Mechanisms for the induction of gastric cancer by *Helicobacter pylori* infection: aberrant DNA methylation pathway

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Abstract Multiple pathogenic mechanisms by which *Helicobacter pylori* infection induces gastric cancer have been established in the last two decades. In particular, aberrant DNA methylation is induced in multiple driver genes, which inactivates them. Methylation profiles in gastric cancer are associated with specific subtypes, such as microsatellite instability. Recent comprehensive and integrated analyses showed that many cancer-related pathways are more frequently altered by aberrant DNA methylation than by mutations. Aberrant DNA methylation can even be present in noncancerous gastric mucosae, producing an “epigenetic field for cancerization.” Mechanistically, *H. pylori*-induced chronic inflammation, but not *H. pylori* itself, plays a direct role in the induction of aberrant DNA methylation. The expression of three inflammation-related genes, *Il1b*, *Nos2*, and *Tnf*, is highly associated with the induction of aberrant DNA methylation. Importantly, the degree of accumulated aberrant DNA methylation is strongly correlated with gastric cancer risk. A recent multicenter prospective cohort study demonstrated the utility of epigenetic cancer risk diagnosis for metachronous gastric cancer. Suppression of aberrant DNA methylation by a demethylating agent was shown to inhibit gastric

cancer development in an animal model. Induction of aberrant DNA methylation is the major pathway by which *H. pylori* infection induces gastric cancer, and this can be utilized for translational opportunities.

Keywords *Helicobacter pylori* infection · Aberrant DNA methylation · Epigenetic cancer risk diagnosis

Introduction

“How does *Helicobacter pylori* infection induce gastric cancer?” has long been a challenging question. For the last two decades, various pathogenic mechanisms of *H. pylori*-associated gastric cancer have been intensively investigated, and three major mechanisms have become clear. First, multiple signaling pathways were shown to be perturbed in gastric epithelial cells by virulence factors of *H. pylori* such as VacA and CagA [1, 2]. This mechanism is closely involved in the *H. pylori* type IV secretion machinery. Second, mutations were shown to be induced by aberrant expression of activation-induced cytidine deaminase (AID) via NFκB activation in gastric epithelial cells due to *H. pylori* infection-induced chronic inflammation [3]. Third, aberrant DNA methylation was shown to be accumulated in gastric mucosa by chronic inflammation caused by *H. pylori* infection [4].

In particular, multiple lines of evidence indicate that the accumulation of aberrant DNA methylation is very important in gastric carcinogenesis. Firstly, aberrant DNA methylation, a representative epigenetic alteration, can cause inactivation of tumor-suppressor genes. Indeed, comprehensive and integrated analyses of gastric cancer have shown that aberrant DNA methylation has a major impact [5, 6]. Secondly, the degree of accumulation of

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aberrant DNA methylation is highly correlated with gastric cancer risk [7, 8]. Furthermore, animal experiments have shown that inhibiting aberrant DNA methylation induction could prevent gastric cancer development [9].

In this review, we provide an overview of the current understanding of the mechanisms by which aberrant DNA methylation is induced by *H. pylori* infection. We also highlight potential applications of aberrant DNA methylation in precision medicine.

Deep involvement of aberrant DNA methylation in gastric cancer

Genetic and epigenetic alterations accumulate during multistep carcinogenesis through exposure to various carcinogenic factors [10]. However, few frequent driver mutations associated with gastric cancer have been identified besides *TP53* and *CDH1*. Although several new driver genes such as *ARID1A* and *RHOA* have been identified by recent exome and whole-genome sequencing [5, 11], such mutations account for less than 15 % of all gastric cancers. Indeed, more than 20 % of gastric cancers present only one or even no mutation [11].

On the other hand, a deep involvement of aberrant DNA methylation in gastric cancer has been highlighted [12]. In 1999, frequent occurrence of aberrant DNA methylation of CpG islands (CpG island methylator phenotype; CIMP) was shown to be associated with microsatellite instability in gastric cancer, as it is in colon cancer [13]. Characteristically, Epstein–Barr virus-positive gastric cancer has been known to display extreme CIMP [14, 15]. These findings were validated by The Cancer Genome Atlas (TCGA) Research Network [5]. Furthermore, a recent integrated analysis of genetic and epigenetic alterations revealed that inactivation of tumor-suppressor genes such as *p16*, *hMLH1*, and *CDH1* and activation of the WNT pathway were more frequently caused by aberrant DNA methylation than by mutations (Fig. 1) [6]. This evidence suggests that aberrant DNA methylation has as much or even more of an impact on gastric carcinogenesis than mutations.

DNA methylation level in noncancerous mucosa and gastric cancer risk

Aberrant DNA methylation can even be present in non-cancerous gastric mucosa, and its levels are influenced by *H. pylori* infection (Fig. 2). The association of aberrant DNA methylation in gastric mucosa with *H. pylori* was reported by Chan et al. for the first time in 2003 [16]. However, at the same time, Kang et al. showed that there was no association between them [17]. These conflicting

findings were considered to be due to nonquantitative DNA methylation analyses. Later, a quantitative methylation analysis focusing on CpG islands of passenger genes clearly demonstrated an association between high methylation levels in gastric mucosae and *H. pylori* infection [7].

At the same time, a cross-sectional study suggested that eradicating *H. pylori* leads to a decrease in DNA methylation levels [7]. Later, temporal analyses showed that eradication of *H. pylori* decreases DNA methylation levels [18–21]. Importantly, among individuals not currently infected with *H. pylori*, DNA methylation levels were much higher in cancer patients than in healthy individuals [7]. Additionally, methylation levels were higher in cases with multiple gastric cancers than in cases with a single cancer [8]. It was therefore suggested that DNA methylation levels in individuals not currently infected with *H. pylori* are closely correlated with gastric cancer risk.

Cell types and genes susceptible to aberrant DNA methylation

Gastric mucosal biopsy samples contain various types of cells in addition to epithelial cells. Therefore, the cell types that aberrant DNA methylation is induced in were unclear. This issue was addressed by observing increased methylation levels of multiple genes in gastric epithelial cells highly purified by the gland isolation technique from the stomachs of Mongolian gerbils (*Meriones unguiculatus*), a widely used animal model for *H. pylori* infection and gastric cancer [4]. Also, in a genome-wide DNA methylation analysis of human gastric mucosa, aberrant DNA methylation was still observed, even after the exclusion of CpG sites methylated in human blood cells, ruling out the possibility of increased methylation due to blood cell-specific methylation (Nanjo et al., unpublished data). These data showed that gastric epithelial cells are the real targets of aberrant DNA methylation induction. Nevertheless, there remains the possibility that aberrant DNA methylation is also induced in other types of cells, such as stromal cells, and that such epigenetic alterations may also be important for gastric cancer development.

Eradicating *H. pylori* decreases DNA methylation levels in gastric mucosae, and the decreased methylation levels persist for a long time [4, 20]. This suggests that aberrant DNA methylation consists of transient and permanent components. Mechanistically, we can speculate that the aberrant DNA methylation induced in stem cells of a gastric gland is a permanent component because methylation status in stem cells is preserved and replicated, thus determining the fraction of cells with methylation. In contrast, methylation induced only in differentiated cells will disappear when they are replaced by new cells without

Fig. 1 Genetic and epigenetic alterations of genes in multiple signaling pathways in 6 normal gastric mucosa and 50 gastric cancer samples. Three growth-promoting and four tumor-suppressor pathways are included. Inactivation of tumor-suppressor genes such as *p16*, *hMLH1*, and *CDH1* and activation of the WNT pathway were more frequently caused by aberrant DNA methylation than by mutations. This figure was modified from [6]

Fig. 2 *H. pylori* infection, DNA methylation induction, and gastric cancer risk. The clinical course of individuals infected with *H. pylori* is illustrated. The *H. pylori* infection occurs in childhood, causing chronic inflammation in the stomach. Chronic inflammation induces aberrant DNA methylation in gastric mucosa. Once *H. pylori* is eradicated, the DNA methylation level decreases somewhat but does not disappear completely. The degree of residual DNA methylation is strongly correlated with gastric cancer risk

methylation derived from a stem cell without methylation, meaning that this methylation induced in differentiated cells represents a transient component [22] (Fig. 3).

A large number of specific genes are aberrantly methylated by *H. pylori* infection [23]. Mechanistically, it is generally known that promoter CpG islands without active transcription of their downstream genes and with a specific histone modification, H3K27me₃, are likely to become methylated [24–26]. In addition to physiological H3K27me₃, aberrant H3K27me₃ can be induced by environmental factors such as chronic inflammation [27]. Therefore, in gastric mucosa, genes that are not expressed naturally or those that are downregulated by *H. pylori* infection are likely to become methylated.

Such genes that are not expressed naturally are considered to play no biological role in gastric mucosae. Therefore, the

methylation of such genes is likely to have no biological consequences in gastric carcinogenesis, and is thus considered a passenger event. On the other hand, although driver genes such as *p16*, *CDH1*, and *MLH1* are expressed in gastric mucosae with diverse expression levels, they are methylated in cancer cells. If we identify genes that are methylated in gastric cancer but expressed in normal gastric mucosae, they are more likely to be driver genes [28].

Mechanisms by which *H. pylori* infection induces aberrant DNA methylation

To verify that *H. pylori* infection induces aberrant DNA methylation, Mongolian gerbils were infected with *H. pylori*, and induction of aberrant DNA methylation in

Fig. 3 Potential target cells for the induction of aberrant DNA methylation. *Left:* gastric mucosa with active *H. pylori* infection. *Right:* gastric mucosa after eradication of *H. pylori*. Chronic inflammation, characterized by infiltration of monocytes/macrophages with neutrophils, induces aberrant DNA methylation. Aberrant DNA methylation is actively induced in differentiated cells, possibly in progenitor cells (transient component), along with some stem cells. When methylation is present in a stem cell, all of the cells derived from the stem cell in a gland are

methylated (permanent component). When methylation is induced in differentiated cells, heterogeneous methylation within a gland is present, and this methylation will disappear when fresh cells without methylation are derived from a stem cell. Without active induction of aberrant DNA methylation, the methylation status of a gland reflects that of its stem cell. The methylation level in the gastric mucosa is assumed to be proportional to the fraction of stem cells with methylation. This figure was modified from [22]

purified gastric gland cells was demonstrated [4]. In addition, eradicating *H. pylori* clearly decreased methylation levels, which were accompanied by diminished histological inflammatory responses (Fig. 4a). Then, to address whether *H. pylori* or the resultant chronic inflammation was responsible for inducing aberrant DNA methylation, inflammatory responses were repressed by cyclosporin A, an immunosuppressive agent, in *H. pylori*-infected gerbils. Although the amount of *H. pylori* was not reduced in the gastric mucosa, the repression completely suppressed the induction of aberrant DNA methylation [9]. Hence, it was concluded that it was not *H. pylori* itself but the inflammatory response triggered by *H. pylori* infection that was directly responsible for the induction of aberrant DNA methylation.

Fig. 4a, b Induction of aberrant DNA methylation by *H. pylori* infection in Mongolian gerbils and the effect of eradication. **a** After gerbils were infected with *H. pylori*, DNA methylation levels in purified gastric epithelial cells, as measured by quantitative methylation-specific PCR (qMSP), increased at ≥ 10 weeks of infection. After eradication, DNA methylation levels were not decreased at 1 week, but were decreased at 10 and 20 weeks. Importantly, DNA methylation levels after eradication were still higher than those in never-infected gerbils. **b** Capacities of various kinds of inflammation to induce aberrant DNA methylation. Persistent inflammation was induced by *H. pylori*, *H. pylori* strain SS1, *H. felis* infection, high concentrations of alcohol, and saturated NaCl. As controls, an MNU group and a nontreatment group were analyzed. In all eight CpG islands analyzed (methylation levels of CpG island HE6 are shown in Fig. 3b), only groups with *H. pylori*, *H. pylori* strain SS1, and *H. felis* infection showed the induction of aberrant DNA methylation. This figure was modified from [4]

The next question was whether any kind of persistent inflammation could induce aberrant DNA methylation. Mongolian gerbils were treated with alcohol or sodium chloride (NaCl), both of which are well known to be inflammation inducers. Aberrant DNA methylation was induced only by *H. pylori* and *H. felis*, but not by high concentrations of alcohol or saturated NaCl (Fig. 4b). *H. pylori* and *H. felis* triggered chronic inflammation as characterized by infiltration of monocytes/macrophages with residual neutrophils, whereas alcohol and NaCl elicited repeated acute inflammation as characterized by major infiltration of neutrophils [29].

Regarding inflammatory response genes, *Il1b*, *Nos2*, and *Tnf* were upregulated consistently in gastric mucosa of *H. pylori*- or *H. felis*-infected gerbils, and were associated with increased DNA methylation levels. Notably, *Il1b* and *Nos2* were also induced in mouse colonic mucosae with dextran sulfate sodium-induced colitis [30]. Consequently, we can conclude that aberrant DNA methylation is induced by specific types of inflammation, and is likely to be associated with the expression of *Il1b*, *Nos2*, and *Tnf*.

Application of aberrant DNA methylation induced by *H. pylori* infection

Aberrant DNA methylation in specific genes is frequent, even in noncancerous tissue, and contributes to carcinogenesis, so it could be used in a variety of applications relating to cancer risk diagnosis and chemoprevention.

Clinical study of epigenetic cancer risk diagnosis

The accumulation of aberrant DNA methylation in non-cancerous tissues has been termed an “epigenetic field for cancerization” or “epigenetic field defect,” especially in inflammation-associated cancers such as gastric cancer [31]. Cross-sectional studies have shown that the degree of a field defect can be assessed using appropriate cancer risk markers, as described above [32, 33]. However, cross-sectional studies inevitably include various types of biases. Recently, a multicenter prospective cohort study for predicting the risk of metachronous gastric cancer demonstrated the utility of an epigenetic cancer risk marker for the first time [34].

In this study, gastric cancer patients were enrolled after endoscopic submucosal dissection (ESD). After assessing the methylation levels of three preselected genes, annual follow-up to detect metachronous gastric cancer was conducted for 3 years by trained endoscopists who were blinded to methylation information. Multivariate analysis showed that the highest quartile of the methylation level of *miR-124a-3*, a marker gene, had a significantly higher HR

Fig. 5 Cumulative incidence of authentic metachronous gastric cancer (i.e., gastric cancer that developed after 1 year of enrollment). Patients were grouped into quartiles (Q1–Q4) based on methylation levels of *miR-124a-3*. Q4 (the highest) had a higher incidence of authentic metachronous gastric cancer than Q1 (the lowest). A multivariate analysis adjusting for hospital, gender, age, *H. pylori* infection before enrollment, pepsinogen index, past history of ER, smoking, and green vegetable intake showed that Q4 *miR-124a-3* methylation had a higher HR than Q1 methylation (95 % CI) (2.30 (1.03–5.10); $p = 0.042$). This figure was modified from [35]

of developing metachronous gastric cancer (Fig. 5) [35], showing that methylation levels can identify groups of patients at high risk for gastric cancer (Fig. 2).

That study achieved the proof-of-concept for epigenetic cancer risk diagnosis, but is unlikely to change clinical practice in relation to following up gastric cancer patients after ESD. In order to optimize a surveillance system based on individual risk, a new large-scale multicenter prospective cohort study (UMIN000016894) for predicting the risk of primary gastric cancer in healthy individuals after *H. pylori* eradication was proposed and is currently underway. The number of such healthy individuals is rapidly increasing in Japan after *H. pylori* eradication therapy was approved for chronic gastritis by the national health insurance [36].

Application to cancer prevention

Epigenetic alterations can be reversed by drug interventions and are therefore potential targets for chemoprevention. Importantly, a possible preventive effect of a DNA demethylating agent has been shown experimentally. Oral administration of a DNA demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), to *H. pylori*-infected gerbils treated with *N*-methyl-*N*-nitrosourea (MNU) reduced the incidence of gastric cancers from 55.2 to 23.3 % (Fig. 6a), which was accompanied by a decrease in methylation levels (Fig. 6b) [9]. However, currently available DNA

Fig. 6a, b Suppression of DNA methylation and inhibition of gastric cancers by 5-aza-dC treatment in *H. pylori*-infected gerbils treated with MNU. **a** Protocol of the carcinogenicity experiment. The incidence of gastric cancer was reduced from 55 % in group 2 of the *H. pylori*-infected gerbils with MNU to 23 % in group 3, which received 5-aza-dC ($p < 0.05$). **b** DNA methylation levels of CpG island HE6 in gastric epithelial cells (average $\pm$ SD). DNA methylation levels were significantly lower in G3 than in G2. * $p < 0.05$. This figure was modified from [9]

demethylating agents are not suitable for use in chemoprevention due to their adverse effects. Therefore, novel DNA demethylating agents with only minor adverse effects need to be developed, or intervention in an extremely high-risk population may be considered.

Conclusions

In this review, we have discussed the major impact of aberrant DNA methylation on gastric cancer and carcinogenesis, and current knowledge of the mechanisms for inducing aberrant DNA methylation. From the perspective of applying this knowledge, epigenetic cancer risk diagnosis is becoming a reality in the clinical setting. Clarification of the molecular mechanisms involved in aberrant DNA methylation induction is expected to provide a new strategy for the chemoprevention of gastric cancer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human or animal subjects performed by any of the authors.

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