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Journal of Carcinogenesis & Mutagenesis

Review Article

Helicobacter pylori Infection, DNA Methylation, and Gastric Carcinogenesis

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ABSTRACT

Helicobacter pylori (*H. pylori*), the major cause of chronic gastritis, peptic ulcers and gastric cancer, infects about 50% of the world population. Although various host and bacterial factors have been suggested, the detailed pathogenic mechanisms remain to be defined. Increasing evidences have demonstrated that epigenetic dysregulation, such as DNA methylation, plays a critical role in gastric carcinogenesis, and is currently under intensive investigation. *H. pylori* infection result in aberrant DNA methylation in a number of gene promoters in gastric mucosa, eradication of *H. pylori* can reverse some hypermethylated genes, but had no effect on others. In some methylated genes, the methylation levels persist even after *H. pylori* eradication, and the fact suggests that DNA methylation accumulation is associated with molecular irreversibleness and gastric diseases progression. DNA methylome and gastric cancer risk analysis indicate that certain gene promoter methylation may serve as potential biomarkers for gastric cancer predication. In addition, *H. pylori cagA* and *vacA* s1m1 genotype are independent variables that are associated with higher methylation level. The levels of methylation can be influenced by the degree and length of infection exposure, and certain host gene polymorphisms are also associated with gene methylation in *H. pylori*-infected subjects. Continued investigation in these areas will be critical to provide insights into the molecular mechanisms of *H. pylori*-induced gastric diseases and develop strategies for disease prevention and intervention. We review recent progress and discuss future research directions in this important area.

Keywords: *Helicobacter pylori*; DNA methylation; Gastrointestinal disease; Gastric cancer

INTRODUCTION

Helicobacter pylori (*H. pylori*), a very common gram-negative bacterium, colonized in mucus layer of human stomach, is the major cause of chronic gastritis, peptic ulcers and gastric cancer. *H. pylori* infects approximately 50% of world population, and is also closely associated with a number of extra-gastrointestinal diseases: such as iron deficiency anemia, idiopathic thrombocytopenic purpura, vitamin B12 deficiency, autoimmune diseases, cerebrovascular diseases, etc. [1,2]. The cancer statistics of 2018 has indicated that gastric cancer is the fifth most frequently diagnosed cancer and the third leading cause of cancer death worldwide [3].

H. pylori possesses a number of virulent factors, such as cytotoxin associate gene A (CagA), vacuolating cytotoxin A (VacA), outer inflammatory protein (OipA), outer membrane protein (OMP), and duodenal ulcer-promoting factor (DupA) etc. [4-6]. Upon infection, *H. pylori* attaches to gastric epithelial cells and triggers numerous cellular, inflammatory, and oncogenic signalings. These activated host cellular signaling pathways include AP-1, NF-κB, TGF-β, Wnt, Stat3, p53 pathways etc. *H. pylori* infection also result in either genetic or epigenetic alternations in various types of cells, such as epithelial cells, fibroblasts, immune cells, stem cells through their interacting microenvironment, and cause dysregulation of important cellular events, such as cell proliferation, apoptosis, cell movement, migration, impairment of DNA repair mechanisms and ultimately oncogenic transformation. Despite intensive investigation, it remains to be defined on how and why long term *H. pylori* infection will lead to the development of pre-cancerous conditions and gastric cancer [4-6].

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Received: July 01, 2020; **Accepted:** July 15, 2020; **Published:** July 22, 2020

Citation: Qi YB, Hu RB, Khan MN, Lei L, Wei PR, Jia BL, et al. (2020) *Helicobacter pylori* Infection, DNA Methylation, and Gastric Carcinogenesis. J Carcinog Mutagen. 11:352.DOI:10.35248/2157-2518.20.11.352.

H. PYLORI VIRULENT FACTORS AND GASTRIC CANCER

H. pylori has a smaller genome, and it has been sequenced several times, the genome of recently sequenced strain B128 7.13 consists of one circular chromosome (1.67 Mbp), one plasmid (6.149 kb), and the chromosome contains 1584 identified open reading frames [7]. *H. pylori cag*PAI is a 40 kb region in its genome, contains 31 open reading frames and encodes type IV secretion system (TFSS), *cag*A gene is localized in the -3'end of the island and encodes a 120-145 kDa CagA protein. *H. pylori* strains that carry *cag*PAI with CagA-,VacA-positive cause severe clinical gastric inflammation, which predispose to either tissue damage or neoplastic transformation, therefore are high-risk strains of gastric cancer, and the role of CagA protein is critical in these processes [6,8-10].

CagA protein is delivered into gastric epithelial cells *via* TFSS, and once inside the cell, CagA undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, it then acts as a non-physiological scaffold/hub protein by interacting with multiple host signaling molecules, most notably the pro-oncogenic phosphatase SHP2 and the polarity-regulating kinase PAR1/MARK [11]. In addition to CagA, bacteria peptidoglycan and DNA can also be delivered by TFSS into epithelial cells, and activate multiple cellular signaling as mentioned above, especially NF-κB signaling, which contributes to the inflammatory process and oncogenic transformation [4-6]. Three layers of evidence have clearly indicated the role of CagA in gastric carcinogenesis: (1) *H. pylori* strains that carry mutated *cag*A gene do not have the tumor initiating effects in animal model [9]; (2) *in vivo* transgenic mice model using artificially synthesized whole sequence of *cag*A gene resulted in the development of gastric cancer and other gastrointestinal and hematological tumors in mice without *H. pylori* infection *per se* [8], suggesting that it has the characteristics of oncogenic protein; (3) CagA-,VacA-positive strains are the major forms of *H. pylori* infection clinically in many areas globally, corresponding to their high prevalence in pre-cancerous lesions and gastric cancer incidences in population-based study [10].

Gastric cancer can generally be subdivided into intestinal and diffuse types according to Lauren classification. (1) Intestinal type of gastric cancer has a distinct ductal structure, and is commonly found in older men and manifests as a Correa cascade, including atrophic gastritis, intestinal metaplasia, dysplasia and cancer. (2) Diffuse type of gastric cancer is characterized with diffuse growth, predispose to have lymph nodes and distant metastasis, closely related to genetic inheritance, more common in young women, but both types of gastric cancer are closely related to *H. pylori* [12]. Although many factors, such as diet, high salt, chemical factors, life style, and changes in stomach flora were suggested to be involved in gastric cancer initiation and development, *H. pylori* infection remains the single most important pathogenic factor for gastric cancer [1,4,5].

H. PYLORI- INDUCED DNA METHYLATION IN GASTRIC CANCER

Epigenetic changes are generally categorized into four areas: DNA methylation, histone modification, chromatin remodeling and miRNAs [13]. Epigenetic dysregulation is a hallmark of cancer, and DNA methylation is the major form of epigenetic modifications in cancer cells. DNA of cancer cells is generally hypomethylated, while promoters of certain genes are hypermethylated, both of which are implicated in carcinogenesis, as this will lead to either activation of oncogenes or inactivation of tumor suppressor genes [13,14]. Epigenetic aberrations induced by *H. pylori* infection have been recognized as a critical component of gastric carcinogenesis [15-17].

Connections of *H. pylori* infection, DNA methylation and gastric cancer have been noted in just recent years, many questions remain to be explored, including the effects of bacterial virulence factors such as CagA, VacA, *cag*PAI on DNA methylation, interaction with other epigenetic modifications, and their potential application as gastric cancer biomarker. *H. pylori* infection result in aberrant DNA methylation in a number of gene promoters in gastric mucosa which have been studied extensively [15-19], including cell growth-related genes p16 (INK4a), p14 (ARF) and APC; DNA-repair genes, hMLH1, BRCA1 and MGMT; cell adherence gene E-cadherin; as well as LOX, FLNC, HRASLS, HAND1, THBD and p41ARC, which are known to be methylated in gastric cancer patients [15-19]. Eradication of *H. pylori* lead to reduced methylation levels of several genes; but in other genes, the methylation levels persist even after *H. pylori* eradication, suggest that DNA methylation accumulation is associated with molecular irreversibleness and disease progression in gastric mucosa [15,20,21].

The long-term effects of *H. pylori* eradication on promotor CpG island hypermethylation in gastric carcinogenesis were recently evaluated. Gene methylation rate and median values of several tumor-related genes including p16, CDH1, and RUNX3 were tested before and one year after *H. pylori* eradication. The results showed that *H. pylori*-infection increased DNA methylation rate over the normal control, and methylation rates decreased at p16 and CDH1 genes after bacteria eradication. In contrast, the methylation levels of RUNX3 gene had no difference even one year after *H. pylori* eradication, indicating that *H. pylori* eradication may reverse some hypermethylated genes, but had no effects on others [21].

H. pylori infection also increases RUNX3 promoter methylation that correlated with distinct stages of gastric cancer progression. In another observation by Lu et al. gastric cancer tissues had the highest RUNX3 methylation rate (75.2%) over the atrophic gastritis (15.9%), intestinal metaplasia (36.7%), gastric adenoma (41.8%), and dysplasia (54.9%) groups [22]. The levels of RUNX3 methylation in blood samples correlated to the methylation levels observed in gastric cancer tissues. These findings support the notion that RUNX3 methylation as a risk factor for carcinogenesis during *H. pylori* infection, and RUNX3 methylation from blood samples might be a valuable biomarker for early gastric cancer detection.

H. pylori infection exert different effects on DNA methylation dependent on gastric disease stages, changes of DNA

methylation in gastric mucosa after *H. pylori* eradication were investigated in LOX, APC and MOS genes [23,24]. The patients were followed-up for an average of 26.0 months (range: 6 to 76 months). *H. pylori* eradication decreased methylation levels in LOX, but not in APC gene. In MOS gene, methylation level decreased following *H. pylori* eradication over the controls groups without intestinal metaplasia (IM); but not in patients with IM or in those with dysplasia or gastric cancer. This effect was also noticed in miRNAs methylation during *H. pylori* infection, as Watari et al. [24] noted that miR-124a-3 methylation is reduced after *H. pylori*-eradication in non-IM patients, but not in IM mucosa. The results indicate that *H. pylori* eradication affect DNA methylation in disease stage and gene-specific manner.

Accumulation of aberrant DNA methylation in normal tissues is associated with the risk of gastric carcinogenesis [25]. Using a Mongolian gerbil model of *H. pylori*-induced gastritis, the degree of infection exposure on methylation burden was analyzed in four CpG islands, including HE6 (exon 2 of Ntrk2 gene), SA9 (exon 1 of Nol4 gene), SB5 (location not identified), and SD2 (promoter of Nptx2 gene), which were previously identified as aberrantly methylated genes by *H. pylori* infection. The methylation levels were increased depending on the infection duration. DNA methylation levels decreased after *H. pylori* eradication, but tended to be higher in gastric mucosae with a longer infection period. DNA molecules with dense methylation, but not in those with sparse methylation, increased depending on the infection period. The data suggest that the level of methylation can be influenced by the degree of exposure to *H. pylori* infection.

In addition to the promoter specific effect and intensity of infection, virulence of bacteria strains and inflammation also affect DNA methylation level. Schneider et al. in 2013 tested DNA methylation levels in gastric biopsies at promoters of EN1, PCDH10, RSPO2, ZIC1 and ZNF610 genes during *H. pylori* infection [26]. The results found that *cag*A positive samples had higher methylation level over the uninfected persons; *cag*A-negative *H. pylori* strains only induced intermediate DNA methylation. *H. pylori vac*A s1m1 genotype is highly associated with *cag*A positivity, and *vac*A s2m2 genotype is associated with *cag*A negativity, and methylation level was not related to the number of EPIYA motifs in CagA proteins. Presence of *cag*A and *vac*A s1m1 in *H. pylori* strain were independent variables associated with higher methylation in these genes. In addition, high levels of mononuclear cell infiltration were significantly related to methylation in PCDH10, RSPO2, and ZIC1 genes. In another study, *H. pylori* CagA-induced tumor suppressor gene MGMT hypermethylation was shown by upregulating DNMT1 *via* AKT-NF- κ B pathway, and MGMT promoter methylation is positively correlated with the presence of CagA in clinical specimens [27].

Gene promoter methylation also appears associated with tumor location and histological type. Alves et al. noted that in cardia tumors, p16(INK4A) showed negative histochemical staining; in noncardia tumors, a significant finding was HMLH1 inactivation by methylation in intestinal type of gastric cancer; while in diffuse subtype, CDKN2A inactivation by methylation was prominent [28]. Tumors with methylated COX-2 and HMLH1 genes were associated with *H. pylori vac*A s1 genotype, and nonmethylated tumors were associated with the presence of *fla*A gene. The results suggest that inactivation of these genes by methylation occurs by distinct pathways related to histological subtype and tumor location, and also depends on the *H. pylori* genotype. In addition, DNA methylation accumulation is even found in gastric mucosa adjacent to cancer after *H. pylori* eradication [20].

Using high-throughput methylation microarray technology, genome-wide methylation profiling of *H. pylori* and cancer-associated DNA methylome changes were analyzed. Woo et al. in 2018 found that *H. pylori* infection were associated with 1,924 differentially methylated positions (DMPs) and 438 differentially methylated regions (DMRs) in gastric biopsy samples, 97.3% of them (1,872 DMPs) were hypermethylated [29]. *H. pylori*-associated DMP/Rs showed marked stability after *H. pylori* clearance, suggesting an "epigenetic memory". In addition, single-nucleotide polymorphism array analysis from patient peripheral blood leukocytes found that 152 DMRs were associated with cancer risk that is independent of *H. pylori* infection status in normal gastric mucosa; *H. pylori* and cancer-specific methylation signatures were minimally affected by this genetic variation. These genes therefore may contribute to the gastric carcinogenesis, and also have the potential to be biomarkers for gastric cancer detection.

DNA METHYLATION AS A BIOMARKER IN GASTRIC CANCER PREDICATION AND DISEASE PROGRESSION

Recent advances start to investigate whether certain gene promoter methylation might be able to serve as biomarkers for gastric cancer and disease stage predication. A variety of samples were tested including serum, plasma and gastric washes. The DNA methylation status of tissue, particularly blood, has been associated with predisposition to gastric cancer, further studies are required to validate the results and their usefulness in clinical practice [16,30-32].

Maeda et al. in 2018 investigated nine candidate marker genes, including FLT3, LINC00643, RPRM, JAM2, ELMO1, BHLHE22, RIMS1, GUSBP5, and ZNF3 as epigenetic markers for gastric cancer risk stratification in individuals after *H. pylori* eradication [16]. The results showed that these genes had significantly higher methylation levels in gastric cancer patients than that in gastric atrophy patients. The candidates had sufficient performance (AUC: 0.70-0.80) and high odds ratios (5.43-23.41). The methylation levels of these markers were not associated with gastric atrophy, gender, or age [16].

Using gastric wash DNA or gastric juice exosomal DNA, methylation level of BARHL2 gene was tested for its usefulness as a marker for detection of early gastric cancer. Yamamoto et al. found high levels of BARHL2 methylation in gastric wash-derived DNA obtained from early gastric cancer patients [32]. Analysis using gastric juice-derived exoDNA samples revealed that BARHL2 methylation yielded an area under the curve of 0.923 with 90% sensitivity and 100% specificity in discriminating gastric cancer patients from controls. Future studies are required to confirm if the gastric wash-derived DNA

and/or gastric juice-derived exoDNA might be useful for early cancer detection in clinical setting.

Development of the intestinal subtype of gastric cancer is marked by progression of histopathologic lesions. In a Colombian study in Andean regions in 2015, gastric biopsy samples were examined on the effects of *H. pylori* eradication and antioxidants treatment on the progression of gastric lesions, the patients were followed for 6, 12, and 16 years [30]. Methylation levels of AMPH, PCDH10, RSPO2, SORCS3, and ZNF610 genes were able to predict progression of gastric lesions independent of the duration of *H. pylori* infection, baseline diagnosis, gender of the patient, or scores for mononuclear leukocytes, polymorphonuclear leukocytes, or intraepithelial lymphocytes. Therefore, DNA methylation levels in AMPH, PCDH10, RSPO2, SORCS3, and ZNF610 may contribute to the identification of persons with gastric lesions likely to progress [30].

Metachronous gastric cancer (MGC) can develop after endoscopic tumor resection. Suzuki et al. in 2014 tested if miR-34b/c, SFRP1, SFRP2, SFRP5, DKK2 and DKK3 genes promoter methylation might be able to predict the risk of MGC development after endoscopic tumor resection, they noted 17 (13%) out of 129 patients developed MGC after curative endoscopic therapy [33]. The cumulative incidence of MGC was significantly higher among patients with elevated miR-34b/c, SFRP2 and DKK2 methylation in their gastric body. MiR-34b/c showed the strongest association with the risk of MGC. In another multicenter prospective cohort study of 826 patients in 2015, miR-124a-3 has been suggested as a better marker for predicting the risk of developing metachronous gastric cancer [34]. Further studies will be required to confirm these results and develop protocols for clinical tests.

GENE POLYMORPHISMS AND DNA METHYLATION IN GASTRIC CANCER

Gene polymorphisms are associated with gastric carcinogenesis. DNA methylation in gastric cancer seems to be influenced by the presence of host gene polymorphisms and by *H. pylori cagA/vacA* s1m1 strains [35].

da Costa et al. investigated the interleukin (IL) polymorphisms in gastric tumor samples during *H. pylori* infection. In cardia tumors, methylation in COX-2 promoter was associated with IL-1RA Allele 2(IL1RN*2) genotype, and the associated genotypes IL1B511T+IL1RN*2 seem to be important in the methylation of COX-2 gene, especially infected by *H. pylori* strains that carries *cagA* and *vacA* s1 [35]. The associated genotypes IL6 CC+TNF GG appear to be involved in the unmethylation of CDKN2A along with *cagA*-positive *H. pylori* infection.

The NF-κB1 polymorphisms, -94 insertion (ins)/deletion (del) (rs28362491) and -449 C>G (rs72696119), were recently investigated for their effects on the aberrant gene methylation during *H. pylori* infection [36]. Methylation status was determined in p14ARF, p16INK4a, DAPK and CDH1 gene promoters in gastric mucosa. The results found that -94 del/del homozygosity was significantly associated with risk for developing CpG island methylation, and the number of methylated genes was significantly higher in -94 del/del homozygotes than that in ins/del and ins/ins (ins carrier) *H. pylori*-infected elder subjects. In addition, the inflammation score was significantly higher in *H. pylori*-infected del/del homozygotes over the ins carriers. Therefore, NF-κB1 -94 ins/del ATTG polymorphism (rs28362491) is associated with increased risk developing age-related gene methylation in non-cancerous gastric mucosa during *H. pylori* infection [37].

Genome-wide association study reveal polymorphisms (rs2294008) in prostate stem cell antigen (PSCA) gene are also associated with gastric cancer especially the diffuse type. PSCA rs2294008 C/T polymorphisms were genotyped in 410 cancer-free subjects in relation to promoter CpG island methylation status of three candidate genes (IGF2, MYOD1, and SLC16A12). Methylation levels of all three genes were significantly higher in subjects with PSCA rs2294008 T/T compared with the PSCA rs2294008 C/C, and *H. pylori* infection enhanced the methylation.

These observations provide evidences that host gene polymorphisms may influence the susceptibility of DNA methylation induction in gastric mucosa. However, the detailed molecular mechanisms and their impact on the cancer susceptibility remain to be explored. Future works are required to validate the results and their usefulness in identifying cancer susceptible candidates for disease prevention.

CONCLUSION

Methylation of CpG islands in gene promoter is one of the most characteristic abnormalities in *H. pylori*-induced gastric cancer; despite recent advance, the detailed molecular mechanisms remain to be investigated. *H. pylori*-induced inflammatory microenvironment, including increased inflammatory cell infiltration, reactive oxygen species, cytokines, growth factors and hormones production together impact on DNA methylation in epithelial cells and facilitate oncogenic transformation. Future studies addressing interactions of these factors and their impacts on other epigenetic events will be critical to uncover their roles in gastric carcinogenesis, and more importantly, will provide options for gastric cancer prevention and intervention.

FUNDING

This work was supported by grants to SZD from National Natural Science Foundation of China, No.U1604174; Henan Provincial Government-Health and Family Planning Commission, No.20170123; Henan Provincial Government-Health and Family Planning Commission Research Innovative Talents Project, No.51282; and Henan Provincial Government-Science and Technology Bureau, No.142300410050.

The funding sources had no role in the design of this study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

ACKNOWLEDGEMENT

Authors are grateful to the staffs of Dept. of Gastroenterology and Hepatology, People's Hospital of Zhengzhou University for their valuable assistance in this work.

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