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TOPIC HIGHLIGHT

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DNA methylation in gastric cancer, related to *Helicobacter pylori* and Epstein-Barr virus

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Abstract

Gastric cancer is a leading cause of cancer death worldwide, and significant effort has been focused on clarifying the pathology of gastric cancer. In particular, the development of genome-wide analysis tools has enabled the detection of genetic and epigenetic alterations in gastric cancer; for example, aberrant DNA methylation in gene promoter regions is thought to play a crucial role in gastric carcinogenesis. The etiological viewpoint is also essential for the study of gastric cancers, and two distinct pathogens, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are known to participate in gastric carcinogenesis. Chronic inflammation of the gastric epithelium due to *H. pylori* infection induces aberrant polyclonal methylation that may lead to an increased risk of gastric cancer. In addition, EBV infection is known to cause extensive methylation, and EBV-positive gastric cancers display a high methylation epigenotype, in which aberrant methylation extends to not only Polycomb repressive complex (PRC)-target genes in embryonic stem cells but also non-PRC-target genes. Here, we review aberrant DNA methylation in gastric cancer and the association between methylation and infection with *H. pylori* and EBV.

Key words: Gastric cancer; Epigenetics; DNA methylation; Epstein-Barr virus; *Helicobacter pylori*

Core tip: Recent technological advances in genome-wide analysis tools have revealed various molecular aberrations in cancer. Although gastric cancer involves multiple genetic and epigenetic alterations, aberrant DNA methylation in gene promoter regions is thought to play a critical role in gastric carcinogenesis. From the etiological viewpoint, two pathogens, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are known to participate in gastric carcinogenesis. Chronic inflammation in the gastric mucosa due to *H. pylori* and EBV infection of gastric epithelial cells has been reported to cause aberrant promoter methylation, which may contribute to the tumorigenic mechanisms of these pathogens.

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INTRODUCTION

Gastric cancer is a leading cause of cancer death worldwide[1]. Malignant tumors, including gastric cancer, are

known to arise through multiple genetic and epigenetic alterations[2], and these molecular changes eventually impact the expression of cancer-associated genes, such as oncogenes and tumor-suppressor genes. Historically, one of the most common genetic alterations in cancer is mutation of the *TP53* gene[3,4]. *TP53* is a core tumor-suppressor gene, and more than half of all gastric cancers demonstrate loss of *TP53* function due to genetic alterations[5]. Another example is *CDH1*, the gene encoding a calcium-dependent cell-to-cell adhesion glycoprotein that is responsible for familial diffuse type gastric cancers due to germline mutations[6]. However, sporadic gastric cancers also display *CDH1* somatic mutations at a constant rate[7]. Moreover, recent whole-genome exome analyses in gastric cancer have identified mutations in several genes, including *ARID1A*, *PIK3CA*, and *FAT4*[8,9].

Although gastric cancer involves various molecular alterations, aberrant promoter methylation plays a major role in gastric carcinogenesis[10-15]. $p16^{INK4A}$ is the most well-known tumor-suppressor gene that is silenced by promoter methylation; the promoter region of $p16^{INK4A}$ is aberrantly methylated in 25%-42% of gastric cancers[10,11,16,17], while mutations or deletions are very rare[16]. *RUNX3* is also a significant tumor-suppressor gene in gastric cancer[18], and approximately half of all gastric cancer cases lose *RUNX3* expression due to hemizygous deletion and promoter hypermethylation, while point mutations are rarely reported. Although mutations in DNA mismatch-repair genes such as *MLH1* and *MSH2* are quite rare in gastric cancers[19,20], promoter methylation of *MLH1* represents a major cause of microsatellite instability (MSI)[21,22], which is observed in 31%-67% of gastric cancers[19,23].

Several scanning methods have been developed to identify novel tumor-suppressor genes silenced by promoter methylation[24-30], and genome-wide analysis has demonstrated unusual clustering of aberrant methylation in a subset of cancer cases. The phenotype presenting atypical methylation of cytosine-phosphate-guanine (CpG) islands, termed the CpG island methylator phenotype (CIMP), was first described in colorectal cancers[31]. Gastric cancer was also evaluated using methylation markers for colorectal cancer CIMP, and CIMP was also found to be present in gastric cancer[10]. Genome-wide analysis of aberrant DNA methylation in gastric cancer was first performed using the methylation-sensitive-representational difference analysis (MS-RDA) method to identify methylation-associated silenced genes, including novel tumor-suppressor genes[32-34]. Using silenced genes as markers, a subset of gastric cancers was demonstrated to harbor unusual accumulation of aberrant methylation in promoter CpG islands[32].

Environmental factors are also significantly related to the induction of aberrant DNA methylation, and etiological studies have provided evidence that two distinct infectious agents, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are closely associated with gastric carcinogenesis[35-37]. Here, we review aberrant DNA methylation in gastric cancer and the association between methylation and infection with these two unique pathogens.

EPIGENETIC MODIFICATION AND DNA METHYLATION

Physiological function of DNA methylation

"Epigenetics", as compared to "genetics", is defined as the study of genomic DNA modifications that are heritable during cell division but do not involve a change in the DNA sequence itself, such as DNA methylation and histone modification[12,13,38]. DNA methylation is the covalent modification of a methyl group on the 5-position of cytosine at CpG dinucleotides[38,39]. CpG islands are genomic regions that contain dense CpG dinucleotides, and they are located in the promoter regions of approximately half of all genes. CpG islands are generally free from DNA methylation, allowing for the expression of downstream genes whose transcription is regulated by histone modification[13].

In normal cellular processes, DNA methylation is used for robust gene silencing, such as genome imprinting[40] and X-chromosome inactivation[41]. Moreover, tissue-specific patterns of methylation or changes in methylation during cellular differentiation have been discovered at CpG-poor promoters[42] and inter- and intragenic CpG islands[43]. In addition, when cells encounter foreign nucleic acid, such as viral DNA, host cells take advantage of DNA methylation as a defensive system to inactivate foreign nucleic acid[44].

Aberrant DNA methylation in cancers

Broadly speaking, aberrant DNA methylation in cancer is divided into two categories: "genome-overall hypomethylation" and "regional hypermethylation". The former, global hypomethylation, was discovered in the 1980s[45] and can be defined as a decrease in 5-methylcytosine content throughout the genome. CpG dinucleotides show heterogeneous distribution, especially in repetitive sequences, which are typically methylated in normal tissue[46,47]. In cancers, these repetitive sequences demonstrate aberrant hypomethylation[48], promoting genomic instability and cancer progression[49-51]. Loss of imprinting is another example of an epigenetic alteration related to aberrant hypomethylation[52], and loss of imprinting in *IGF2* was shown to be involved in the early events of carcinogenesis and was associated with increased colorectal cancer risk[53,54]. A subset of male germ line-specific genes, specifically the *MAGE* gene families, was discovered to be a cancer antigen in malignant melanoma[55]. These genes are repressed by promoter methylation in normal somatic tissues but are activated through promoter hypomethylation in several types of cancers[56,57].

The latter type of DNA methylation, regional hypermethylation, arises in CpG islands[58-60]. Aberrant methylation of promoter CpG islands leads to inappropriate transcriptional silencing, and this phenomenon is

regarded as one of the major mechanisms for inactivating tumor-suppressor genes[2,12-14]. Promoter methylation in tumor-suppressor genes has been discovered in various cancers, including *RB* in sporadic retinoblastoma[61], *VHL* in renal cell carcinoma[62], *CDH1* in hepatocellular carcinoma[63], and $p16^{INK4A}$ in various cancers[64].

In embryonic stem (ES) cells, the Polycomb repressive complex (PRC) plays a significant role in reversibly repressing gene expression. In ES cells, these PRC-target genes are frequently methylated compared to non-PRC-target genes in various cancers[65]. Our comprehensive methylation analysis of gastric cancer revealed significant enrichment of aberrant methylation in PRC-target genes in a subset of gastric cancers with a high-methylation epigenotype[37]. However, another subset of gastric cancer demonstrated an extensively high methylation epigenotype that displayed extended methylation in both PRC-target genes and non-PRC-target genes. This phenotype was detected in EBV-positive gastric cancer, and it will be discussed in detail later in this review.

Among the factors known to cause aberrant DNA methylation in non-cancerous tissues, aging is known to promote the accumulation of DNA methylation[66,67]. Indeed, age-dependent promoter methylation could explain the association between cancer and aging[68]. Recent whole-genome bisulfite sequencing comparing newborn and centenarian genomes demonstrated that centenarian DNA had a lower DNA methylation content throughout the genome and showed the more hypomethylated CpGs in promoters, exonic, intronic, and intergenic regions, whereas a greater level of DNA methylation was observed in CpG island promoters[69]. Another report showed that replicative senescent human cells exhibited features similar to the cancer epigenome, such as widespread DNA hypomethylation and focal hypermethylation[70]. Epidemiological studies have also revealed that the epigenetic status is influenced by various environmental factors[67] and can be associated with cancer incidence or prognosis[71,72].

Among environmental factors, chronic inflammation is a significant inducer of aberrant DNA methylation, as demonstrated by the analysis of non-cancerous tissues, such as colonic mucosae with ulcerative colitis[73], liver tissue with chronic hepatitis[74], esophageal mucosae with inflammatory reflux esophagitis[75], and gastric mucosae with chronic gastritis[76]. In a mouse colitis model induced by dextran sodium sulfate, aberrant CpG island methylation in colonic epithelial cells was shown to accumulate gradually on a monthly basis[77]. Interestingly, even in severe combined immunodeficiency (SCID) mice lacking functional T and B lymphocytes, DNA methylation was induced at the same level as in the background strain of mice, suggesting that functional T and B lymphocytes are not essential for methylation accumulation.

H. PYLORI AND ABERRANT DNA METHYLATION

Two distinct pathogens, *H. pylori* and EBV, are known to be involved in gastric carcinogenesis. First, we will discuss the association between chronic inflammation due to *H. pylori* and DNA methylation.

H. pylori, discovered in 1983 by Marshall BJ and Warren JR[78], is a helix-shaped Gram-negative bacterium present in the stomach of approximately half of the world's population[79,80]. Recent prospective cohort studies indicate that *H. pylori* infection plays an essential role in various disorders, including gastric cancer[35,36], chronic gastritis[78], intestinal metaplasia[81,82], and gastric lymphoma[83]. In 1994, the World Health Organization concluded that "*H. pylori* is a definite carcinogen" based on epidemiological evidence[79,80].

Two pathways have been proposed to play a role in gastric carcinogenesis resulting from *H. pylori* infection: the direct interaction of *H. pylori* with the gastric epithelium and its indirect involvement through chronic inflammation. Ultimately, however, both of these pathways cooperate to promote gastric carcinogenesis.

Direct interaction of H. pylori with gastric epithelium

The direct mechanism by which *H. pylori* contributes to gastric carcinogenesis is attributed to its pathogenicity. Most Gram-negative bacteria exert pathogenicity through the acquisition of an exogenous gene cluster, termed a pathogenicity island (PAI). *H. pylori* also contains a *cag* PAI, which consists of an approximate 40-kbp stretch of DNA encoding approximately 30 genes, including those of the type Ⅳ secretion system[84]. The pathogenicity of *H. pylori* depends on whether it contains cytotoxin-associated gene A (CagA) protein or not. Almost all strains of *H. pylori* in East Asia contain the CagA protein, whereas this frequency in Western strains is limited to 60%. The pathogenicity of the CagA protein is exerted via injection into gastric epithelial cells through type Ⅳ secretion systems (Figure 1). The CagA protein contains a conserved motif in the C-terminus, the EPIYA motif, which dictates the severity of its pathogenicity. East Asian strains of CagA exert more aggressive cytotoxicity compared to Western strains[85].

Host cellular responses against injected CagA protein display several patterns. These include (1) enhanced cell motility that induces a growth-factor-like phenotype, termed hummingbird, in host gastric cells[86]; (2) disruption of the epithelial apical-junctional complex[87]; and (3) epithelial proliferative and proinflammatory responses associated with the development of chronic gastritis and gastric cancer[88]. Therefore, CagA plays a key role in gastric carcinogenesis, although the direct involvement of CagA or other components of *H. pylori* in the induction

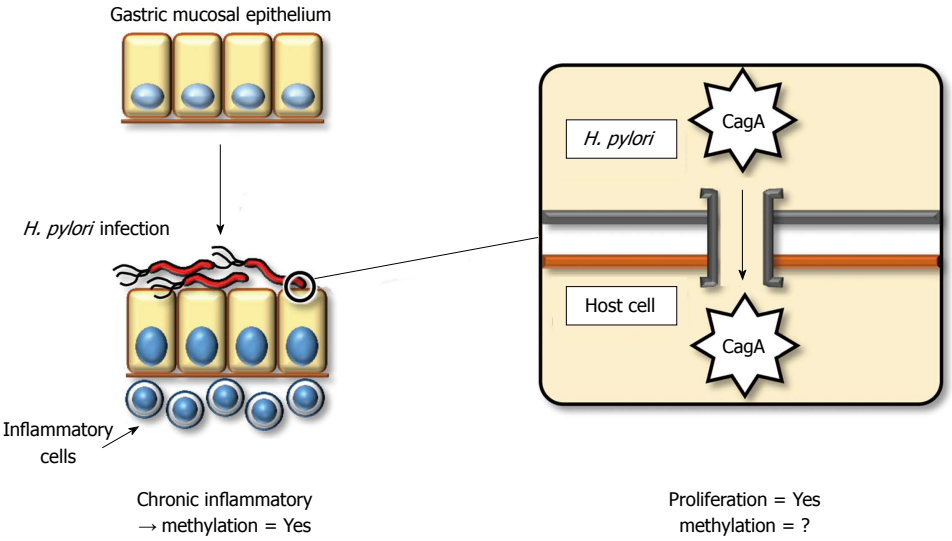

Figure 1 Schematic representation about infectious condition and pathogenicity of *Helicobacter pylori*. Pathogenicity of *Helicobacter pylori* (*H. pylori*) is exerted through injection of CagA into gastric epithelial cells using type Ⅳ secretion system. Inflammatory cell infiltration due to *H. pylori* might be more significant factor in induction of aberrant DNA methylation (left). Injection of CagA leads to proliferation of epithelial cells, but it is still unclear whether it plays an important role in methylation induction in epithelial cells (right).

of DNA methylation has not been clarified.

Chronic inflammation induced by H. pylori

H. pylori indirectly promotes pathogenicity by inducing chronic inflammation. Chronic inflammation generally involves the accumulation of molecular damage through a variety of mechanisms, such as DNA damage by free radicals[89] or aberrant expression of activation-induced cytidine deaminase (AID)[90]. Moreover, chronic inflammation induces aberrant DNA methylation[74]. Rather than *H. pylori* infection itself, inflammatory cell infiltration by *H. pylori* might be a more significant factor for the induction of aberrant DNA methylation[91]. In a Mongolian gerbil model, suppression of aberrant DNA methylation by 5-aza-dC treatment reduced, but did not entirely prevent, the incidence of *H. pylori*-induced gastric cancers[92]. This result demonstrates that aberrant DNA methylation contributes to *H. pylori*-related gastric carcinogenesis, although some direct influences of *H. pylori*, without aberrant DNA methylation, may also be significant.

H. pylori infection induces aberrant promoter methylation in tumor-suppressor genes, including $p16^{INK4A}$, *LOX*, and *CDH1*[34,93]. Although eradication of *H. pylori* can reduce the level of promoter methylation, a certain amount of methylation remains[91,94]. This observation suggests that not only fully differentiated gastric epithelial cells but also stem/progenitor cells might acquire aberrant methylation. In human ulcerative colitis and hepatitis, increased expression of *IL-1β*, *IL-8*, *NOS2*, and *TNF* was observed[95-98], and these genes may represent a common factor associated with the induction of aberrant DNA methylation during chronic inflammation. In particular, *IL-1β* is thought to be significant, as a specific single-nucleotide polymorphism of *IL-1β* is associated with increased gastric cancer risk and increased incidence of *CDH1* promoter methylation in gastric cancers[99,100]. Furthermore, the role of *IL-1β* in *H. pylori*-induced gastric inflammation and DNA methylation was confirmed using IL-1 receptor type 1 knockout mice[101].

EBV AND ABERRANT DNA METHYLATION

EBV is another pathogen known to be involved in gastric carcinogenesis.

EBV and gastric carcinogenesis

EBV is a gamma-herpes virus consisting of a double-strand DNA genome approximately 170 kbp in length. EBV may cause infectious mononucleosis during initial infection, and more than 90% of adult individuals become EBV carriers[102], as this virus can be maintained asymptomatically in a latent form in memory B lymphocytes. However, EBV displays the characteristics of an oncogenic virus; indeed, it was initially discovered in human neoplastic cells, specifically a Burkitt's lymphoma cell line, in 1964[103]. Subsequently, EBV was associated with several types of malignant tumors, such as nasopharyngeal carcinoma[104], Hodgkin lymphoma[105], and opportunistic lymphoma in immunocompromised individuals[106,107]. Moreover, a subgroup of gastric cancer patients infected with EBV was discovered in 1990[108], and this unique subgroup is distributed throughout the world, without regional or racial deviation, at a rate of 7%-15%[109, 110].

EBV-positive (EBV^+) gastric cancers show distinct clinicopathological features. First, EBV^+ gastric cancers demonstrate EBV infection in almost all neoplastic cells of the tumor, which has been confirmed by *in situ* hy-

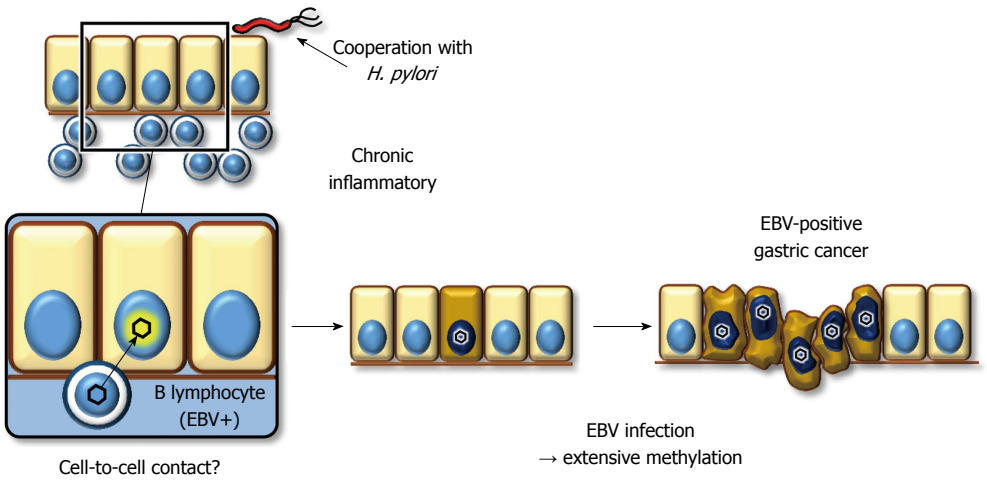

Figure 2 Schematic representation about infectious condition and pathogenicity of Epstein-Barr virus. Direct cell-to-cell contact between B lymphocyte and gastric epithelial cell may perhaps be the most likely model to infect with Epstein-Barr virus (EBV) into epithelial cells *in vivo* (left). EBV infection induces extensive promoter methylation, which should contribute to tumorigenesis. *H. pylori: Helicobacter pylori.*

bridization for a non-coding small RNA, EBER, which is abundantly expressed in the nuclei of infected neoplastic cells. In addition, the clinical features of EBV$^+$ gastric cancers differ from EBV-negative (EBV) gastric cancers due to their male predominance, proximal location, and relatively favorable prognosis[111]. Histopathologically EBV$^+$ gastric cancers demonstrate characteristic features of a poorly differentiated adenocarcinoma with marked infiltration of lymphocytes into the stromal tissue, which has been reported as "gastric cancer with lymphoid stroma"[112].

EBV has a double-stranded DNA genome that exists in a linear form in viral particles. After EBV enters the host cell, the viral DNA circularizes *via* the fusion of terminal repeats at both ends, and it maintains its circular form in the nuclei of latently infected cells without integration into the host genome[113]. Southern blot analysis for terminal repeats has demonstrated that EBV present in neoplastic cells is mono- or oligo-clonal, even in advanced stages[114-116]. Moreover, all of the cancerous cells are positive for EBER-*in situ* hybridization in all cases of EBV$^+$ gastric cancer. This fact indicates that EBV infection occurs at the initial, or a very early stage, of carcinoma development, and it implies a profound association of EBV with gastric carcinogenesis.

The mechanism underlying EBV infection in the gastric mucosal epithelium remains unclear, while the viral receptor molecule for CD21 in B lymphocytes is not expressed on epithelial cells[117]. Because co-cultivation of virus-producing lymphocytes demonstrates a much greater efficiency of infection (up to 800-fold) compared to cell-free infection, direct cell-to-cell contact between B lymphocytes and gastric epithelial cells is the most likely model to explain how EBV infects epithelial cells *in vivo* (Figure 2)[118]. This hypothesis supports histopathological data showing that the background mucosa of EBV$^+$ gastric cancer presents atrophic gastritis with lymphocyte infiltration due to *H. pylori* infection[119]. However, it remains unclear whether chronic inflammation with *H. pylori* is a prerequisite for EBV to infect gastric epithelial cells.

DNA methylation in EBV-positive gastric cancer

EBV$^+$ gastric cancer forms a distinct subgroup of gastric cancer. Previous reports have indicated that promoter methylation is observed more frequently in EBV$^+$ gastric cancers than in EBV gastric cancers, despite analyzing a limited number of cancer-associated genes[120-122]. We performed a comprehensive analysis of promoter methylation in clinical gastric cancers and found that gastric cancers clustered into three distinct subgroups. Interestingly, EBV$^+$ gastric cancers displayed an extremely high methylation phenotype, termed the EBV$^+$ epigenotype[37]. Moreover, genes specifically methylated in EBV$^+$ gastric cancers were shown to expand not only within PRC-target genes in ES cells but also to non-PRC-target genes. This result implies that EBV$^+$ gastric cancer is methylated *via* a unique mechanism(s). Subsequently, to clarify the causal role of EBV infection, we performed *in vitro* EBV infection experiments in low-methylation MKN7 gastric cancer cells to determine whether these cells would acquire extensive methylation and, as a result, the EBV$^+$-specific methylation epigenotype. The induced methylation repressed multiple genes, including multiple tumor-suppressor genes, suggesting a role for EBV in tumorigenesis[37].

The inducer of aberrant DNA methylation remains elusive. EBV exists in three latent forms defined by the expression pattern of latent genes. Lymphoblastoid cell line (LCL) and transformed primary B lymphocytes infected with EBV express all latent genes, LMPs (1, 2A, 2B), EBNAs (1, 2, 3A, 3B, 3C, LP), EBERs (1, 2), and BARTs, and this expression program is referred to as type Ⅲ latency. In contrast, Burkitt's lymphoma shows type I latency, with the minimum expression of EBNA1, EBERs, and BARTs only. Type Ⅱ latency, in which LMP1 and LMP2 are expressed in addition to

latency I genes, is observed in EBV-associated Hodgkin lymphoma, peripheral natural killer/T-cell lymphoma, and nasopharyngeal carcinoma. EBV$^+$ gastric cancer shows type I (or II) latency and expresses EBNA1, EBERs, BARTs, and LMP2A[105,111,123].

Several studies have elucidated the function of latent genes for promoter methylation. LMP1 was reported to down-regulate *CDH1* gene expression and induce cell migration using cellular DNA methylation machinery[124,125]. LMP2A plays an essential role in epigenetic abnormalities by inducing promoter methylation of *PTEN*[126]. EBER1 and EBER2 are small non-coding RNAs of approximately 170 bases in length that are abundantly expressed in the nuclei of latently infected cells, up to 10^7 copies per cell. Although some oncogenic properties of EBERs have been reported, such as the contribution of efficient growth transformation of B lymphocytes[127,128] or the induction of insulin-like growth factor 1 (IGF-I) acting as an autocrine growth factor in gastric cancer or nasopharyngeal carcinoma cells[129,130], the distinct influence of epigenetic modification remains unclear. Moreover, while these viral genes may play a role in aberrant methylation, methylation induction at the genome-wide scale, which can result from EBV infection, has not been demonstrated through the forced expression of any viral gene[37].

Rather than viral factors, host cellular mechanisms may play more important roles in the induction of aberrant methylation. In type I latency, while host cells induce dense methylation in the viral genome to silence most viral genes, the host genome itself is also extensively hypermethylated[131,132]. In type III latency, such as LCL, neither the viral genome nor the host cellular genome is significantly hypermethylated[132], and this observation implies that a host-driven mechanism that induces DNA methylation in the viral genome may affect methylation of the host genome. Recent exome sequencing analysis demonstrated that *ARID1A* was frequently mutated in EBV$^+$ gastric cancer[8,133], and other chromatin remodelers were also mutated[9]. While it is not known whether mutation of these genes is causally associated with aberrant methylation in EBV$^+$ gastric cancer, these chromatin remodelers may play a role in protecting the epigenomic status of the host genome from the pressure of methylation induction. Further investigation is necessary to clarify the roles of host cellular factors in methylation induction.

CONCLUSION

In gastric carcinogenesis, aberrant promoter methylation plays a major role by inactivating tumor-suppressor genes. Two pathogens, *H. pylori* and EBV, may contribute to carcinogenesis through the induction of aberrant methylation in gastric epithelial cells, although further study is necessary to elucidate the detailed molecular mechanisms underlying the induction of aberrant promoter methylation in response to infection with these two pathogens. Understanding these mechanisms could clarify the process of gastric carcinogenesis, and application of this knowledge for clinical use could aid in diagnosis, risk management, and prevention. Epigenetic aberrations can accumulate at early stages of carcinogenesis, preceding genomic mutations in polyclonal tissues; aberrant DNA methylation is therefore a powerful biomarker for the early detection of cancers and/or cancer risk. Moreover, from prophylactic or therapeutic viewpoints, aberrant DNA methylation could represent an attractive target due to its reversible nature. For example, a patient with persistent DNA methylation after *H. pylori* eradication might be a candidate for demethylation therapy to prevent gastric cancer. Moreover, in EBV$^+$ gastric cancers, aberrations in chromatin remodeling factors in background fields may promote EBV infection or carcinogenesis and could represent a target for the prevention of EBV$^+$ gastric cancer.

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