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Review

DNA methylation as a marker for the past and future

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Aberrant methylation of CpG islands in promoter regions can permanently inactivate tumor-suppressor genes, as mutations and chromosomal abnormalities do. In gastric cancers, *CDKN2A*, *CDH1*, and *MLH1* are inactivated more frequently by aberrant methylation than by mutations, and novel tumor-suppressor genes inactivated by promoter methylation are being identified. We recently found that *Helicobacter pylori* (*HP*), a potent gastric carcinogen, induces aberrant methylation in gastric mucosae. When a panel of CpG islands was examined, some CpG islands were consistently methylated in gastric mucosae of individuals with *HP* infection, while others were resistant. The amount of methylated DNA molecules in the gastric mucosae (methylation level) fluctuated while active *HP* infection was present, but decreased after it was no longer present. Among individuals without active *HP* infection, methylation levels in the gastric mucosae were higher in individuals with gastric cancers than in those without. DNA methylation is emerging as a promising marker for past exposure to carcinogens and future risk of cancers.

Introduction

Epigenetic alterations, like mutations and chromosomal abnormalities, can be causally involved in carcinogenesis.[1] Epigenetic information, which consists of DNA methylation status and histone modification status, is stably inherited over cell generations, and the high fidelity of DNA methylation status has been well characterized.[2,3] It is also known that DNA methylation in promoter CpG islands represses transcription of the downstream genes, in cooperation with an altered histone modification status, such as deacetylation.[1,4] Therefore, epigenetic information is physiologically important for development and tissue differentiation, and an aberrant status of epigenetic information is involved in the development of cancers and possibly other disease conditions.[5]

Aberrant DNA methylation is more frequently present in gastric cancers than mutations,[6] and possibly also in colorectal cancers.[7] We can take advantage of the frequent presence of aberrant methylation in cancers by using it as a clue to identify novel tumor-suppressor genes,[4] as a marker to detect cancer cells,[8,9] and as a therapeutic target.[10] Another advantage of using aberrant DNA methylation as a marker instead of mutations is that it is possible to detect even a single aberrantly methylated DNA molecule embedded in 1000 unmethylated DNA molecules.[8] Studies have detected cancer-specific methylation in stool (colorectal cancers), pancreatic juice (pancreatic cancers), and many other types of samples.[9]

The use of aberrant methylation as a marker is likely to be expanded to the detection of past exposure to carcinogens and future risk of cancer development. In this review, we focus on the identification of tumor-suppressor genes inactivated by promoter methylation and the expanding use of DNA methylation as a marker, enabling us to know the past and the future.

Tumor-suppressor genes inactivated by promoter methylation

The *CDKN2A* (*p16*), *CDH1* (*E-cadherin*), and *MLH1* tumor-suppressor genes can be inactivated by methylation of their promoter CpG islands, as well as by mutations or chromosomal losses. It has been repeatedly reported that all three genes are inactivated more frequently in gastric cancers by promoter methylation than by mutations.[6] Moreover, *RUNX3* is inactivated almost solely by promoter methylation.[11] In colorectal cancers,

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inactivation of *CDKN2A*, *MLH1*, *HIC1*, *SFRP1*, and many other genes by promoter methylation has been reported.[7] Notably, methylation of some tumor-suppressor genes, such as *SFRP1*, whose inactivation enhances Wnt signaling, has been observed in the very early lesions of colon carcinogenesis, aberrant crypt foci, and is considered to provide a milieu that facilitates the selection of cells with further alterations in tumor-related genes.[7] Some researchers believe that aberrant methylation takes place before genetic alterations.

Based upon the important roles of aberrant methylation in gastrointestinal and other cancers, procedures have been developed to perform genomewide screenings for aberrant methylation in cancers.[4] In the late 1990s, the number of promoter CpG islands that were known to be methylated in cancers was limited. Therefore, once a methylated promoter CpG island was identified, its downstream gene was speculated to be important in carcinogenesis. However, it is now known that many CpG islands are methylated in cancers;[4,12] for example, 421 ± 75 genes are inactivated in the AGS gastric cancer cell line, and it is unlikely that all or even most CpG islands methylated in cancers are involved in carcinogenesis. Rather, transcriptional repression is considered to be an important trigger of methylation of a CpG island,[13,14] and "out-of-use" methylation is frequently observed.

Nevertheless, identification of genes inactivated by methylation of promoter CpG islands is a powerful approach to the identification of novel tumor-suppressor genes. To avoid false positives, we must pay careful attention as to whether or not the gene is functionally expressed in normal counterpart cells, or whether the gene can be induced, even if it is not expressed in steady conditions, by cell cycle acceleration or cellular stresses. We then introduce the inactivated gene into cancer cell lines that do not express the gene, and make the gene express at its physiological level. If the expression at the physiological level suppresses the growth of cancer cells, then the gene is a strong candidate for a novel tumor-suppressor gene. As tumor-suppressor genes, we have identified *Lysyl oxidase* (*LOX*) in gastric cancers,[15] and *PRDX2* in melanomas.[16] Genomewide screening procedures have also identified *SOCS1* in hepatocellular carcinomas,[17] *SFRP1* in colorectal cancers,[18] *RELN* in pancreatic cancers,[19] and so on. These genes are expected to serve as novel targets for cancer diagnoses and therapeutics.

Factors inducing methylation of CpG islands

Considering the deep and wide involvement of aberrant DNA methylation of CpG islands in human cancers, identification of the inducing factors can be expected to provide novel targets for cancer prevention. However, for decades only limited information has been available on these factors. Aging was first revealed to be an inducing factor of *ER* methylation in colonic mucosae.[20] Later, *CDKN2A* exon 1 was found to be frequently methylated in the colonic mucosae of patients with ulcerative colitis, and chronic inflammation was suggested to promote methylation.[21] Noncancerous liver tissues of patients with chronic hepatitis or liver cirrhosis harbor cells with methylation of *CDKN2A* exon 1 and some marker loci.[22] This suggests that chronic inflammation can induce methylation of promoter CpG islands in noncancerous tissues.

To analyze the effect of *Helicobacter pylori* (*HP*) infection on the induction of DNA methylation, we collected gastric mucosae from 154 healthy volunteers with or without *HP* infection.[23] The methylation level, the fraction of DNA molecules methylated for a specific gene, was measured by quantitative methylation-specific polymerase chain reaction (quantitative MSP), and was considered to reflect the fraction of cells with methylation of the gene. We selected eight regions of seven genes, *CDKN2A* (promoter and exon 1), *LOX* (promoter), *FLNc* (promoter), *HRASLS* (promoter), *HAND1* (promoter), *THBD* (promoter), and *p41ARC* (exon 8), which were known to be methylated in gastric cancers. The result was very clear; methylation levels in *HP*-positive individuals were 5.4- to 303-fold higher than those in *HP*-negative individuals (Fig. 1, modified from Maekita et al.[23]).

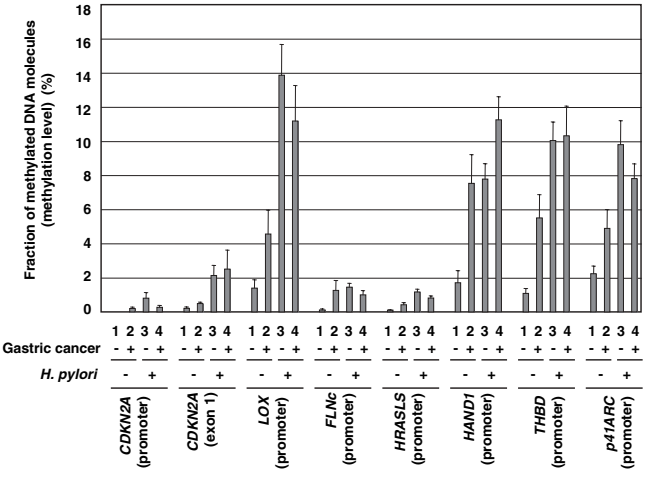

Fig. 1. Methylation levels in the noncancerous gastric mucosae of individuals with and without *Helicobacter pylori* (*HP*) infection and with and without gastric cancer. The fraction of methylated DNA molecules was quantified for eight regions of seven genes using DNA from the antral noncancerous gastric mucosae. Methylation levels increased in individuals with *HP* infection whether or not they had gastric cancer. When methylation levels were compared among individuals without *HP* infection, they were clearly higher in gastric cancer patients than in healthy volunteers. *Error bars*: standard errors

Mechanisms of methylation induction

Ulcerative colitis, hepatitis virus infection, and *HP* infection are now on the list of factors that induce methylation in noncancerous tissues. Therefore, chronic inflammation, or at least some specific chronic inflammation, can induce methylation of CpG islands. One possible mechanism of the induction is increased rates of cell replication. However, the occurrence of methylation of a CpG island is estimated to be very rare under stable cell culture conditions,[3] and it seems difficult to attribute the increased methylation under chronic inflammation only to increased rates of cell replication. It is more likely that some abnormality in the methylation machinery is induced by chronic inflammation. A possible clue is the fact that a proinflammatory allele of interleukin 1β is associated with a risk of gastric cancer development, especially when possible *HP* infection is present,[24,25] suggesting that some cytokine signals can induce abnormality in the methylation machinery.

To examine whether certain promoter CpG islands are preferentially methylated by *HP* infection, we selected promoter CpG islands of 48 genes that can be methylated in gastric cancer cell lines.[12] When methylation of these 48 genes was examined in the gastric mucosae of individuals with or without *HP* infection, some of the genes were consistently methylated in individuals with *HP* infection while others were completely resistant to methylation (Fig. 2) (manuscript in preparation). This result shows that CpG islands of some genes are preferentially methylated by *HP* infection, and that some molecular mechanisms underlie this preferential induction.

Decreased transcription or the absence of transcription is also known to trigger methylation of promoter CpG islands.[13,14] A careful analysis using the *GSTP* gene showed that its promoter CpG island tends to be methylated when its promoter activity is diminished and transcription is impaired.[13] It is likely that transcription levels of various genes are decreased in chronic inflammation, and the decrease, in cooperation with the abnormality in the methylation machinery, seems to promote methylation of promoter CpG islands (Fig. 3). However, neither the decrease in the transcription level nor the abnormality in the methylation machinery is sufficient to induce methylation because most little-transcribed genes remain unmethylated even when *HP* infection is present. Possible factors that determine the targets of aberrant methylation include histone modifications and differential responses among individuals to *HP* infection.[26]

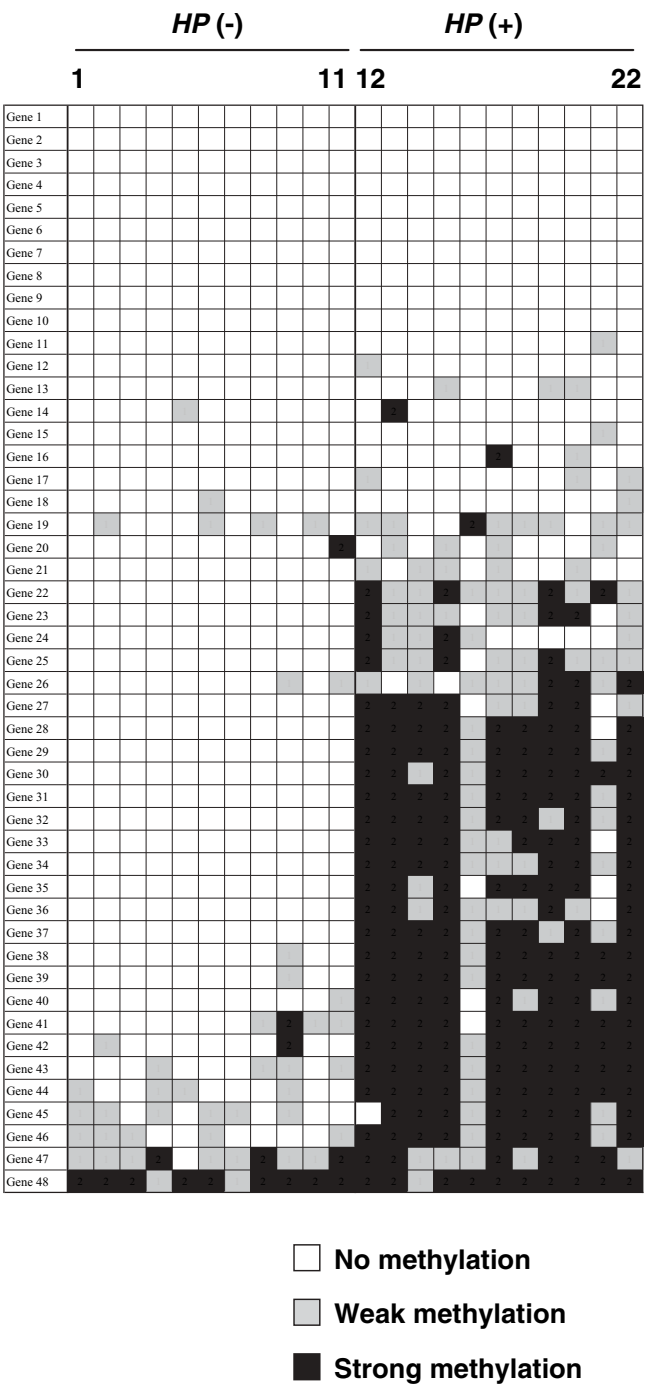

Fig. 2. Preferential methylation of CpG islands of certain genes in *HP*-infected gastric mucosae. Methylation of promoter CpG islands of 48 genes was examined by methylation-specific polymerase chain reaction in 11 healthy volunteers without *HP* infection and in 11 healthy volunteers with *HP* infection. Black, gray, and white boxes show the presence of strong and weak methylation and the absence of methylation, respectively. Genes 1–10 were completely resistant to methylation, while genes 28–37 were specifically methylated in healthy volunteers with *HP* infection

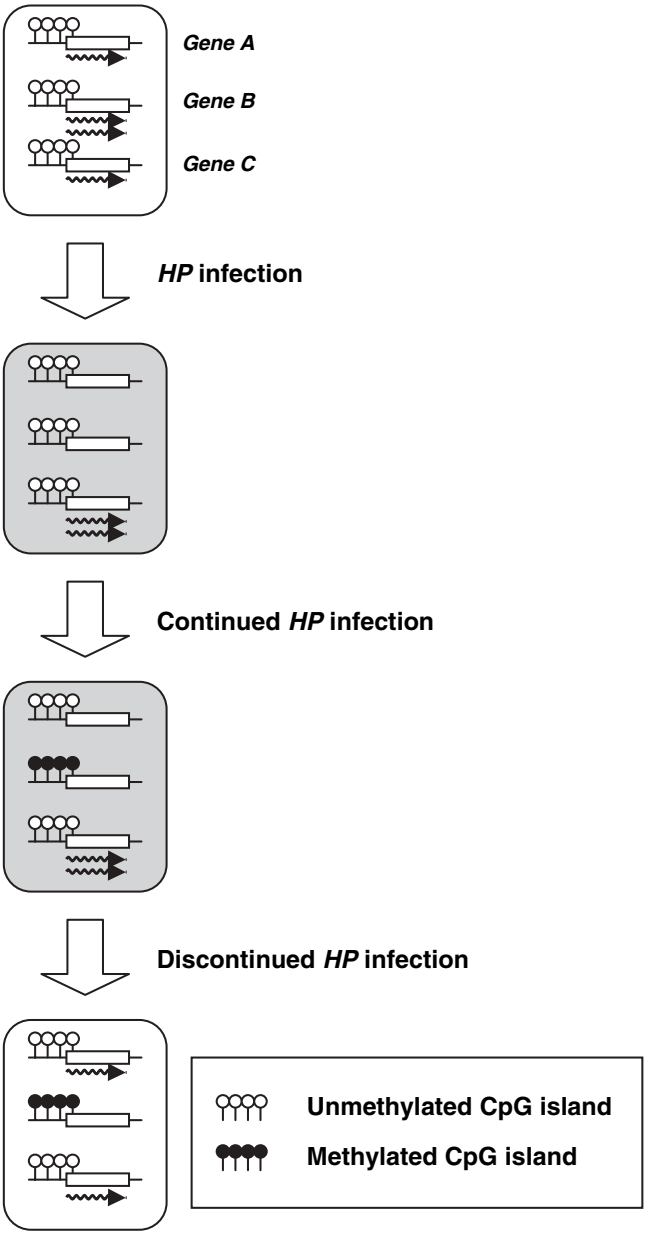

Fig. 3. A model for preferential methylation induction in CpG islands of genes. When *HP* infection takes place, transcription levels (*wavy arrows*) of various genes change. Abnormality might also be induced in the methylation machinery (*shading*). These two factors can each trigger preferential methylation of certain genes, but they are not sufficient

Association of methylation in gastric mucosae with gastric cancer risk

HP infection potently induces aberrant DNA methylation in gastric mucosae. *HP* is a potent gastric carcinogen, and aberrant DNA methylation is deeply involved in gastric cancers. This raises the question as to whether gastric mucosae with aberrant DNA methylation are predisposed to cancers. In other words, do gastric mucosae with aberrant DNA methylation provide a milieu, or field defect, where a cell with further alterations in tumor-suppressor genes can gain a clear growth advantage?

To address this question, we measured methylation levels in the gastric mucosae of healthy volunteers and in the noncancerous gastric mucosae of patients with a differentiated-type gastric cancer.[23] Eight regions of seven genes were analyzed, as in the analysis of methylation induction by *HP* infection. Patients with gastric cancers are known be at higher risk of developing a second gastric cancer,[27] and their noncancerous gastric mucosae can thus be regarded as having a higher risk of developing gastric cancers. Since *HP* infection potently induces methylation, the two groups were further classified according to their current *HP* infection status. Therefore, methylation levels were measured in the noncancerous gastric mucosae of four groups: *HP*-negative healthy volunteers, *HP*-positive healthy volunteers, *HP*-negative patients with gastric cancer, and *HP*-positive patients with gastric cancer. It must be noted that most individuals in the third group did not have *HP* infection at the time of the analysis, but were considered to have had past exposure to *HP*.

It was found that methylation levels were high in individuals with *HP* infection, whether they were healthy volunteers or gastric cancer patients (representative results are shown in Fig. 1). It was also found that, among *HP*-negative individuals, methylation levels were 2.2- to 32-fold higher in the noncancerous gastric mucosae of gastric cancer patients than in the gastric mucosae of healthy individuals. These findings suggest two important concepts. First, some fraction of the increase of methylation levels by *HP* infection is temporary, so methylation levels will decrease when *HP* infection is no longer present. This concept explains why *HP*-negative gastric cancer patients had lower or equal methylation levels than individuals with *HP* infection. Second, the accumulation of aberrant methylation in the gastric mucosae does constitute a field defect for gastric cancers, which was clear in the *HP*-negative individuals.

Methylation dynamics in the gastric mucosae

The decrease of methylation levels in individuals with past *HP* exposure can be explained by several different mechanisms. First, methylation in gastric epithelial cells might disappear as a result of demethylation in stem cells. Considering that DNA demethylation is chemically difficult and that the existence of DNA demethylase has not been established, this seems unlikely. Second, some of the increased methylation might have originated from infiltrating inflammatory cells. We

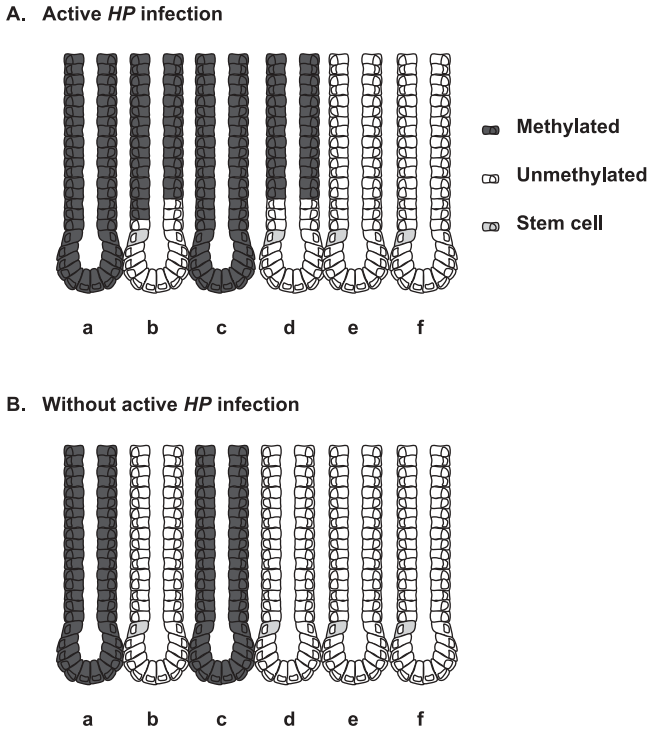

Fig. 4A,B. A model for methylation induction in nonstem cells. Active *HP* infection potently and temporarily induces methylation. **A** With active *HP* infection: Methylation is actively induced in nonstem cells, possibly in progenitor cells, and a gland can be composed of cells with methylation and cells without methylation (glands *b* and *d*). When methylation is present in stem cells, all the cells in a gland are methylated (glands *a* and *c*). **B** Without active *HP* infection: An entire gland reflects the methylation status of its stem cell, and the methylation level in the gastric mucosa is proportional to the fraction of stem cells with methylation. Based on this model, a gland composed of cells with and without methylation under active *HP* infection (glands *b* and *d* in **A**) can be replaced by cells without methylation when *HP* infection ceases (**B**), causing the methylation level to decrease

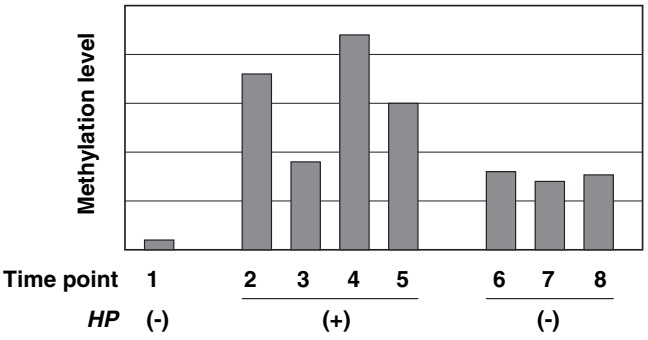

Fig. 5. A time-course model of methylation levels in the gastric mucosae with *HP* infection. Our analysis of methylation levels before and after eradication therapy showed that the methylation level decreased when eradication was successful, whereas it either increased or decreased when eradication failed. The data indicated that *HP* infection potently increases methylation levels, compared with before infection (time point 1), that methylation levels fluctuate during *HP* infection (time points 2–5), and that methylation levels decrease to a steady level when *HP* infection ends (time points 6–8). The methylation level in an individual without *HP* infection is considered to be proportional to the fraction of stem cells with methylation, and thus reflects the risk of cancer development

measured methylation levels in the mononuclear cells in the peripheral blood, and found that methylation of the eight regions of the seven genes was almost entirely absent (data not reported). Third, methylation could be induced in nonstem cells of the gastric epithelium by *HP* infection, and, once the *HP* infection was gone, the nonstem cells would be replaced by cells newly supplied from stem cells without methylation (Fig. 4). In this model, methylation levels in *HP*-positive individuals reflect a complex mixture of stem cells with methylation and nonstem cells with methylation. In contrast, methylation levels in *HP*-negative individuals are directly proportional to the fraction of stem cells with methylation. This model most suitably explains the lower methylation levels in individuals with past *HP* exposure and the association between methylation levels and cancer risk in *HP*-negative individuals. Naturally, this model needs experimental verification.

If methylation levels decrease when *HP* infection ceases, does therapeutic eradication of *HP* decrease methylation levels? We analyzed methylation levels in the same individuals before and after *HP* eradication and found that methylation levels significantly decreased after successful eradication (manuscript in preparation). Also, methylation levels fluctuated in individuals with *HP* infection (Fig. 5). This fluctuation can be explained by the dynamic induction of methylation and turnover of cells with methylation, and it is compatible with the third model in which methylation is induced in nonstem cells by *HP* infection.

Clinical use: a methylation marker for past exposure and future risk

HP infection induces methylation of CpG islands of preferential genes. Although some fraction of the induced methylation is temporary, the rest remains permanently in the gastric mucosae. This strongly suggests that methylation patterns of selected genes can serve as a marker for past exposure to *HP*. Since methylation is generally induced in specific genes by nonrandom processes, there is a good chance that this concept of a methylation marker for past exposure to *HP* infection in the gastric mucosae can be expanded to methylation markers for past exposure to various carcinogens in various tissues. Analysis of more samples with a defined

exposure to carcinogens is necessary, and animal models will be of critical value for this purpose.

The methylation levels in the gastric mucosae are clearly higher in gastric cancer patients than in healthy individuals. This again strongly suggests that methylation levels of specific genes can serve as markers of future risk for gastric cancers. To investigate this possibility, we analyzed methylation levels in the noncancerous gastric mucosae of patients with *multiple* gastric cancers, who were considered to have a high risk of developing more gastric cancers.[27] We confirmed that, if selected genes were analyzed, methylation levels increased over levels in healthy volunteers, from patients with a single gastric cancer to patients with multiple gastric cancers (Nakajima et al., submitted). This use of methylation markers in the colonic mucosae of patients with ulcerative colitis for future cancer risk has already been proposed[21,28] and might also be expanded to other various cancers.

Quantification of methylated DNA molecules in individuals is critically important for predicting individual risk, and cannot be achieved by qualitative analysis with conventional methods such as MSP. Further technological developments are also necessary for future routine clinical use of methylation markers.

Epilogue

We here focused on the applicability of epigenetics to gastrointestinal cancers. If we look at cancers at other organ sites, epigenetics is already making an impact on therapeutics. A demethylating agent is the only drug whose efficacy was demonstrated in myelodysplastic syndrome by a randomized control trial.[29] Moreover, epigenetics is having an impact on decision making in therapeutics. Neuroblastomas with methylation of multiple CpG islands have an entirely different prognosis from those without.[30,31] Translational epigenetics is already here.

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