■ REVIEW ■

# CpG Island Hypermethylation in Gastric Carcinoma and Its Premalignant Lesions

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Gastric cancers arise through a multistep process characterized by the progressive accumulation of molecular alterations in which genetic and epigenetic mechanisms have been implicated. Gastric cancer is one of the human malignancies in which aberrant promoter CpG island hypermethylation is frequently found. *Helicobacter pylori* and Epstein-Barr virus, which are known carcinogens for gastric cancer, are closely associated with enhanced hypermethylation of CpG island loci in gastric non-neoplastic epithelial cells and cancer cells, respectively. Aberrant CpG island hypermethylation occurs early in the multistep cascade of gastric carcinogenesis and tends to increase with the step-wise progression of the lesion. Approximately 400 genes that are actively expressed in normal gastric epithelial cells are estimated to be inactivated in gastric cancers as a result of promoter CpG island hypermethylation. In this review, a variety of information is summarized regarding CpG island hypermethylation in gastric cancer.

The field of epigenetics describes information transmission through cell division of heritable changes in a phenotype that does not involve DNA sequence changes. CpG island hypermethylation, histone modification, and transmitted chromatin structure are the underlying mechanisms for epigenetic transmission, and CpG island hypermethylation is a key component for altered gene expression associated with human cancers. CpG islands are DNA segments that are at least 0.5 kb in size, rich in G:C and CpG content, and found in approximately 70% of human gene promoters.[1] Promoter CpG islands are usually unmethylated in normal cells, with the exception of those on an inactive X chromosome or associated with imprinted genes. Although the cause is unclear, promoter CpG island hypermethylation can occur in association with cancer development and aging. Promoter CpG island hypermethylation is found in virtually all human cancer tissue types and acts as an important mechanism for the inactivation of tumor suppressor genes and tumor-related genes.[2,3] Gastric cancer is one of the human cancers in which promoter CpG island hypermethylation is frequently found.[2,4] In our preliminary study, which analyzed 41 candidate genes in major types of human cancers and cancer-associated normal tissues, gastric cancer demonstrated a significantly higher number of methylated genes than those of other human cancer tissue types (Fig. 1). In addition, gastric cancer-associated normal mucosa also exhibited the highest number of methylated genes when compared to normal tissue from other organs, including the lung, breast, colon, and liver (Fig. 1). These facts suggest the possibility that aberrant CpG island hypermethylation is more involved in the carcinogenesis of gastric cancer than in that of other human cancer tissue types.

## GASTRIC CANCER METHYLATION CHANGE

### How many genes are known to be methylated in gastric cancer?

In 1996, Lee *et al.*[5] reported for the first time that p16 was inactivated in gastric cancer by promoter CpG island hypermethylation rather than by genetic mutation. Since then, many researchers have demonstrated cancer-specific hypermethylation and inactivation of candidate genes in gastric cancer tissues and have correlated this hypermethylation with clinicopathologic features. However, the number of genes demonstrated to be inactivated by promoter CpG island hypermethylation was limited until the application of array-based genome-scale DNA me-
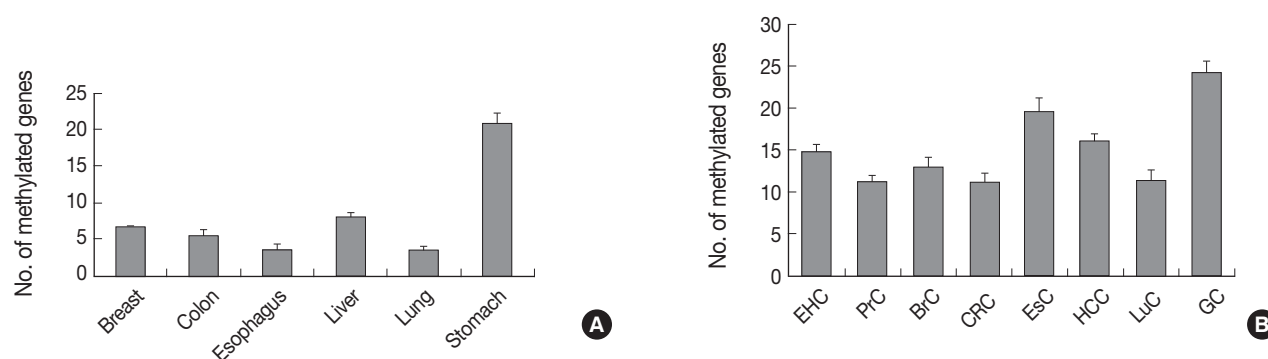
**Fig. 1.** Bar graphs display the number of methylated genes in cancer-associated normal tissue (A) and cancer tissue (B). The error bars indicate the standard error of the mean. Forty-one genes are analyzed for their methylation status in tissue samples from eight human cancer tissue types and six types of cancer-associated normal tissue using the MethyLight assay. EHC, extrahepatic bile duct cancer; PrC, prostate adenocarcinoma; BrC, breast adenocarcinoma; CRC, colorectal adenocarcinoma; EsC, esophageal adenocarcinoma; HCC, hepatocellular carcinoma; LuC, lung adenocarcinoma; GC, gastric adenocarcinoma.

thylation analysis for gastric cancers. With the application of 5-aza-2´-deoxycytidine (DAC) treatment and oligonucleotide microarrays, Yamashita *et al.*[6] estimated that approximately 421 genes are silenced by promoter CpG island hypermethylation in a gastric cancer cell line (AGS). Considering the fact that promoter CpG island hypermethylation is exaggerated in a cancer cell line compared with primary cancer tissue and that the AGS cell line has an increased rate of *de novo* methylation because of over-expression of DNMT3b, the actual number of genes silenced by promoter CpG island hypermethylation can be estimated to be less than 421 in primary gastric cancers. Our team also performed bead array-based expression analysis of gastric cancer cell lines before and after DAC treatment with subsequent confirmation of CpG island hypermethylation of the candidate genes by methylation-specific PCR. We found 140 novel genes that are silenced by promoter CpG island hypermethylation in primary gastric cancer tissue (Jung *et al.* in preparation).

## The timing of hypermethylation in multistep gastric carcinogenesis

Promoter CpG island hypermethylation is now recognized to be an important mechanism responsible for the inactivation of tumor suppressor genes or tumor-related genes. If promoter CpG island hypermethylation of some genes plays an important role in the malignant transformation of gastric epithelial cells, this pattern of hypermethylation should be found in premalignant lesions of the stomach, including gastric adenomas and intestinal metaplasia. In order to determine the frequency and timing of hypermethylation during multistep gastric carcinogenesis, Kang *et al.* analyzed multistep lesions of the stomach, in regards to their methylation status, in five genes[7] or 12 genes[8] using me-

thylation-specific PCR or in 25 genes using MethyLight analysis;[9] they demonstrated that promoter CpG island hypermethylation occurs early in multistep gastric carcinogenesis and accumulates during progression of the gastric lesion along the multistep carcinogenesis pathway. During multistep gastric carcinogenesis, there is a steep rise in the number of methylated genes when progressing from chronic gastritis to intestinal metaplasia, which was a consistent finding in a series of studies.[7-9] Regardless of the status of *Helicobacter pylori* infection, the number of methylated genes in intestinal metaplasia was significantly higher than that found in chronic gastritis without intestinal metaplasia.[9] This suggests that intestinal metaplasia is an epigenetically altered lesion. However, even in chronic gastritis without intestinal metaplasia, promoter CpG island hypermethylation occurs in association with *H. pylori* infection[10] and aging.[11,12]

### *Helicobacter pylori* infection-associated DNA hypermethylation

*H. pylori* has been designated as a human class I carcinogen for gastric malignancy by the International Agency for Research on Cancer. Although the exact mechanism of *H. pylori*-associated gastric carcinogenesis is unknown, long-standing bacterial infection, perpetuated chronic inflammation, and sustained mucosal epithelial cell proliferation are thought to produce a carcinogenic environment. Chan *et al.*[10] were the first to demonstrate *H. pylori*-associated hypermethylation in the gastric epithelia, which was supported by subsequent studies demonstrating that the eradication of *H. pylori* infection results in a reversal of the methylation status of multiple CpG island loci.[13-15] Thus, it is plausible that aberrant methylation induced by *H. pylori* infection may contribute to *H. pylori* infection-associated gastric car-

cinogenesis. It has been reported that interleukin 1 beta can modulate CpG island methylation through the activation of DNA methyltransferase.[16] In an *in vitro* study, interleukin 1 beta siRNA blocked *H. pylori*-induced methylation of the *CDH1* promoter CpG island locus in a gastric cancer cell line.[17] In an animal model experiment by the Ushijima team, *H. pylori* infection resulted in the induction of CpG island hypermethylation of candidate genes, and the eradication led to marked decreases in methylation levels in the candidate genes. However, the suppression of inflammation by treatment with the immunosuppressive drug cyclosporine blocked the induction of DNA methylation in the candidate genes. These findings suggest that the infection-associated inflammatory response, rather than *H. pylori* itself, was responsible for the induction of altered DNA methylation.[18] In a subsequent gerbil study, neutrophilic inflammation caused by treatment with ethanol or NaCl did not induce DNA methylation in candidate genes, whereas chronic inflammation caused by *H. pylori* or *H. felis* infection led to altered methylation in candidate genes. This finding suggests that it is not the inflammation itself, but rather specific types of inflammation, that are necessary for methylation induction.[19]

## Aging-related hypermethylation vs inflammation-related hypermethylation

Challenging traditional thought regarding the lack of CpG island methylation in normal tissues, a recent study indicated that 4-8% of CpG island loci are methylated in the genomic DNA of human blood, brain, muscle, and spleen tissue.[20,21] Additionally, normal cells have been shown to acquire hypermethylation in an aging-related manner: aging-related methylation was first demonstrated for the oestrogen receptor (*ER*) gene by Issa *et al.*[22] and has subsequently been demonstrated in multiple genes by Ahuja *et al.*[23] In the stomach, Waki *et al.*[11] reported aging-related methylation of *CDH1, MLH1*, and *p16* in non-neoplastic gastric epithelia. However, because CpG island hypermethylation can be induced by chronic inflammation in the stomach and because the prevalence of *H. pylori* infection increases with age,[24] the interplay between aging and chronic inflammation is complicated by *H. pylori* infection. Chan *et al.*[25] found that *CDH1* methylation was associated with age in the stomach, the presence of chronic gastritis, and *H. pylori* infection using a univariate analysis, but *H. pylori* infection was the only independent factor associated with *CDH1* methylation in the multivariate analysis. Furthermore, Maekita *et al.*[26] reported that there was no aging-related hypermethylation in healthy gastric mucosa. However, a current study by our team supports the presence of aging-related hypermethylation in the gastric mucosa because the number of methylated genes was significantly higher in *H. pylori*-negative adult stomach samples than in *H. pylori*-negative pediatric samples.[27] In WI-38 human embryonic lung fibroblasts, *p16* was found to undergo spontaneous promoter CpG island hypermethylation during passage of these cells in culture,[28] which supports the presence of age-related hypermethylation. Thus, we cannot exclude the possibility that aging-related methylation occurs in non-neoplastic gastric epithelia without *H. pylori* infection.

## Field cancerization

Many studies have shown that methylation levels or frequencies of multiple genes are higher in gastric mucosa from gastric cancer patients than in mucosa from non-cancer subjects.[11,26,29] In a stomach with enhanced CpG island hypermethylation, the affected cells may have a growth-selective advantage imparted by the expressional loss of the methylated genes, which may predispose the cells to acquiring further genetic or epigenetic defects that lead to neoplasia. Synchronous multiple gastric cancers, constituting 4-9% of gastric cancers, occur at an older age and are more commonly associated with an extensive distribution of intestinal metaplasia in the background mucosa compared with single gastric cancer.[30,31] In a recent study, non-neoplastic gastric mucosa from synchronous gastric cancer patients was found to have more hypermethylated genes than non-neoplastic gastric mucosa from patients with a single gastric cancer, which suggests that enhanced hypermethylation in the background gastric mucosa might contribute to the development of multiple gastric cancers.[32] Multiple gastric cancers are not only a genetic model but also an epigenetic model for field cancerization. Based on the findings that methylation levels in gastric mucosa are significantly increased in cases with a single gastric cancer and even more so in cases with multiple gastric cancers, it has been suggested that quantitative information regarding methylation levels of specific genes may serve as a biomarker for predicting an individual's risk for developing gastric cancer.[32,33] However, it should be noted that increased methylation levels or frequencies in gastric mucosa from gastric cancer patients may reflect a higher prevalence of *H. pylori* infection and intestinal metaplasia (compared with gastric mucosa from non-cancer subjects).[34,35] Because both *H. pylori* infection and intestinal metaplasia are closely linked with increased CpG island hypermethylation, further clarification is needed regarding whether non-neoplastic gastric mucosa without intestinal metaplasia from *H. pylori*-negative gastric cancer patients harbors more hy-

permethylated genes than non-neoplastic gastric mucosa from *H. pylori*-negative non-cancer subjects. In Park *et al.*'s study,[9] no significant differences were observed in the number of methylated genes or methylation levels of 25 individual genes in chronic gastritis tissue from cancer and non-cancer patients after normalization of the confounding factors of *H. pylori* and intestinal metaplasia. Because synchronous multiple gastric cancers tend to be associated with widespread intestinal metaplasia when compared with single gastric cancers, cancer-associated gastric mucosa might show higher methylation frequencies or higher methylation levels of multiple CpG island loci in multiple synchronous gastric cancer patients than in single gastric cancer patients.

### Prognostic implications of individual gene methylation

Promoter CpG island hypermethylation can be utilized as a tumor biomarker for the detection of tumor cells in gastric juice or serum or for the prediction of clinical outcomes. A dozen DNA methylation markers have been reported to be closely associated with worse or better clinical outcomes in gastric cancer patients; *MAL* or *COX2* methylation was correlated with better clinical outcomes in gastric cancer patients,[36,37] whereas *DAPK, TMS1, IQGAP2, SOX2, CACNA2D3, DKK-3, TFPI2,* and *Cystatin* methylation has been correlated with worse clinical outcomes in gastric cancer patients.[38-44] However, most of the studies that have evaluated these DNA methylation markers for their prognostic implication used methylation-specific polymerase chain reaction (MSP). Although MSP is a highly sensitive method for detecting one methylated allele in 10,000 unmethylated alleles,[45] it is unreliable for detecting low levels of methylation. Another issue related to these DNA methylation markers is that a validation study was not performed to prove their utility as a prognostic marker. The final issue concerns concordance among individual gene hypermethylation: hypermethylation of one specific gene tends to be concordant with that of another individual gene; thus, better or worse survival observed in gastric cancers with hypermethylation of an individual gene may not be attributed to hypermethylation of that particular gene. Rather, survival may be related to concordant hypermethylation of multiple CpG island loci, namely CpG island methylator phenotype (CIMP).

### CIMP-positive gastric cancer

CIMP refers to a subset of malignancies that is characterized by widespread hypermethylation of multiple promoter CpG island loci. Since the CIMP concept was first introduced for the molecular pathways of colorectal cancers (CRCs) by Dr. Issa's group,[46] many investigators have attempted to characterize the clinicopathological and molecular features of CIMP-positive CRCs and have found a close association with proximal colon location, older age at onset, poor differentiation, microsatellite instability (MSI), and *BRAF* mutations.[47-49] Similarly, the presence of CIMP-positive gastric cancers has been reported by the same group,[50] which did not find distinct clinicopathologic features of CIMP-positive gastric cancers, with the exception of a close association with MSI. Since then, several researchers have attempted to characterize the clinicopathological features of CIMP-positive gastric cancers using variable methodologies for DNA methylation analysis and their own CIMP marker panels; this has led to controversial results.[51-56] Despite variable findings, relatively common findings include close associations of CIMP-positive gastric cancers with diffuse type-histology and better clinical outcomes. Recently, with the exclusion of MSI-positive gastric cancers and Epstein-Barr virus (EBV)-positive gastric cancers from the analysis, Park *et al.*[57] found characteristic clinicopathologic features of CIMP-positive gastric cancers that were defined as tumors with methylation of 13 or more markers during the analysis of the 16 cancer-specific DNA methylation markers; these defined CIMP-positive gastric cancers tended to show distinct clinicopathologic features, including a proclivity toward diffuse or mixed-type histology, poor differentiation, infiltrative gross types, and higher cancer stages.

### EBV-associated gastric cancer

In addition to *H. pylori*, EBV has also been recognized as a gastric cancer-causing infectious agent.[58,59] EBV-associated gastric cancer, comprising nearly 10% of gastric cancers, are characterized by a younger age at onset, male predominance, proximal location, frequent association of lymphoid stroma, and a better prognosis.[60] EBV-associated gastric cancer is a prototype of the CIMP-positive types of gastric cancer and exhibits consistently higher frequencies and levels of methylation in examined cancer-related methylation markers,[53,57,61] which is independent of whether the EBV-associated gastric cancer is histologically lymphoepithelioma-like or ordinary.[60] Because the number of methylated cancer-specific methylation markers is far higher in EBV-associated gastric cancers than in EBV-negative gastric cancers, EBV-associated aberrant hypermethylation is a global event. However, *MLH1* methylation and resultant MSI are, if ever, rarely observed in EBV-associated gastric cancers. Although it can be speculated that the methylation machinery of EBV-associated gastric cancer is capable of recogniz-

ing its own methylation targets, it may be hypothesized that the presence of both CIMP and MSI could cause a growth disadvantage and subsequently lead to the negative selection of gastric cancer cells containing both CIMP and MSI. Tumor cells from EBV-associated gastric cancers display *DNA methyltransferase I* over-expression,[62] which is closely associated with *interleukin-1-beta* (*IL1B*) over-expression[60] or *latent membrane protein 2A*-associated phosphorylation of *signal transducer and activator of transcription 3* (*STAT3*) in EBV-associated gastric cancers.[63] *IL1B* is capable of increasing the expression of *DNMT1* via the production of nitric oxide, and phosphorylated *STAT3* binds to the *DNMT1* promoter and induces transcription.

## MSI-positive gastric cancer

The frequency of MSI-positive gastric cancer varies from 8% to 37%. In gastric cancer, MSI is mainly caused by promoter CpG island hypermethylation of the *MLH1* gene. Somatic mutations of mismatch repair genes are very rare in sporadic gastric cancers. Thus, known clinicopathological features of MSI-positive gastric cancer, including female sex, older age of onset, antral location, ulcerofungating gross type by Borrmann's classification, intestinal type by Lauren classification, expanding type by Ming classification, and better survival[64] represent features of sporadic MSI-positive gastric cancer. Since gastric cancer is an extracolonic lesion in Lynch syndrome, we have encountered MSI-positive gastric cancer without *MLH1* methylation. Recently, our team has compared clinicopathological features between MSI-positive gastric cancers with and without *MLH1* methylation (Kim *et al.* in preparation). Of the known clinicopathological features for MSI-positive gastric cancer, female preponderance, older age of onset and antral location do not correspond with Lynch syndrome-associated MSI-positive gastric cancer.[65] Several studies have shown that *MLH1* methylation occurs in premalignant stages, including intestinal metaplasia and gastric adenoma. Thus, *MLH1* methylation has been considered an early event during multistep gastric carcinogenesis.[7,9,66,67] However, a recent study of Ling *et al.*[68] has shown that MSI can develop from MSI-low or the absence of MSI due to time-dependent accumulation of DNA methylation during progression of early stage-gastric cancer, which indicates that silencing of *MLH1* due to promoter hypermethylation may appear as a later event during multistep gastric carcinogenesis. Unfortunately, the study did not investigate whether later acquisition of *MLH1* methylation occurs as a phenomenon of CIMP. *MLH1* methylation is unlikely to occur as an isolated sporadic event without concurrent hypermethylation of multiple gene promoter CpG island loci.

## Epigenetic regulation of microRNA

A new class of small non-coding RNAs known as microRNAs (miRNA) have recently been discovered. Mature miRNAs, 21 to 30 nucleotide-sized, are cleaved from 70- to 100-nucleotide hairpin miRNA precursors in the cytoplasm by the RNase III enzyme Dicer. Base-pairing between the miRNA strand and the 3′ untranslated regions of its target mRNAs (potentially hundreds of genes) directs RNA-induced silencing complex to either cause mRNA degradation or translational repression. Through these molecular mechanisms, miRNAs serve as key regulators of gene expression involved in crucial cellular processes, including development, proliferation, cellular differentiation, and apoptosis. Although the generation of miRNAs and their mode of action in regulating gene function have been intensively studied, the regulation of miRNA expression remains largely unclear. The means by which miRNA is regulated is somewhat complicated. Recently, Saito *et al.* demonstrated for the first time that *miR-127* is regulated by DNA hypermethylation[69] and since then, a dramatically increased number of studies have documented the epigenetic regulation of miRNAs.[70] However, in gastric cancer, approximately 113 miRNAs have been demonstrated to be dysregulated compared with normal gastric mucosal tissues but a dozen miRNA genes have been shown to be downregulated by aberrant hypermethylation, including *miR-512-5p*,[71] *miR-375*,[72] *miR-212*,[73] *miR-181c*,[73] *miR-196b*,[74] *miR-137*,[75] *miR-129-2*,[76] *miR-124a-1*, *miR-124a-2*, *miR-124a-3*,[77] *miR-34b*, and *miR-34c*.[78] In particular, Ando *et al.*[77] demonstrated that *miR-124a-1*, *-2*, and *-3* are frequently methylated in primary gastric cancer and in normal gastric mucosal tissues from healthy individuals with *H. pylori* infections. Among *H. pylori*-negative individuals, methylation levels are significantly higher in non-cancerous gastric mucosal tissues from gastric cancer patients than gastric mucosal tissues from healthy individuals, which suggest that methylation of miRNA genes comprises a field defect contributing to the pathogenesis of gastric cancer.[77] Suzuki *et al.*[78] also reported that *miR-34b/c* methylation is significantly associated with *H. pylori* infection among healthy individuals.

## Future perspective

Although it is well known that EBV-positive gastric cancer is featured with extensive hypermethylation of multiple genes, the mechanism leading to genome-wide hypermethylation is still unknown. Because EBV-positive gastric dysplasia or ade-

noma has never been reported, it seems likely that EBV-infected epithelial cells transform directly into malignant cells. DNMT1 elevation in association with EBV infection does not provide a satisfactory explanation for genome-wide extensive hypermethylation because *in vitro* transfection of *DNMT1* in cell lines or DNMT1 elevation in association with *H. pylori* infection also does not lead to such extensive hypermethylation as seen in EBV-positive gastric cancer. If we can elucidate the mechanism of EBV-associated extensive hypermethylation, we will have a better understanding of how promoter CpG island hypermethylation occurs.

Although gastric cancer has shown a higher number of genes methylated compared with colorectal cancer, clinicopathological features of CIMP-positive gastric cancer are still obscure and marker panels diagnosing CIMP-positive gastric cancer are not established yet, which is in contrast to the situation in colorectal cancer. It is imperative to develop CIMP panel markers enabling the diagnosis of CIMP-positive gastric cancer and then to characterize clinicopathological features of CIMP-positive gastric cancer. In case these are accomplished, we expect to identify the precursor lesions of CIMP-positive gastric cancer and to delineate multistep morphological progression of CIMP-positive tumors.

## CONCLUSION

Although molecular pathways and morphological pathways are not well established for gastric cancers when compared with colorectal cancers, rapid development of methylation analysis technology will enable us to take a glimpse at the landscape of epigenetic alterations occurring at each step of multistep gastric carcinogenesis, which will provide molecular insights on morphological progression pathways. In addition, epigenetic studies may offer great potential for the identification of tumor biomarkers that can be utilized to detect and diagnose gastric cancer at its earliest stages, to accurately assess an individual's risk for gastric cancer, or to predict the response to chemotherapy or clinical outcomes.

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