When a promoter CGIs of a tumor-suppressor gene is aberrantly methylated, it leads to inactivation of the gene and can be causally involved in cancer development [26,27,28]. Therefore, aberrant methylation of promoter CGIs is considered to be equivalent to point mutations and chromosomal deletions.

Recent genome-wide studies on aberrant DNA methylation in cancers revealed that a large number of genes are methylated in their promoter CGIs in a single cancer cell [29,30,31]. Methylation of some genes, such as *CDKN2A*, *CDH1*, *MLH1*, *RUNX3*, *LOX*, and *MiR-124a*, is considered to be causally involved in cancer development [24,32,33], and these genes are designated as drivers. Methylation of other genes, such as *HAND1*, *FLNc*, and *THBD*, are unlikely to be involved in cancer development considering their low expression in normal gastric mucosae and known functions [12], and these genes are designated as passengers. The distinction between the drivers and passengers is just as in mutations.

10.3.2. Epigenetic Field for Cancerization, or Epigenetic Field Defect

Aberrant methylation of some genes, especially that of passenger genes, is accumulated in a large fraction of epithelial cells of the stomach, reaching up to several tens % [12,13,15]. The accumulation levels of methylation of specific passenger genes correlate with those of methylation of driver genes, and can be quantified more precisely because their methylation levels are high (figure 10-2).

Healthy people without *H. pylori* infection have very low methylation levels in their gastric mucosae, but gastric cancer patients without *H. pylori* infection have 5- to 300-fold higher methylation levels in their non-cancerous gastric mucosae (not in cancer tissues) (figure 10-2) [12].

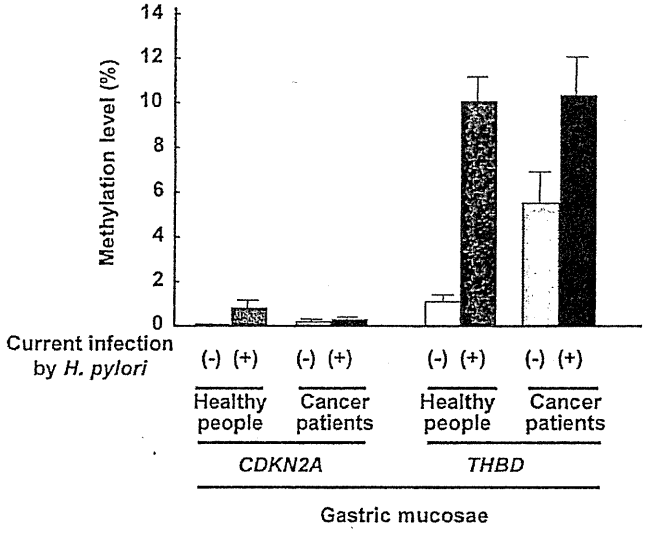

Figure 10.2. DNA methylation levels of *CDKN2A* and *THBD* in gastric mucosae of healthy people and cancer patients with and without *H. pylori* infection. Modified from Maekita et al [12]. Among healthy people, methylation levels were high in individuals with *H. pylori* infection, suggesting that *H. pylori* infection induce aberrant DNA methylation. Among individuals without *H. pylori* infection, gastric cancer patients showed higher methylation levels than healthy people, indicating that accumulation of aberrant DNA methylation is involved in formation of field defect.

Further, among individuals without *H. pylori* infection, patients with multiple gastric cancers have higher methylation levels in their non-cancerous gastric mucosae than those with a single gastric cancer, showing methylation levels in gastric mucosae are correlated with gastric cancer risk [13]. This shows that accumulation of aberrant DNA methylation of various genes in non-cancerous gastric mucosae forms an epigenetic field for cancerization, or epigenetic field defect.

10.3.3. Temporal Profile of Formation of Epigenetic Field Defect

Individuals with *H. pylori* infection have a very high methylation level irrespective of their cancer status and cancer risk. Since the vast majority of gastric cancer patients without current *H. pylori* infection are known to have had this infection in their past [14], the very high methylation level in individuals with *H. pylori* infection is expected to decrease when *H. pylori* infection discontinues. Temporal analysis of methylation levels in individuals who underwent eradication therapy for *H. pylori* confirmed that methylation levels decrease after eradication [34,35]. In other words, a methylation level in gastric mucosae is composed of two components – a temporary component that disappears when *H. pylori* infection discontinues and a permanent component that persists even after *H. pylori* infection discontinues.

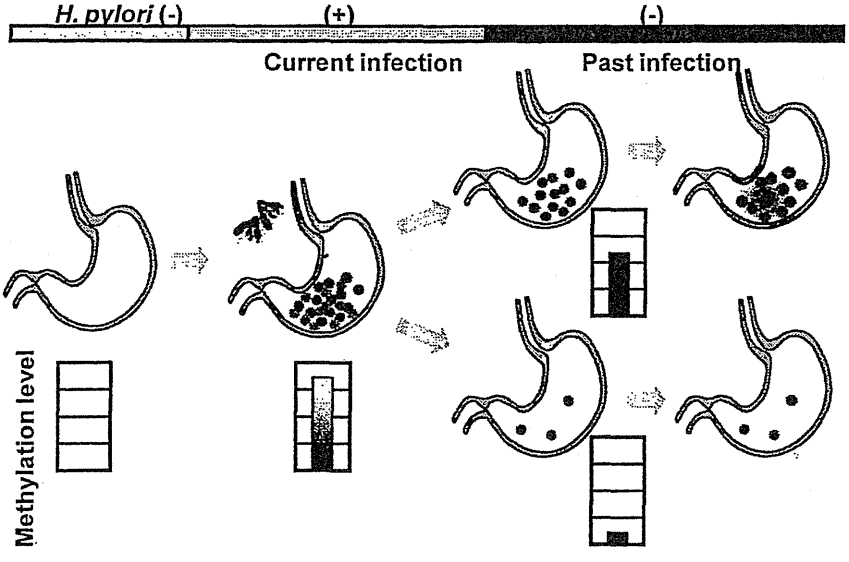

Figure 10.3. Induction of temporary and permanent components of methylation by *H. pylori* infection, and correlation between the permanent component and gastric cancer risk. Almost no methylation is present in gastric mucosae of individuals without any *H. pylori* infection. In gastric mucosae of individuals with *H. pylori* infection, very high levels of aberrant DNA methylation is induced, consisting of temporary and permanent components. When *H. pylori* infection discontinues by eradication or progression of atrophy, the temporary component, which is likely to be methylation in progenitor or differentiated cells, will disappear. In contrast, the permanent component, which is likely to be methylation in stem cells, will remain, and its level correlates with gastric cancer risk.

Based on these findings, a methylation level in gastric mucosae in one's life can be inferred to be very low before *H. pylori* infection takes place, to be very high while *H. pylori* infection is present, and to decrease when *H. pylori* infection discontinues (figure 10-3). If

one's methylation level decreases to a low level, this indicates that his gastric mucosa has limited epigenetic damage in stem cells and has a low risk for gastric cancers. If one's methylation level shows little decrease, this indicates that his gastric mucosa has already accumulated a lot of epigenetic damage in stem cells and has a high risk for gastric cancers.

10.3.4. Mechanisms of Methylation Induction by *H. Pylori* Infection

The observations in humans described above strongly indicate that *H. pylori* infection induces aberrant methylation in gastric mucosae, but lack demonstration of a causal relationship.

Now, the causal role of *H. pylori* infection in methylation induction has been demonstrated by use of Mongolian gerbils. Infection of gerbils with *H. pylori* induced aberrant methylation in gastric mucosae while little methylation was induced in non-infected age-matched gerbils [15].

Mechanisms how *H. pylori* infection induces aberrant DNA methylation were also unclear in humans. *H. pylori* has endogenous methyltransferases and the type IV secretion system that allows its endogenous proteins to infect epithelial cells [36,37], and there was a possibility that bacterial methyltransferase was directly involved in methylation induction.

However, such a direct role was excluded since suppression of inflammation without decreasing number of *H. pylori* in the stomach markedly suppressed methylation induction [15]. It is still unknown what component of inflammation induced by *H. pylori* is responsible for methylation induction.

10.3.5. Gene Specificity in Methylation Induction

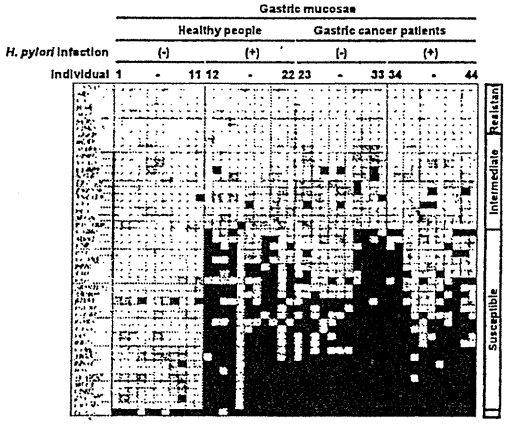

Figure 10.4. The presence of target gene specificity in methylation induction. Modified from Nakajima et al [38]. The presence of aberrant DNA methylation was analyzed by a high-sensitivity method, methylation-specific PCR, in gastric mucosae of healthy people and gastric cancer patients with and without *H. pylori* infection. Filled box, high levels of methylation detected; hatched box, low levels of methylation detected; and open box, no methylation detected. Some genes were easily methylated by *H. pylori* infection, and their methylation persisted in gastric cancer patients. The other genes were resistant to methylation induction.

Both driver and passenger genes are methylated in gastric mucosae, but there is a gene specificity in methylation induction [38]. A panel of genes was analyzed for methylation induction in gastric mucosae of individuals with and without *H. pylori* infection, and it was found that only a fraction of the genes were methylated in individuals with *H. pylori* infection (figure 10-4).

Similar target gene specificity has been found in esophageal mucosae of tobacco smokers [39]. Target genes for methylation induction are known to be determined by low transcription, the presence of a specific histone modification (trimethylation of lysine 27 of histone H3), and the lack of stalled RNA polymerase II [40,41].

10.4. Genetic Changes in Gastric and Other Tissues Associated with Gastric Cancers

Germline mutations and genetic polymorphisms are known to be associated with increased inborn risk of gastric cancers, and thus can be considered to be involved in the formation of "genetic field defect" of gastric cancers. Germline mutations have high penetrance and odds ratios, but are very rare. In contrast, genetic polymorphisms are commonly observed in general populations, but their effects on gastric cancer susceptibility are very weak.

10.4.1. Germline Mutations Associated with Gastric Cancers

Germline mutations of *CDH1* (E-cadherin) were first found in a large family from New Zealand in which diffuse-type gastric cancers took place at an early age [42,43]. *CDH1* germline mutations are very rare, but have been found in other areas in the world [44]. An individual with a *CDH1* germline mutation starts to accumulate a number of small foci of signet ring cells, most of which have methylation of the wild-type allele, and a small fraction of the foci develop into diffuse type cancers [45].

Since the penetrance of *CDH1* germline mutations is very high, prophylactic gastrectomy is a treatment option [42].

In families with hereditary nonpolyposis colorectal cancer (HNPCC), caused by germline mutations of mismatch repair genes, such as *MLH1*, *MSH2*, and *MSH6*, there used to be cases of gastric cancers. The gastric manifestation used to be common in older generations of HNPCC families [46], and is common in Asian populations who have high incidence of gastric cancers[47]. Patients with familial adenomatous polyposis, which is caused by *APC* germline mutations, often present gastric polyps, and also have increased risk for gastric cancers [48].

10.4.2. Genetic Polymorphisms Associated with Gastric Cancer

Genes whose genetic polymorphisms are most widely analyzed are pro-inflammatory cytokine, IL1b, and its receptor antagonist, *IL1RN*. It was initially reported that a single nucleotide polymorphism (SNP) in the *IL1b* promoter was associated with approximately 10-

fold higher risk of gastric atrophy and 2- to 3-fold higher risk of gastric cancers [49]. A SNP in *IL1RN* was also associated with increased gastric cancer risk. Many studies followed this initial study, and a meta-analysis reports that overall gastric cancer risk associated with *IL1b* and *IL1RN* are 1.26 and 1.20 folds, respectively [50].

It is noteworthy that *IL1b* is one of the candidate cytokines involved in methylation induction [15], and that frequent methylation in gastric cancers (CGI methylator phenotype; CIMP) was associated with a SNP in *IL1b* [51]. Taken together, the SNPs in cytokines can be involved in the susceptibility in methylation induction, and thus in gastric cancer susceptibility.

A SNP in the first exon of *PSCA* was identified by a large-scale genome-wide association study, and was associated with 1.62-fold increased risk of diffuse-type gastric cancers [52]. A meta-analysis showed that a SNP in *EGFR* is associated with 1.54-fold increased risk of gastric cancers [53].

Another meta-analysis supported that there is 1.42-fold increased risk for a SNP in folate metabolizing enzyme, *MTHFR* [54]. SNPs in *IL8*, *IL10*, and *TP53* might be risk factors for gastric cancer, but definitive conclusions cannot be made [55,56,57].

10.4.3. Somatic Genetic Changes Associated with Field Defect

Microsatellite instability (MSI) is caused by inactivation of mismatch repair genes, such as *MLH1* and *MSH2*. Especially, *MLH1* is known to be inactivated by its promoter methylation, and one of the important drivers involved in epigenetic field defect. By analysis of cancer tissues, cancers of patients with multiple gastric cancers have been reported to exhibit a higher incidence of MSI than cancers of patients with a single gastric cancer [58]. Since the major mechanism of *MLH1* inactivation is its promoter methylation [24], the presence of MSI in cancer tissues indicates that the patient has high levels of methylation and thus epigenetic field defect. It is difficult to analyze the presence of MSI in non-cancerous gastric mucosae since the fraction of cells with MSI is expected to be very small, and individual gastric glands with MSI are expected to have different types of microsatellite mutation. However, *MLH1* methylation can be detected using a sensitive method, methylation-specific PCR (MSP), and was shown to be present in non-cancerous gastric mucosae of gastric cancer patients with MSI [59].

Activation-induced cytidine deaminase (AID) is a member of the cytidine-deaminase family that acts as a DNA- and RNA-editing enzyme. Infection of gastric epithelial cells with *H. pylori* is known to induce aberrant expression of AID via the IkB kinase-dependent nuclear factor-kB activation pathway. Upregulation of AID is reported to lead to increased *TP53* mutations in gastric epithelial cells *in vitro*, and it seems to contribute to formation of field defect for gastric cancers [60].

Conclusion

Gastric cancer is closely associated with *H. pylori* infection. It induces aberrant methylation of various but specific genes in gastric mucosae mainly through inflammation, and produces an epigenetic field defect (figure 10-5).

Methylation levels of some genes are correlated with gastric cancer risk, and are promising cancer risk markers. *H. pylori* infection can also induce AID expression, which leads to induction of mutations in gastric epithelial cells. Germline mutations of *CDH1*, *MLH1*, and *APC* are involved in gastric cancer susceptibility, but all these are very rare. In contrast, SNPs of some cytokines, such as *IL1b* and *IL1RN*, are common but only weakly associated with gastric cancer risk. These could be involved in the differential responses to *H. pylori* infection and thus in how "efficiently" epigenetic field defect is formed.

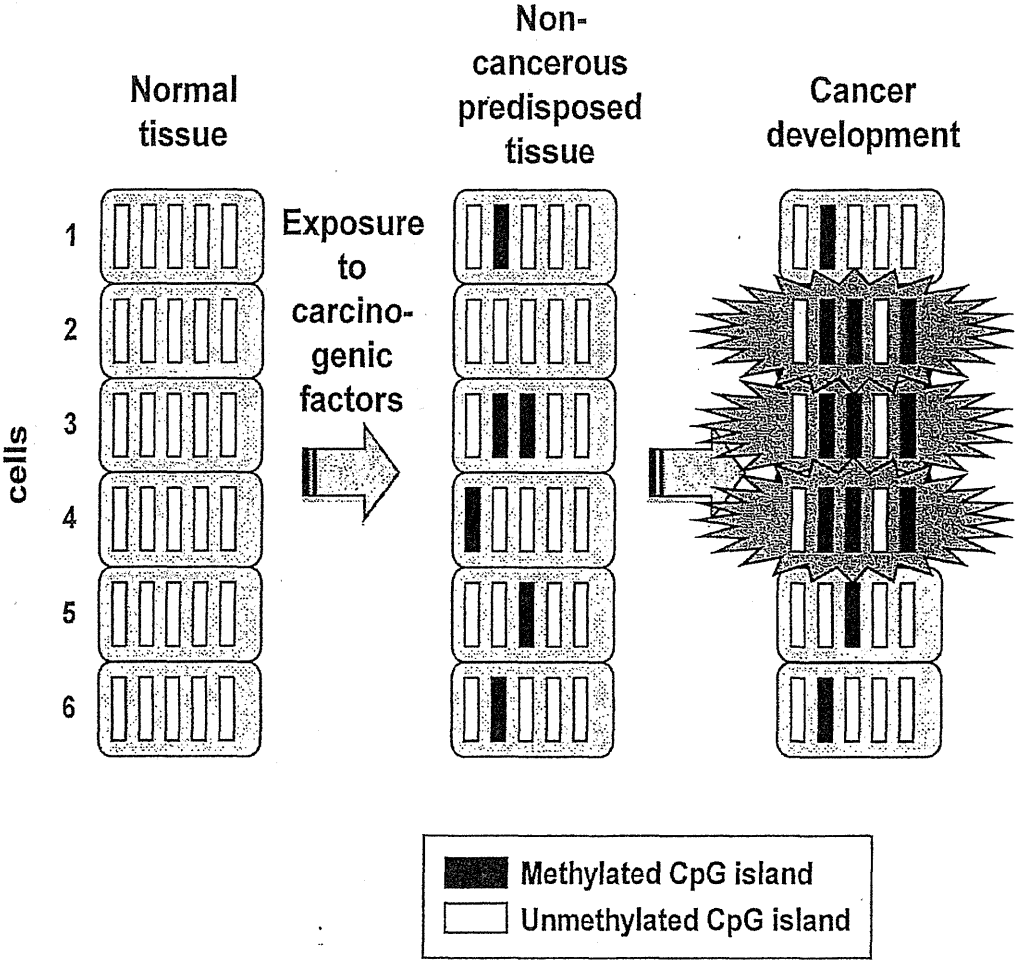

Figure 10.5. Formation of epigenetic field defect and cancer development. If a normal tissue is exposed to an inducer of aberrant DNA methylation, such as *H. pylori* infection, methylation of various but specific genes is induced (non-cancerous predisposed tissue). Both driver and passenger genes (genes A, B, and C) are methylated, but driver genes usually have very low methylation levels. If a predisposed cell harbors an additional hit (e. g. methylation of gene E), a cancer is considered to develop.

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Epigenomic Analysis in Toxicology

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1 INTRODUCTION

The epigenome, the totality of epigenetic modifications in a cell, plays a fundamental role in development, differentiation, and reprogramming (Law and Jacobsen, 2010). Like the genome and unlike transcriptome and proteome in a cell, the epigenome is replicated upon somatic DNA replication (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004; Margueron and Reinberg, 2010). At the same time, unlike the genome, the epigenome undergoes dynamic changes during development, differentiation, and reprogramming (Bird, 2007; Cedar and Bergman, 2009). In other words, the epigenome is established as a consequence of interactions between the genome and environmental input (Gan *et al.*, 2007), and serves as a cellular memory once established.

From a toxicological viewpoint, agents that induce aberrations in the epigenome are of serious concern. Once an aberrant epigenome is established by some factors, the aberrant epigenome is inherited at somatic cell divisions even if the aberrant status is hazardous to the cell or host. It is well established now that aberration of the epigenome can be causally involved in cancer development and progression (Jones and Baylin, 2007), and it is expected that aberration of the epigenome could be involved in a broader range of acquired disorders (Jones *et al.*, 2008; Robertson, 2005). This chapter will introduce what is the epigenome, how it is altered in cancers and other disorders, what induces epigenetic alterations, and essential techniques for epigenome analysis.

2 EPIGENETIC MODIFICATIONS AND EPIGENOME

Epigenetic modifications include DNA methylation and histone modifications. DNA methylation is well known for its high fidelity at somatic cell replication (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004), and thus is considered as the central player in maintenance of long-term cellular memory in mammalian cells. Histone modifications are more diverse, and individual modifications seem to have their own roles and fidelity in somatic cell replication.

2.1 DNA Methylation

DNA methylation in epigenetics refers to physiological methylation at the 5 position of cytosines at some CpG sites (Figure 1a). This methylation is different from pathological DNA methylations at O^6 and $N7$ positions of guanines, which are abnormal adducts produced by alkylating agents and important in the field of toxicology. DNA methylation at CpG sites is characterized by its inheritance upon somatic cell division, and critical roles in regulation of gene transcription.

2.1.1 Maintenance of DNA Methylation Statuses

When a CpG site is methylated, cytosines on both strands are methylated (Figure 1b). At DNA

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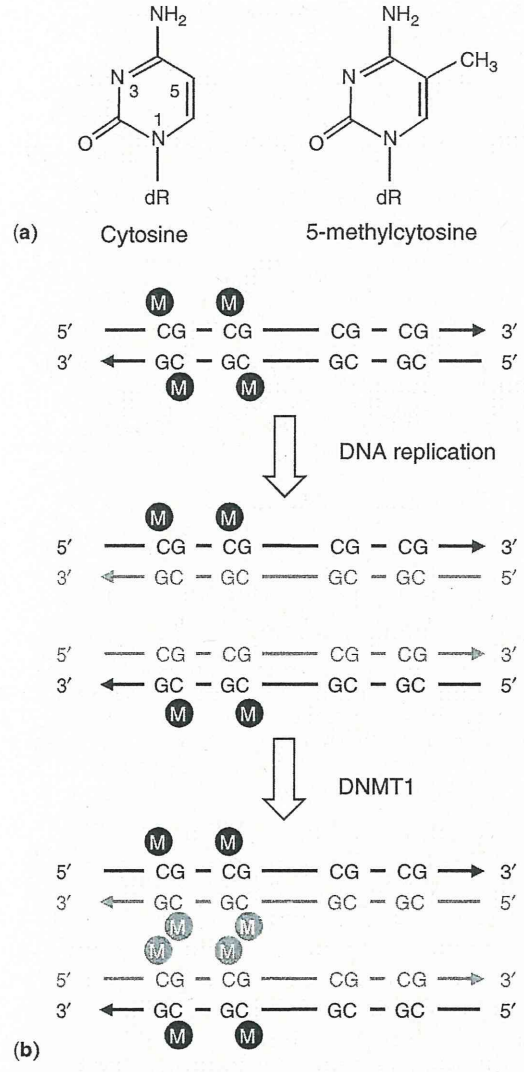

Figure 1. Characteristics of DNA methylation: (a) structure of 5-methylcytosine; (b) maintenance of DNA methylation at somatic cell replication. DNMT1 restores fully methylated statuses by methylating hemi-methylated CpG sites at DNA replication. Methylated or unmethylated statuses are inherited with high fidelity.

replication, cytosines in a newly synthesized DNA strand do not contain methyl groups, and hemi-methylated CpG sites are temporarily formed. However, a maintenance methylase, DNA methyltransferase 1 (DNMT1), associated with a replication fork (Hermann, Goyal and Jeltsch, 2004), restores those hemi-methylated CpG sites into fully methylated CpG sites. DNMT1 has much lower activity on unmethylated CpG sites, and unmethylated CpG sites are kept unmethylated. Therefore, DNA methylation patterns are replicated at somatic DNA replication with a high fidelity (~99.9%), especially in CpG islands (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004).

DNA methyltransferases are essential machineries to establish and maintain DNA methylation. As mentioned above, DNMT1 has the major role in maintaining DNA methylation upon DNA replication, and homozygous knockout of *Dnmt1* is lethal in mid-gestation (Li *et al.*, 1992). In contrast, two *de novo* methylases, DNMT3A and DNMT3B, are involved in establishment of genome-wide DNA methylation patterns (Okano, Xie and Li, 1998; Hermann, Goyal and Jeltsch, 2004). While Dnmt3a cannot methylate nucleosomal DNA, Dnmt3b can (Takeshima *et al.*, 2006). Homozygous knockout of *Dnmt3a* causes lethality after birth (Okano *et al.*, 1999), and Dnmt3a is essential in establishment of genomic imprinting (Kaneda *et al.*, 2004). Homozygous knockout of *Dnmt3b* causes lethality before birth, and germline mutations of *DNMT3B* cause a recessive inherited disorder, ICF syndrome, in humans (Okano *et al.*, 1999).

2.1.2 *Gene Silencing Caused By DNA Methylation of Promoter CpG Islands*

DNA methylation of a CpG island in a gene promoter region has been known to be consistently associated with transcriptional repression of its downstream gene (Baylin and Ohm, 2006; Ushijima, 2005). This was further supported by recent genome-wide analyses of DNA methylation and gene expression (Weber *et al.*, 2007; Rauch *et al.*, 2009; Yamashita *et al.*, 2009). As a mechanism how DNA methylation of a CpG island in a promoter region causes silencing of its downstream gene, the role of nucleosome formation, as discussed below, is currently believed to be important (Li *et al.*, 2007), in addition to induction of inactive histone modifications and inhibition of binding of methylation-sensitive transcription factors.

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2.1.3 *Gene Body Methylation and Increased Transcription*

Recent genome-wide analyses also showed that methylation of CpG islands in gene bodies is often associated with increased gene transcription (Hellman and Chess, 2007; Rauch *et al.*, 2009; Yamashita *et al.*, 2009). Since the association is much weaker than that between methylation of promoter CpG islands and gene repression, the association observed in gene bodies is considered to have no direct cause-consequence relationship.

2.2 Nucleosomes and Histone Modifications

DNA in the nucleus is not naked, and forms nucleosome structures along with core histones. The critical importance of histone modifications and nucleosomes in transcriptional regulation is now recognized.

2.2.1 *Nucleosome and Nucleosome-Free Region in Promoters*

A core nucleosome is made of 146 ± 2 base-pair stretches of DNA around the histone octamer for 1.65 turns in a left-handed superhelix (Figure 2) (Luger *et al.*, 1997). A linker is made of a short stretch of DNA and linker histone H1, and connects two nucleosomes. Thus one nucleosome contains a core and a linker, and is approximately 200 base-pairs long. The histone octamer consists of two of each of four core histone proteins, H2A, H2B, H3, and H4. It is known that DNA in nucleosomes is resistant to micrococcal nuclease and *Sss*I methylase activity, which is experimentally important.

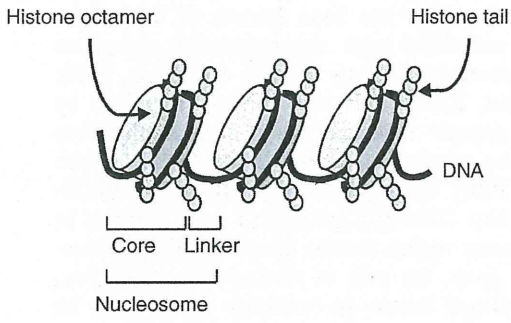

Figure 2. Structure of nucleosome. DNA wraps around the histone octamer, forming a core nucleosome. Core nucleosomes are connected by a linker, and the core and linker forms a nucleosome. Histone tails protrude from the histone octamer.

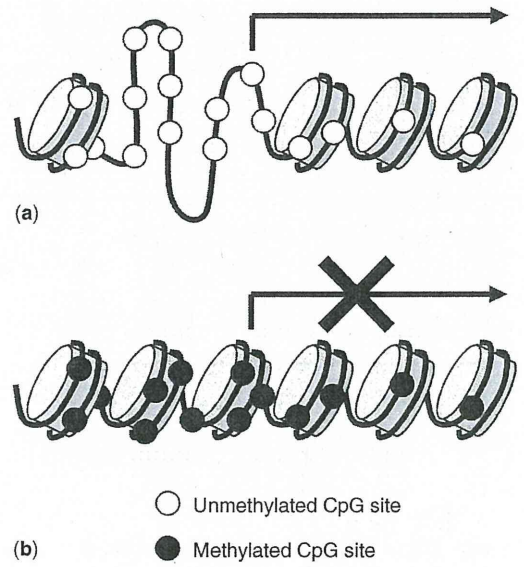

Figure 3. Nucleosome-free region (NFR) and its role in transcription: (a) an approximately 200 bp region upstream of a transcription start site (TSS) lack a nucleosome, and is designated as a NFR. RNA polymerase II and other transcription factors are considered to bind to the NFR; (b) if a NFR in a promoter CpG island is methylated, a nucleosome is formed, and transcription from the NFR is markedly impaired.

It is now known that an approximately 200 bp region just upstream of a transcription start site (TSS) lacks a nucleosome, forming a nucleosome-free region (NFR) (Figure 3a) (Lee *et al.*, 2004; Li *et al.*, 2007; Ozsolak *et al.*, 2007). When a NFR of a CpG-rich promoter is unmethylated, no nucleosomes are formed there, and transcription can be initiated. In contrast, if a NFR is methylated, a nucleosome is formed in the region, and transcription is markedly impaired (gene silencing by promoter methylation) (Figure 3b) (Lin *et al.*, 2007).

2.2.2 *Histone Modifications and Their Roles in Transcription Regulation*

All core histones are composed of a histone fold domain and a structurally undefined tail region (Zheng and Hayes, 2003). Tail regions of histones

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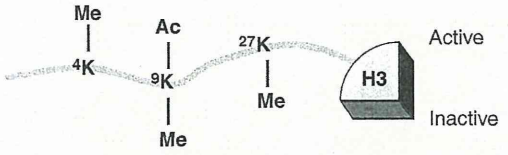

Figure 4. Representative histone modifications. Methylation of H3K4 and acetylation of H3K9 are associated with increased gene transcription, and methylation of H3K9 and H3K27 are associated with gene silencing.

H2A, H2B, H3, and H4 protrude from the histone octamer, and their chemical modifications play important roles in gene regulation (Ruthenburg *et al.*, 2007). Histone acetylation can be observed on the tails of four kinds of histones, and is usually associated with active gene transcription. Histone acetyltransferases (HATs) acetylate histones, and histone deacetylases (HDACs) deacetylate histones (Minucci and Pelicci, 2006). Four classes of HDACs are known, and HDAC1, HDAC2, and HDAC4 are considered to be good targets of HDAC inhibitor drugs.

In addition to histone acetylation, histone methylation at specific lysine and arginine residues is now known to have specific meanings (Figure 4) (Ruthenburg *et al.*, 2007). Especially, methylation of lysine 4, 9, and 27 of histone H3 (H3K4, H3K9, and H3K27, respectively) is associated with active or inactive gene transcription (Barski *et al.*, 2007). At transcription start sites, trimethylation of H3K4 (H3K4me3) is strongly associated with active transcription, and H3K27me3 is associated with silencing of a group of genes (Barski *et al.*, 2007; Kondo *et al.*, 2008). In transcribed regions, H3K4me1, H3K4me2, and H3K4me3 are associated with active transcription, H3K27me2 and H3K27me3 are associated with inactive transcription, and H3K9me2 and H3K9me3 are weakly associated with inactive transcription. The methylation statuses of histones are finely regulated by histone methyltransferases (Kouzarides, 2007; Hublitz, Albert and Peters, 2009) and demethylases (Shi, 2007; Klose and Zhang, 2007).

2.3 Interplay Between DNA Methylation and Histone Modifications

DNA methylation and histone modifications are often dependent upon each other. For example, DNA methylation is recognized by multiple proteins, such as MeCP2 and MBDs, and these proteins recruit histone deacetylases (Richards and Elgin, 2002) and a histone methyltransferase, SUV39H1 (Fujita *et al.*, 2003), which is known to be involved in formation of a heterochromatin structure (Stewart, Li and Wong, 2005). Deacetylated histones are known to be positively charged and to associate tightly with DNA, inhibiting accession of transcription complexes to DNA. On the other hand, H3K9me3 is recognized by heterochromatin protein 1 (HP1), and HP1 recruits DNMT3A and DNMT3B (Fuks, 2005). It helps that inactive histone modification is re-enforced by DNA methylation.

3 EPIGENOME ALTERATIONS IN CANCERS AND OTHER DISORDERS

Epigenomes of normal cells are precisely established and maintained according to developmental stages (Meissner *et al.*, 2008; Rauch *et al.*, 2009). The vast majority of CpG islands are kept unmethylated, and repetitive sequences, which consist of more than 40% of the genome (Lander *et al.*, 2001), are heavily methylated. In cancer cells, an altered epigenome, characterized by "global hypomethylation and regional hypermethylation", is observed (Figure 5).

3.1 Global Hypomethylation

Global hypomethylation, defined as a decrease in 5-methylcytosine content in the genome, is proposed to be present in almost all types of cancer cells (Feinberg and Tycko, 2004). Global hypomethylation is closely associated with hypomethylation of repetitive sequences (Feinberg and Tycko, 2004; Kaneda *et al.*, 2004), but can involve demethylation of normally methylated CpG islands. Demethylation of normally methylated promoter CpG islands leads to aberrant transcription of cancer-testis antigen genes, such as melanoma antigen genes (*MAGE*s) (de Smet *et al.*, 1999), and potentially oncogenes. Also, hypomethylation of a differentially methylated region (DMR) of *IGF2*, known as loss of imprinting, can lead to increased expression and tumor development (Cui *et al.*, 2002). A mouse strain with global hypomethylation demonstrated increased rates of chromosomal loss

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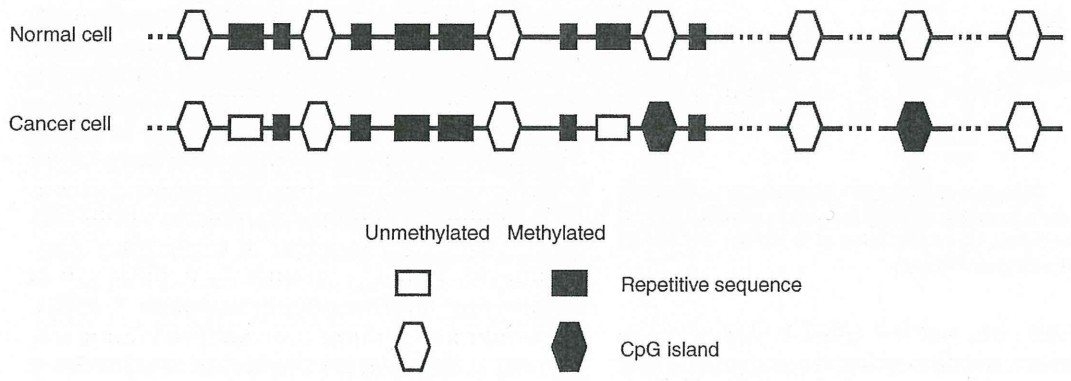

Figure 5. Epigenomic alterations in cancers. Normally methylated repetitive sequences are hypomethylated, and a fraction of normally unmethylated CpG islands are methylated.

(Chen *et al.*, 1998) and increased incidences of lymphomas, colonic microadenomas and liver tumors (Chen *et al.*, 1998; Eden *et al.*, 2003; Yamada *et al.*, 2005). At the same time, global hypomethylation led to suppression of macroscopic tumors of the intestine (Laird *et al.*, 1995; Yamada *et al.*, 2005).

3.2 Regional Hypermethylation – Aberrant Methylation of CpG Islands

"Regional hypermethylation" denotes methylation of CpG islands that are normally unmethylated. If such methylation is induced in the promoter CpG island of a tumor-suppressor gene, the gene is permanently silenced, and the silencing can be causally involved in cancer development and progression (Baylin and Ohm, 2006; Jones and Baylin, 2007). Now, many tumor-suppressor genes involved in various cellular processes, such as cell cycle regulation (*CDKN2A*), WNT signalling (*SFRP* family and *CDH1*), and DNA repair (*MLH1* and *MGMT*), are known to be inactivated by promoter methylation (Baylin and Ohm, 2006). In some cancer types, such as gastric cancers, tumor-suppressor genes are inactivated more frequently by promoter methylation than by mutations (Ushijima and Sasako, 2004). Importantly, CpG islands aberrantly methylated in cancers are not limited to those in promoter regions, and can be present in CpG islands in gene bodies. Methylation of such regions is often associated with increased gene expression (Ushijima, 2005; Rauch *et al.*, 2009; Yamashita *et al.*, 2009).

3.3 Driver Methylation and Passenger Methylation

Now it is known that several hundred to one thousand promoter CpG islands are methylated in cancer cells (Rauch *et al.*, 2009; Yamashita *et al.*, 2009). Most of the genes methylated in cancers have no or only low expression, have H3K27me3 modification, and lack stalled RNA polymerase II in normal counterpart cells (Takeshima and Ushijima, 2010; Takeshima *et al.*, 2009). Therefore, it is considered that most of the promoter CpG islands aberrantly methylated in cancers are not causally involved in carcinogenesis, but methylated in association with it. As mutations are classified as driver and passenger mutations, methylation causally involved in carcinogenesis is designated as "driver methylation", and methylation that simply accompanies the process is designated as "passenger methylation".

3.4 Aberrant Histone Modifications

Histone modifications are also known to be altered in cancers. The global decrease in acetylation of lysine 16 and trimethylation of lysine 20 of histone H4 is known as a hallmark of cancer cells (Fraga *et al.*, 2005a). A global decrease in H3K4me1, H3K9me2, and H3K9me3 and acetylation of histone H3 and H4 are reported in prostate cancer cells (Ellinger *et al.*, 2010; Seligson *et al.*, 2009). A decrease in H3K4me2, H3K9me2, and acetylation of H3K18 is present in pancreatic cancers,

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and is the most significant predictor of overall survival (Manuyakorn *et al.*, 2010). In addition to these alterations, EZH2, a histone methyltransferase involved in H3K27me3, is known to be overexpressed in breast and prostate cancer cells (Kleer *et al.*, 2003; Varambally *et al.*, 2002). In accordance with EZH2 overexpression, H3K27me3 is increased in many genes in prostate cancer cells (Kondo *et al.*, 2008). Since H3K27me3 is involved in gene silencing independently of DNA methylation (Kondo *et al.*, 2008), it is expected that H3K27me3 can be causally involved in gene silencing of tumor-suppressor genes.

3.5 Epigenetic Field for Cancerization

Aberrant DNA methylation is present in non-cancerous tissues of cancer patients, forming an epigenetic field for cancerization (epigenetic field defect) (Figure 6) (Ushijima, 2007). High levels of methylation of specific CpG islands, if appropriately selected, are observed in non-cancerous tissues of cancer patients, but not in the corresponding tissues of age-matched individuals (Maekita *et al.*, 2006). The methylation level is correlated with risk of cancer development (Nakajima *et al.*, 2006), and the accumulation can be considered to be associated with cancer development. In an animal model, it was clearly demonstrated that aberrant DNA methylation was induced as a result of exposure to an environmental factor, and accumulation is associated with cancer development (Niwa *et al.*, 2010). Epigenetic field defects are now attracting attention as a target for cancer risk diagnosis and cancer prevention.

3.6 Comparison Between Point Mutations and Aberrant DNA Methylation

Aberrant DNA methylation of promoter CpG islands, especially in NFRs, is now accepted as an equivalent of inactivating mutations, such as inactivating point mutations and chromosomal losses. However, when compared with point mutations, sharp contrasts have been clarified (Table 1) (Ushijima and Asada, 2010). The number of alterations in a cancer is estimated to be approximately 80 for mutations and several hundred to 1,000 for methylation (Gao *et al.*, 2008; Hayashi *et al.*, 2007; Keshet *et al.*, 2006; Rauch *et al.*, 2008; Wood *et al.*, 2007; Yamashita *et al.*, 2009). The fraction of cells with alterations in non-cancerous (thus polyclonal) tissues is very small for mutations (usually at 1×10^{-5}/cell) and can be large for methylation

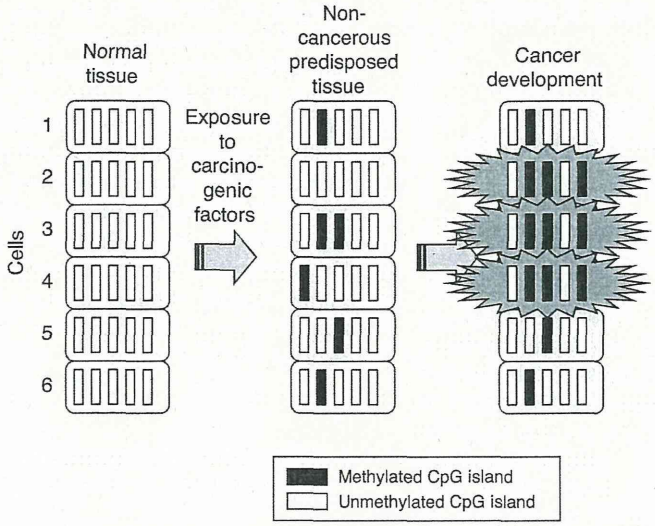

Figure 6. Epigenetic field for cancerization. By exposure to carcinogenic factors, methylation of various, but specific genes, involving both passenger and driver genes, is induced in normal appearing tissues. However, the accumulation level is correlated with cancer risk, and the status is designated as an epigenetic field for cancerization or epigenetic field defect.

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Table 1. Comparison between aberrant DNA methylation and point mutations.

	Point mutation	DNA methylation	References
Number of alterations per cancer cell	~80	Several hundred to 1,000	(Gao *et al.*, 2008; Hayashi *et al.*, 2007; Keshet *et al.*, 2006; Rauch *et al.*, 2008; Wood *et al.*, 2007; Yamashita *et al.*, 2009)
Frequency of alterations of a specific gene in non-cancerous tissues	10^{-5}/cell up to 10^{-3}/cell	0.1 to several % up to several 10%	(Maekita *et al.*, 2006; Nagao *et al.*, 2001)
Target gene	Random	Specific	(Costello *et al.*, 2000; Keshet *et al.*, 2006; Loeb, 2001; Wood *et al.*, 2007)
Reversibility	Irreversible	Reversible	(Gan *et al.*, 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al.*, 2008; Wood *et al.*, 2007)

Modified from (Ushijima and Asada, 2010).

(up to several 10% of cells) (Maekita *et al.*, 2006; Nagao *et al.*, 2001). Regarding target genes, mutations are induced mostly in random genes, but methylation is induced in specific genes depending on tissues and inducers (Costello *et al.*, 2000; Keshet *et al.*, 2006; Loeb, 2001; Wood *et al.*, 2007; Nakajima *et al.*, 2009; Oka *et al.*, 2009). Although mutations are essentially irreversible, methylation is potentially reversible, and is now used as a therapeutic target (Gan *et al.*, 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al.*, 2008; Wood *et al.*, 2007). As discussed in Section 4, inducers of aberrant DNA methylation are markedly different from those of mutations.

3.7 Possible Involvement of Epigenomic Alterations in Acquired Disorders Other Than Cancers

Epigenomic alterations are known to be responsible for some inborn disorders other than cancers, such as Rett syndrome (inborn mutations of *MeCP2*), ICF syndrome (inborn mutations of *DNMT3B*), and Beckwith-Wiedemann syndrome (imprinting disorder). From toxicological viewpoints, involvement of epigenomic alterations in acquired human disorders other than cancers is of great interest. As described above, aberrant methylation of specific genes can be present in up to several 10% of cells in non-cancerous tissues, different from mutations. Even if one of 10^5 cells in a tissue had lost expression of specific genes by mutations, it does not harm the function of the tissue. However, it is well expected that, if 10% of cells in a tissue had lost expression of specific genes by methylation, it could harm the function of the tissue.

Epigenomic differences become larger as monozygotic twins grow older, and this could explain different disease susceptibility between twins (Fraga *et al.*, 2005b). Monozygotic twins with and without multiple sclerosis had exactly the same genome and transcriptome, but a slightly different epigenome (Baranzini *et al.*, 2010). Glucocorticoid receptor is reported to be aberrantly methylated in the hippocampus of suicide victims (McGowan *et al.*, 2009). Activating epigenetic changes are induced in the nuclear factor kappaB (NF-κB) subunit *p65* gene in aortic endothelial cells after transient high glucose, and the epigenetic changes and altered gene expression persists during subsequent normoglycemia (El-Osta *et al.*, 2008). Involvement of epigenetic alterations in autoimmune disorders and atopic disorders is also proposed (Maciejewska Rodrigues *et al.*, 2009; van Panhuys, Le Gros and McConnell, 2008). The mechanistic basis and evidence in human and animal studies strongly indicate that epigenomic alterations are involved in common acquired human disorders.

4 INDUCERS OF EPIGENETIC ALTERATIONS

Epigenetic alterations play a major role in cancer development, as described, and possibly in other disorders (Robertson, 2005; Ushijima and

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Asada, 2010). Nevertheless, only limited information is available on the factors that induce epigenetic alterations, including aging, inflammation, virus infection, one carbon metabolism, and chemicals (Ushijima and Okochi-Takada, 2005). These inducers are also in a sharp contrast with those of mutations, such as mutagenic chemicals, radiation, and ultraviolet light. Little information is available on how epigenetic alterations are induced.

4.1 Interpretation of Changes in Epigenetic Modifications

Epigenetic modifications can regulate gene transcription, but can be regulated by it at the same time. Therefore, we have to be cautious in interpreting the meaning of changes of epigenetic modifications. An agent may target epigenetic modifications first, and the epigenetic changes can then lead to permanent changes in gene expression (left panel in Figure 7). This change of epigenetic modifications corresponds to mutations, and can be designated as epigenetic alterations. Inducers of epigenetic modifications are of great concern from a toxicological viewpoint.

At the same time, an agent may induce gene expression changes first, and then the expression changes can lead to changes in epigenetic modifications, such as histone acetylation statuses (right panel in Figure 7). Such changes in epigenetic modifications might be inherited upon cell division, or might not be inherited. It is often observed that DNA methylation of a CpG island in an exon is induced when expression of the gene is reduced, or that DNA methylation of the CpG island is reduced when its expression is induced (see sections 2.1.2 and 2.1.3).

Even limited to regions within a promoter CpG island, methylation outside a NFR is often observed while the NFR is kept unmethylated (Graff *et al.*, 1997; Issa *et al.*, 2001; Abe *et al.*, 2002). A gene is usually kept to be transcribed even if regions outside the NFRs are methylated (Ushijima, 2005). This shows that methylation outside NFRs is relatively easily induced, but does not cause gene silencing.

4.2 Aging

Issa *et al.* (1994) first reported that a *Not*I site in exon 1 of estrogen receptor (*ESR*) was methylated in normal colon mucosa in association with aging (Issa *et al.*, 1994). The age-dependent methylation

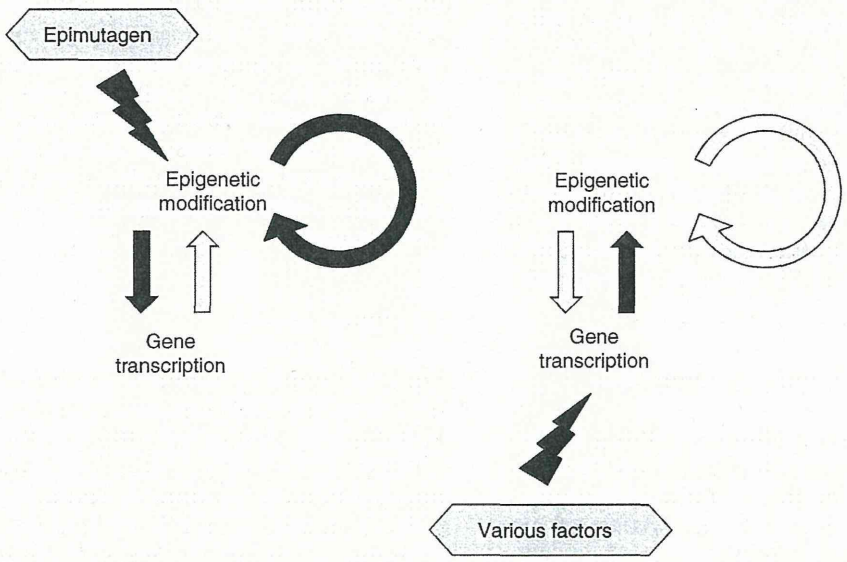

Figure 7. Direct and indirect effects on epigenetic modifications by exogenous factors. *bona fide* epimutagens (defined in Section 4.6) target epigenetic modifications first, and their alterations are inherited and lead to changes in gene transcription. On the other hand, many chemicals induce changes in gene transcription first, and the changes can be accompanied by changes in epigenetic modifications.

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was later confirmed by many investigators using human and animal samples (Abe *et al.*, 2002; Waki *et al.*, 2003). It was later shown that age-dependent methylation takes place in specific CpG islands (type A CpG islands) (Ahuja *et al.*, 1998; Toyota *et al.*, 1999). However, it is often observed that, even within the same CpG island, only peripheral regions are methylated but its central regions, which correspond to NFRs in promoter CpG islands, are kept unmethylated (Graff *et al.*, 1997; Issa *et al.*, 2001; Abe *et al.*, 2002). This suggests that mechanisms for methylation induction are different between NFRs in central regions of CpG islands. As a mechanism of age-dependent methylation, an increase in the cumulative number of cell proliferations is considered to give a higher chance of induction of "aberrant" DNA methylation (Issa *et al.*, 2001; Issa *et al.*, 1994).

4.3 Chronic Inflammation

Among the poorly characterized inducers, the best-characterized inducer is possibly chronic inflammation. Aberrant DNA methylation is known to be present in colonic tissues with long-standing ulcerative colitis (Hsieh *et al.*, 1998; Issa *et al.*, 2001; Toyota *et al.*, 2002), in the liver with chronic hepatitis (Kondo *et al.*, 2000), and in gastric tissues exposed to *Helicobacter pylori* (*H. pylori*) infection (Maekita *et al.*, 2006; Park *et al.*, 2009). In addition to these associations, we recently demonstrated using an animal model that inflammation triggered by *H. pylori* infection, not *H. pylori* itself, is indeed the cause of methylation induction (Niwa *et al.*, 2010). Exact mechanisms of how chronic inflammation induces aberrant DNA methylation are still unknown, but expression levels of *Tnfa*, *Il1b*, *Cxcl2*, and *Nos2* are well correlated with methylation induction.

4.4 Viral Infection and Exogenous DNA

It was noted decades ago that viral DNA is methylated upon infection into mammalian cells (Doerfler *et al.*, 1995). It was shown that cells transgenic for an adenovirus type have methylation of not only the transfected viral DNA but also cellular DNA (Muller, Heller and Doerfler, 2001), and the presence of exogenous DNA was suggested to induce methylation of even endogenous genes. The Epstein-Bar (EB) virus infection is occasionally associated with human gastric cancers, and such cancers are known to have more methylated CGIs than gastric cancers without EB virus infection (Kang *et al.*, 2002; Chang *et al.*, 2006). As a potential mechanism, it was recently reported that DNMT1 is activated by EBV latent membraneprotein 2A (Hino *et al.*, 2009). Liver tissues infected by Hepatitis virus C have methylation of multiple genes (Nishida *et al.*, 2008). An adult T-cell leukemia virus was also shown to induce methylation of endogenous genes (Yasunaga *et al.*, 2004). All these indicate that viral infection and exogenous DNA are inducers of aberrant DNA methylation of endogenous genes.

4.5 Disturbances in One Carbon Metabolism

Disturbances in one carbon (methyl group) metabolism, due to deficiency of folate, vitamin B_{12}, or choline, can influence DNA methylation status by limiting availability of the methyl donor, *S*-adenosylmethionine (Poirier, 2002). In animal experiments, methyl supplementation in maternal diet during pregnancy affected methylation levels of a transposable element of offsprings, and the resultant phenotype persisted for a life time (Waterland and Jirtle, 2003). In human, malnutrition during intrauterine and neonatal periods is known to be associated with the development of obesity, type 2 diabetes, and other related co-morbidities (Kalhan, 2009). This strongly indicates that disturbances of one carbon metabolism can induce changes in DNA methylation, which predispose individuals to disease conditions.

4.6 Chemicals

Some chemicals are considered to induce epigenetic alterations, and are designated as "epimutagens" (Holliday, 1991; MacPhee, 1998; Holliday and Ho, 2002). One of the most well characterized epimutagens is a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), which is widely used in laboratories and has now been approved as a therapeutic drug for myelodysplastic syndrome (Jones, 1985; Issa *et al.*, 2005; Issa and Kantarjian, 2009). 5-Aza-dC is incorporated into DNA strands and

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Table 2. List of chemicals reported to alter epigenetic statuses.

Action	Chemical	Characteristics	Reference
DNA hypermethylation	Butyrate	Short-chain fatty acid	(Boffa, Mariani and Parker, 1994)
	4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	Tobacco-specific carcinogen	(Pulling *et al.*, 2004)
	Phenobarbital	Antiepileptic agent	(Bachman, Phillips and Goodman, 2006)
	Vinclozolin	Antiandrogenic compound	(Anway *et al.*, 2005)
	Diethylstilbestrol	Synthetic estrogen	(Bromer *et al.*, 2009)
DNA hypomethylation	5-Azacytidine, 5-Aza-2′-deoxycytidine	Cytidine analog	(Egger *et al.*, 2004)
	5-Fluoro-2′-deoxycytidine	Cytidine analog	(Jones and Taylor, 1980)
	5,6-Dihydro-2′-azacytidine	Cytidine analog	(Curt *et al.*, 1985)
	Zebularine	Cytidine analog	(Cheng *et al.*, 2003; Holleran *et al.*, 2005)
	Ethionine	Methionine analog	(Shivapurkar, Wilson and Poirier, 1984)
	Arsenic compound	Metal compound	(Zhao *et al.*, 1997;Reichard, Schnekenburger and Puga, 2007)
	Valproic acid	Antiepileptic agent	(Detich, Bovenzi and Szyf, 2003)
	Procainamide	Antiarrhythmic agent	(Lee *et al.*, 2005; Segura-Pacheco *et al.*, 2003)
	Procaine	Anesthetic agent	(Villar-Garea *et al.*, 2003)
	Hydralazine	Antihypertensive agent	(Segura-Pacheco *et al.*, 2003)
	6-Mercaptopurine	Anticancer agent	(Hogarth *et al.*, 2008)
	6-Thioguanine	Anticancer agent	(Hogarth *et al.*, 2008)
	Psammaplins A	Antibiotic agent	(Pina *et al.*, 2003)
	(-)-Epigallocatechin-3-*O*-gallate (EGCG)	Major polyphenol from green tea	(Fang *et al.*, 2003)
	RG108	DNMT inhibitor	(Brueckner *et al.*, 2005)
	SGI-1027	DNMT1 inhibitor	(Datta *et al.*, 2009)
	Bisphenol-A	Synthetic estrogen	(Bromer *et al.*, 2010)
Alterations of histone modifications	Butyrate	short-chain fatty acid	(Stadtman and Barker, 1949)
	Trichostatin A	Microbially derived compound	(Yoshida *et al.*, 1990)
	Valproic acid	Antiepileptic agent	(Kramer *et al.*, 2003)
	Suberoylanilide hydroxamic acid (SAHA)	Hydroxamic acid	(Kelly *et al.*, 2003)
	Depsipeptide	Microbially derived compound	(Furumai *et al.*, 2002)
	Nickel compound	Metal compound	(Chen *et al.*, 2006)
	Chromium compound	Metal compound	(Zhou *et al.*, 2009)
	Arsenic compound	Metal compound	(Zhou *et al.*, 2009)
	Cobalt compound	Metal compound	(Li *et al.*, 2009)
	Cocaine	Crystalline tropane alkaloid	(Maze *et al.* 2010

traps DNMT1, which is subsequently degraded by proteasome (Ghoshal *et al.*, 2005). This leads to depletion of DNMT1 in a cell, and passive DNA demethylation is resultantly induced. There are many other chemicals reported to induce changes in epigenetic modifications (Table 2), but their direct action or indirect action through gene expression changes should be carefully evaluated.

5 EPIGENOMIC ANALYSIS IN TOXICOLOGY

Epigenomic alterations are deeply involved in carcinogenesis and possibly in other disorders. In addition, there are a large number of non-mutagenic carcinogens (Snyder and Green, 2001), some of which exert their carcinogenic action by inducing

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