of both mRNA expression and the enzymatic activity of DNMTs [79]. Introduction of oncogenic Ha-ras into GAP transfectants has been found to increase the levels of DNA methylation and DNMT activity. Moreover, transient transfection CAT assays have demonstrated that the DNMT promoter in Y1 cells is activated by AP-1 and inhibited by down-regulators of Ras signaling [79]. In addition to Y1 cells, it has been reported that over-expression of unmutated Ha-ras in human T cells causes an increase in DNMT expression, and that DNMT is decreased by inhibitory signaling via the ras-MAPK pathway [80].

The apoptosis-promoting protein Par-4 has been shown to be down-regulated in Ras-transformed NIH 3T3 fibroblasts through the Raf/MEK/ERK MAPK pathway. The par-4 promoter is methylated in Ras-transformed cells through a MEK-dependent pathway, and treatment with a DNMT inhibitor restores the levels of both the Par-4 mRNA transcript and protein, suggesting that the Ras-mediated down-regulation of Par-4 occurs through promoter methylation [81]. In fact, it has been revealed that Ras transformation is associated with up-regulation of DNMT1 and DNMT3 expression [81].

3.7 DNA METHYLATION AND HISTONE MODIFICATIONS

DNA methylation determines chromatin configuration and regulates the expression levels of genes in cooperation with histone modifications [82,83]. Covalent histone modifications mark active promoters (methylation of lysine 4 of histone H3 [H3K4] and acetylation of histone H3 lysine 27 [H3K27]), active enhancers (H3K4 methylation, H3K27 acetylation), actively transcribed genes (H3K36 methylation), or heterochromatin regions (H3K9 methylation, H3K27 methylation) [82,83]. When methyl-CpG-binding proteins, such as MeCP2 and MBD2, bind to methylated CpG dinucleotide, their transcriptional repression domain recruits a co-repressor complex containing histone deacetylases (HDACs) [84]. On the other hand, histone methyltransferases, such as G9A and SUV39H1, are required to recruit DNMTs [85].

Transcriptionally repressive chromatin modifications within the promoters of tumor-suppressor genes silenced by DNA methylation are known to resemble the chromatin modifications of these genes in normal embryonic stem cells, e.g. polycomb (PcG) complex binding and H3K27 methylation. These genes also have an active marker, H3K4 methylation, in normal stem cells, and this bivalent state is converted to a primary active or repressive

chromatin conformation after differentiation cues have been received [86]. During carcinogenesis, such modifications may render the genes vulnerable to errors, resulting in aberrant DNA methylation. These PcG complexes have been shown to directly interact with DNMTs, and possibly to promote cancer-specific gene silencing. EZH2, the PcG proteins in the polycomb repressive complex 2/3 (PRC2/3) that catalyzes the trimethylation of H3K27, may be a key player [87]. Overexpression of EZH2 is correlated with tumor progression and poorer prognosis in various cancers [88,89]. Depletion of EZH2 in cancer cells leads to growth arrest [90]. CBX7, another PcG protein, is a constituent of PRC1, and has also been shown to read the repressive histone marks, H3K9me3 and H3K27me3 [91]. Similarly to EZH2, CBX7 is able to recruit DNA methylation machinery to gene promoters and facilitate gene silencing during the development of cancers.

It has long been known that individual cancers each consist of heterogeneous cell populations. The recently proposed cancer stem cell hypothesis has emphasized that only certain subpopulations, known as cancer stem cells, cancer-initiating cells or tumor-propagating cells, have tumorigenic potential. These cancer-initiating cells are usually resistant to chemotherapy and radiotherapy, leading to treatment failure. Moreover, they may be capable of forming metastatic foci in distant organs. Despite the existence of such subpopulations, the cancer stem cell hypothesis continues to generate controversy. Since the PcG complex targets similar sets of genes in embryonic stem cells and cancer cells, much effort should be focused on how epigenetic mechanisms participate in the generation of cancer-initiating cells [20,23].

3.8 SUBCLASSIFICATION OF HUMAN CANCERS BASED ON DNA METHYLATION PROFILING

Almost all cancers are heterogeneous diseases composed of distinct clinicopathological subtypes. DNA methylation profiles may, at least partly, represent the molecular basis of each subtype [92,93]. Recently, analysis on a genome-wide scale has become possible using DNA methylation-sensitive restriction enzyme-based or anti-methyl-cytosine antibody affinity techniques that enrich the methylated and unmethylated fractions of genomic DNA [94]. These fractions can then be hybridized to DNA microarrays. Such DNA methylation profiling may provide new insight into disease entities and help to provide more accurate classifications of human cancers [23]. Such subclassification may yield clues for clarification of distinct mechanisms of carcinogenesis in various organs, and identify possible target molecules for prevention and therapy in patients belonging to specific clusters.

For example, progressive accumulation of genetic and epigenetic abnormalities has been best described in colon cancers. Clustering analyses based on either epigenetic (DNA methylation of multiple CpG island promoter regions) profiling or a combination of genetic (mutations of BRAF, KRAS, and p53 and microsatellite instability [MSI]) and epigenetic profiling have revealed distinct molecular signatures. Colon cancers were clustered into CIMP1, CIMP2, and CIMP-negative groups based on DNA methylation data [95]. CIMP1 is characterized by MSI and BRAF mutations and rare KRAS and p53 mutations. CIMP2 is associated with KRAS mutations and rare MSI, BRAF, or p53 mutations. CIMP-negative cases have a high rate of p53 mutation and lower rates of MSI or mutation of BRAF or KRAS. Together, the data show that colon cancers can be grouped into three molecularly distinct disease subclasses [95]. These three groups also differ clinically: CIMP1 and CIMP2 are more often proximal, CIMP1 has a good prognosis because it consists mostly of MSI-high cancers, and CIMP2 has a poor prognosis. Moreover, these groups may have distinct precancerous lesions that can be diagnosed endoscopically, such as serrated adenomas for CIMP1, and villous adenomas for CIMP2.

We focused on renal carcinogenesis and examined the DNA methylation status of C-type CpG islands of multiple tumor-related genes using bisulfite conversion. Even in non-cancerous