Helicobacter pylori-induced inflammation and epigenetic changes during gastric carcinogenesis

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Abstract
The sequence of events associated with the development of gastric cancer has been described as "the gastric precancerous cascade". This cascade is a dynamic process that includes lesions, such as atrophic gastritis, intestinal metaplasia and dysplasia. According to this model, Helicobacter pylori (H. pylori) infection targets the normal gastric mucosa causing non-atrophic gastritis, an initiating lesion that can be cured by clearing H. pylori with antibiotics or that may then linger in the case of chronic infection and progress to atrophic gastritis. The presence of virulence factors in the infecting H. pylori drives the carcinogenesis process. Independent epidemiological and animal studies have confirmed the sequential progression of these precancerous lesions. Particularly long-term follow-up studies estimated a risk of 0.1% for atrophic gastritis/intestinal metaplasia and 6% in case of dysplasia for the long-term development of gastric cancer. With this in mind, a better understanding of the genetic and epigenetic changes associated with progression of the cascade is critical in determining the risk of gastric cancer associated with H. pylori infection. In this review, we will summarize some of the most
relevant mechanisms and focus predominantly but not exclusively on the discussion of gene promoter methylation and miRNAs in this context.

Key words: Helicobacter pylori; Methylation; Gastric cancer; Epigenetics; MicroRNA

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Core tip: Helicobacter pylori infection increases the risk of developing gastric cancer. Intestinal type gastric cancer is characterized by a histological cascade in which aberrant methylation of CpG islands and deregulation of microRNAs are observed. An exacerbated host response and bacterial virulence factors contribute to these epigenetic changes by enhancing DNA methyl transferase activity via nitric oxide production and silencing of tumor suppressor genes and miRNAs. Interestingly, methylated Reprimo DNA is detectable in blood samples and is potentially useful as an early detection marker. Finally, also the role of gamma glutamyl transpeptidase related mechanisms in the loss of the anti-apoptotic protein Survivin and gastric carcinogenesis is discussed.


H. PYLORI-INDUCED INFLAMMATION,
EPIGENETICS AND GASTRIC CANCER

Persistent inflammation is a prevalent contributing factor to the development of many types of cancer[11-13]. For instance, infection with bacterial and viral agents is known to promote cancer in different epithelia[14]. Among the many potentially relevant intermediates produced during inflammation are molecules, such as cytokines, chemokines, free radicals (ROS and NOS), prostaglandins, growth factors and matrix metalloproteinases (Figure 1). These induce different epigenetic changes, including DNA methylation and histone modifications, which contribute to several steps important in tumorigenesis and progression involving leukocyte recruitment, neo-angiogenesis, proliferation, survival, invasion, and finally metastasis of tumor cells[13,15]. Specifically, H. pylori-induced gastric carcinogenesis has been associated with chronic inflammation, characterized by infiltration of neutrophils and macrophages to the gastric epithelium, which favor the accumulation of pro-inflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS). Accordingly, host factors exacerbating inflammatory responses are also responsible for the final outcome. For instance, polymorphisms present in IL-8, TNF-α and IL-1β promoter regions are associated with more severe signs of inflammation associated, an increased risk of developing gastric cancer[16-19]. H. pylori-associated chronic inflammation is linked to...
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...silencing of tumor suppressor genes via epigenetic modification[20]. Methylation of CpG islands in the promoter regions of tumor suppressor genes and deregulation of some onco-miRs have emerged as highly relevant players in the context of understanding *H. pylori*-linked gastric carcinogenesis.

Importantly, studies in animal models of *H. pylori*-associated carcinogenesis further support this hypothesis. For instance, a study in the Mongolian gerbil model, demonstrated that *H. pylori* infection promotes DNA methylation as a consequence of chronic inflammation[21]. Interestingly, bacterial eradication did not decrease significantly overall DNA methylation in the gastric epithelium. However, upon suppression of inflammatory responses in gerbils treated with Cyclosporin A, the increased level of methylation was blocked without affecting bacterial colonization[22]. Following a similar rationale, the same authors described that prevention of DNA methylation using 5-aza-2-deoxycytidine decreased the incidence of gastric cancer induced by *H. pylori* infection[23]. However, in this study development of gastric cancer was not completely prevented, suggesting *H. pylori* could promote gastric cancer via additional mechanisms beyond aberrant DNA methylation, perhaps by favoring the development of genetic mutations. Likewise, a clinical study using human gastric samples showed that higher levels of DNA methylation in some genes typically hypermethylated in gastric cancer correlated with more severe gastric inflammation and more advanced precancerous lesions[23].

The effect of *H. pylori* induced-inflammation on epigenetic DNA modifications has also been observed in vitro. Katayama and colleagues[24] showed that *H. pylori* induces iNOS expression and NO production in macrophages, and the co-culture of macrophages and gastric cells promotes RUNX3 methylation in the gastric cells, without a requirement for direct interaction between gastric cells and the bacteria[24]. Alternatively, Huang et al[25] showed that E-cadherin gene methylation occurred in a manner independent of immune cells, because the direct interaction between *H. pylori* and gastric cells increased methylation of this gene. Furthermore, DNA methyl transferase (DNMT) activity was enhanced by increasing NO levels due to iNOS induction in host cells triggered by IL-1β secretion. Moreover, the participation of interleukin-1β in *H. pylori*-induced gastric inflammation and DNA methylation was confirmed using a receptor type 1 knockout (IL-1R1−/−) mouse model in which gastritis, NO production and methylation levels were reduced[26]. Despite some discrepancies between these studies, both point towards the importance of the pro-inflammatory molecule NO as a key mediator in the *H. pylori*-induced methylation of genes relevant to the genesis of gastric cancer. In agreement with this conclusion, *H. pylori* infection of INOS−/− mice lead to a decreased incidence of gastric cancer compared with wild type controls[27].

**DNA DAMAGE AND *H. PYLORI* INFECTION**

During gastric cancer progression and similar to other types of cancer, chronic inflammation leads to epigenetic changes characterized by the hypermethylation of driver tumor-suppressor genes, as well as passenger genes, and the hypomethylation of repetitive DNA sequences generating genomic instability[20,28]. Consistent with these observations, dietary supplementation with folic acid (a methyl donor) prevented the decrease in global DNA methylation as well as gastric dysplasia and mucosal inflammation observed during *H. pylori*-associated carcinogenesis in mice[29]. However, for an important proportion of gastric cancers mutations in tumor suppressor genes are also detected, indicating that this type of cancer originates not only as an epigenetic disease. For instance, mutations, particularly in genes like TP53, APC, CTNNB1, CDH1 and KRAS are frequently observed in gastric cancer[30].

Inflammatory processes initiated in response to *H. pylori* infection are accompanied by the formation of reactive oxygen and nitrogen species (ROS/NOS), predominantly by activated neutrophils, macrophages and gastric cells in response to bacterial factors[31,32] (Figure 1). As a consequence, elevated levels of oxidized proteins and fragmented DNA are detected in the gastric mucosa[31]. Nitric oxide produced by inducible oxide synthase (iNOS) increases early in the infected mucosa and, as a consequence, DNA adducts, such as 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG or 8-OHdG) are readily detected in humans and animal models[33,34]. Another mechanism that contributes to mutagenesis of host DNA is the *H. pylori*-induced aberrant NF-κB-dependent expression of activation-induced cytidine deaminase (AID), an enzyme that functions as a DNA- and RNA-editing enzyme in response to *H. pylori* infection[35]. Uppregulation of AID favored nucleotide alterations in the TP53 tumor suppressor gene in gastric cells in vitro, and similar alterations were also detected in the gastric mucosa of infected patients[35,36]. Studies in animal models indicate that oxidative stress is an important contributing factor to gastric carcinogenesis. For instance, nutritional supplementation with sulforaphane, a natural compound and activator of the antioxidant transcriptional factor Nrf2, was shown to protect mice against gastric pathologies induced by *H. pylori* infection[37]. Furthermore, similar results were obtained upon supplementation with antioxidants like ascorbic acid in humans[38]. A possible explanation for these observations is that ROS/NOS are responsible for methylation of antioxidant enzyme genes[39]. Also, it is important to consider that ROS accumulation produces directly epigenetic changes. For instance,
free radical adducts have been shown to alter DNA cytosine methylation\(^{[39]}\) and NO can enhance DNMT activity\(^{[25]}\).

**H. pylori Virulence Factors Associated with Inflammatory Responses and Epigenetic Changes in the Gastric Mucosa**

The experiments in Mongolian gerbils infected with *H. pylori* showing that the presence of the immunosuppressor drug Cyclosporine A reduces the ability of *H. pylori* to induce changes in the methylation status in gastric cells would suggest that *H. pylori* is not directly responsible for triggering aberrant methylation and that these alterations may be attributed to the inflammatory response. However, this point is still controversial because the *H. pylori* genome encodes a large number of cytosine and adenosine-specific type II DNA methyl transferases that would be expected to eventually methylate the host DNA because no additional factors are required for enzymatic activity. Relevant examples in this context is m6A or m4C modifications that normally are not considered in current studies\(^{[40,41]}\).

Also, epidemiological and basic research data all point towards a number of bacterial factors as being responsible for the observed gastric inflammation, deregulation of cellular signaling pathways and cancer. Some 60% of Western *H. pylori* isolates bear a *cag* pathogenicity island (*cag*PAI)\(^{[11]}\), which encodes among other components a type IV secretion system (T4SS)\(^{[42]}\) that is able to transfer macromolecules, peptidoglycans and DNA into the host cells\(^{[42]}\) (Figure 1). The presence and functionality of the *cag*PAI correlates very strongly with the development of peptic ulcer and gastric cancer\(^{[43]}\). Interestingly, NF-κB activation is found to be dependent on an intact T4SS secretion system\(^{[44]}\).

For instance, one study showed that peptidoglycans injected by the T4SS apparatus were responsible for NF-κB activation via the intracellular pathogen recognition receptor NOD1\(^{[45]}\).

As mentioned previously, Mongolian gerbils infected

![Figure 1 Helicobacter pylori-linked inflammation promotes methylation of genes associated with tumor suppression. A: Helicobacter pylori (*H. pylori*) induces, via injection of the virulence factors CagA and peptidoglycan (PGN), NF-κB activation, promoting a pro-inflammatory response that increases the expression of cytokines (IL-1β, IL-8) and inducible nitric oxide synthase (iNOS). The latter generates nitric oxide (NO) a mediator of inflammation. Furthermore, the *H. pylori*-linked activation of NF-κB promotes miR-155 expression, a microRNA associated with gastric pathologies; B: In addition to the direct effects of *H. pylori* on gastric epithelial cells, the infection activates macrophages, which increase NO production and NO levels in gastric epithelial cells. NO can activate DNA methyltransferase 1 (host DNMT1) to promote DNA methylation in several gene promoter regions; C: Also *H. pylori* DNA Methyltransferases (*Hp* DNMTs) may directly promote host DNA methylation in the promoter regions of several genes thought to function as tumor suppressors in gastric cancer. Moreover, infection is associated with promoter methylation and decreased expression of miR210, a miRNA which targets mRNAs implicated in stem cell survival, stalling of the DNA repair system, induction of angiogenesis and cellular differentiation, among others.](image-url)
with \textit{H. pylori} undergo histological changes similar to those observed in human gastric carcinogenesis\cite{54}. In a recent study, the inactivation of the peptidoglycan deacetylase (PgdA) enzyme in a particularly carcinogenic \textit{H. pylori} strain (\textit{H. pylori} 7.13, isolated following animal passage)\cite{67}, showed that this peptidoglycan modification enhanced the pro-inflammatory activity of this molecule\cite{48}. However, it should be noted that this observation is controversial, because in another study PgdA inactivation contributed to bacterial survival by mitigating the immune response\cite{49}. Together, these studies demonstrate once again the relevance of T4SS functionality in the promotion of inflammation and gastric cancer, although the precise contribution of peptidoglycan-dependent activation of the intracellular receptor NOD1 to aberrant methylation requires further investigation.

On the other hand, the T4SS is also implicated in the injection of another important carcinogenic factor, the immuno-dominant cytotoxin CagA (cytotoxin associated gene A), which is perhaps the best characterized factor in \textit{H. pylori-associated gastric cancer development}\cite{50} (Figure 1) Accordingly, patients infected with CagA+ strains develop more pronounced inflammation and higher levels of IL-8 and IL-1β\cite{51}. CagA (125-145 KDa) is also encoded in the cagPAI and is present in 60% of \textit{H. pylori} isolates. After bacterial adhesion, this factor is injected into host cells by the T4SS\cite{52,53}. Once attached to the inner leaflet of the plasma membrane, multimeric CagA is phosphorylated by c-Src and c-Abl kinases on tyrosine residues present in the Glu-Pro-Ile-Tyr-Ala (EPIYA)- motifs of the C-terminal region of the protein\cite{52,53}. Four different EPIYA motifs (A, B, C and D) exist, which determine the genetic variability of CagA due to differences in the number and the sequences of these motifs. In particular, the D motif is exclusively found in East-Asian CagA+ strains, where its presence is considered a major risk for developing gastric cancer\cite{54}. The oncogenic activity of CagA has been demonstrated in a variety of settings where sustained expression of this factor promotes primary gastric cell immortalization\cite{55}, intestinal trans-differentiation\cite{56}, epithelial-mesenchymal transition\cite{56}, loss of cell-cell adhesion\cite{57}, extracellular matrix remodeling\cite{58}, metastasis\cite{59} and gastrointestinal and hematopoietic neoplasms in mouse models\cite{60}. Signaling pathways may be altered by CagA activity both in phosphorylation-dependent and -independent ways\cite{61}. Phosphorylation-dependent downstream signaling processes include the recruitment of Src homology 2 (SH2)-domain containing molecules\cite{62}. The ensuing events perturb the actin cytoskeleton, and in doing so trigger elongation and scattering of infected host cells \textit{in vitro}, a morphological change referred to as the “hummingbird phenotype”\cite{63}. Phosphorylation-independent signaling induces pro-inflammatory events, mitogenic responses and disruption of cell-cell junctions\cite{64}. Interestingly, the Grb2 adapter can interact with CagA in a phosphorylation-dependent and -independent manner. Furthermore, transgenic expression of CagA mimics the eukaryotic Grb2-associated adaptor protein in Drosophila melanogaster\cite{64}. This interaction promotes Ras activation and in turn stimulates the Raf-MEK-ERK signalling cascade. Particularly, this axis appears to be involved in the CagA phosphorylation-independent persistent activation of transcription factors, such as NF-κB (inflammation)\cite{65-67}. Interestingly, in infected patients, the presence of CagA+ strains is associated with increased levels in the methylation of certain promoters when compared to CagA- infections\cite{68}. Similar observations have also been reported \textit{in vitro}. For instance, ectopic CagA expression can promote silencing of let-7 microRNA by enhancing histone and DNA methylation\cite{69}.

The cytotoxin VacA, another potentially relevant secreted bacterial factor, induces vacuole formation in cells treated with purified or recombinant forms of the protein\cite{70,71}. Initially, its activity was associated with apoptosis of gastric cells\cite{72}, the inhibition of T and B cell proliferation\cite{73} and induction of autophagy\cite{74} \textit{in vitro}. Despite the many functions attributed to VacA \textit{in vitro}, their relevance \textit{in vivo} is questionable\cite{75}. Of note, vacuolization of cells has been observed in human biopsies and oral administration of partially purified toxin produces mucosal damage in mice\cite{76}. However, infection of gnotobiotic piglets and Mongolian gerbils with isogenic \textit{H. pylori} mutants lacking VacA causes indistinguishable degrees of gastritis in both cases\cite{77}. Despite such results, epidemiology data indicate that the presence of more active variants of the toxin is associated with increased inflammation, peptic ulcers and even cancer\cite{78,79}. Also, a group recently demonstrated that an \textit{H. pylori} strain bearing a more active VacA induced metaplasia in mice\cite{80}. Furthermore, VacA alleles with elevated activity are associated with enhanced secretion of pro-inflammatory cytokines such as TNF-α, MIP-1α, IL-1β, IL-8, IL-10 and IL-13 \textit{in vitro} and expression of the \textit{s1m1} allele correlates with increased inflammation in the gastric mucosa\cite{76,81,82}. In summary, it remains unclear whether VacA effects are relevant during early steps of carcinogenesis in promoting gastric epithelial damage or whether this factor promotes aberrant epigenetic changes during gastric progression and further research is required to resolve this issue.

THE EPIGENETIC BASIS OF THE GASTRIC PRECANCEROUS CASCADE

The control of gene expression via epigenetic mechanisms, including DNA methylation and microRNAs, are important regulatory events that modulate all pathways in the cellular network\cite{83}. Moreover, these epigenetic modifications are considered excellent candidates to explain how environmental factors impact on the genome and cell function and increase the risk of cancer.
DNA methylation in gastric cancer and the precancerous cascade

DNA methylation, a process whereby cytosine bases are modified with a methyl group in the 5’ position when they are followed by a guanine in the DNA sequence, is increasingly considered to be of great importance in gastric carcinogenesis. Currently, a list of gastric cancer genes is emerging that are altered by DNA methylation and considered important in the progression of the cascade. However, only a few reports address the role of DNA hypermethylation in the gastric precancerous cascade. Importantly, higher methylation levels are detected in intestinal metaplasia than in atrophic gastritis samples. However, no differences were observed in the methylated status between intestinal metaplasia and dysplasia. Further studies have shown methylation in multiple genes in H. pylori-induced chronic gastritis in comparison with healthy donors, suggesting that H. pylori infection potently induces aberrant DNA methylation.

As mentioned above, aberrant DNA methylation can be considered as a mechanism to explain how environmental factors alter the susceptibility of individuals to gastric cancer. To support this idea, Chan et al. and Leung et al. evaluated changes in DNA methylation of the promoter region of the CDH-1 gene before and after the eradication of H. pylori, and in both studies complete regression of DNA methylation after H. pylori eradication was observed.

Genes modified by methylation upon H. pylori infection

Genes linked to intercellular junctions: As indicated above, extensive evidence is available linking gastric cancer to the silencing of the CDH1 gene by aberrant methylation. E-cadherin is a well-established tumor suppressor and represents an important component of adherent junctions. A number of studies have pointed to the existence of a connection between the H. pylori infection status and CDH1 methylation in pre-cancer lesions. For instance, H. pylori infection and aberrant DNA methylation of CpG islands in the CDH1 promoter correlated with dyspeptic patients. Perri et al. confirmed this observation, demonstrating that the CDH-1 promoter is also hypermethylated in gastritis patients infected with H. pylori, and that this was reduced following bacterial eradication. Interestingly, an in vitro study showed that H. pylori infection augmented E-cadherin promoter methylation via activation of the IL-1β receptor, iNOS induction and subsequently increased DNM1 activity as a consequence of NO production (Figure 1).

Vezatin (VEZT), another Adherent junction protein, is also silenced by gene promoter hypermethylation and is proposed to represent a novel tumor suppressor in gastric cancer. As for CDH-1, the VEZT promoter is found to be hypermethylated in biopsies from H. pylori-positive chronic gastritis patients when compared with the non-infected control group. Additionally, an in vitro study demonstrated that H. pylori infection of the non-transformed gastric epithelial GES-1 cell line was associated with VEZT promoter methylation and a reduction in Vezatin mRNA levels was also observed. Aberrant methylation of the VEZT promoter is also observed in other types of cancer, and re-expression of the protein reduces malignancy of these cells, indicating that Vezatin might be an important target during H. pylori-associated aberrant methylation and gastric carcinogenesis.

Connexin 32 (Cx32) and Connexin 43 (Cx43) are structural components of gap junctions between epithelial cells. Wang and colleagues demonstrated that expression of these connexins was decreased in gastric pre-neoplastic lesions associated with H. pylori infection when compared to non-infected individuals and that these changes were closely associated with methylation in the respective promoter regions. Furthermore, hypermethylation of Cx32 and Cx43 promoters increased during the progression from pre-neoplastic to neoplastic lesions in infected patients.

Genes linked to cell cycle regulation: CDKN2A, another important tumor suppressor gene that is normally methylated in gastric cancer, encodes the p16 (INK4A) protein, which is involved in cell cycle arrest in the G1 phase. Maekita et al. showed that methylation of this promoter was significantly elevated in the mucosa of H. pylori-positive compared with H. pylori-negative healthy volunteers. Additionally, another study demonstrated that methylation levels of CDKN2A in the gastric mucosa did not differ from those observed in intestinal metaplasia patients, indicating that CDKN2A promoter methylation is an early event during H. pylori-associated gastric carcinogenesis. Furthermore, eradication of H. pylori infection reverted the aberrant methylation status of this gene.

Genes linked to DNA repair: The gene hMHL-1 (the human homolog of E. coli MutL), encoding a DNA repair protein in humans, is also hypermethylated in the promoter region following H. pylori gastric infection. However, this event appears to occur in later stages of cancer progression, as revealed in a study of intestinal metaplasia lesions. In a similar study, higher levels of CpG methylation were observed for the promoter of a gene encoding another DNA repair protein, O6-methylguanine DNA methyltransferase (MGMT), in the gastric mucosa of gastritis patients compared to control patients. Following H. pylori eradication, methylation was reduced from 70% to 48% of the cases, coincident with significantly reduced CpG methylation and increased MGMT expression. Furthermore, in this same study, the authors demonstrated that MGMT methylation was remarkably higher in those patients infected with CagA bearing strains.

A recent study showed that hMHL-1 and MGMT promoters are hypermethylated in biopsies from H. pylori-positive gastric cancer patients. As for CDH-1, the VEZT promoter is found to be hypermethylated in biopsies from H. pylori-positive chronic gastritis patients when compared...
pylori-associated chronic gastritis adult patients, but not in biopsy samples from infected children, and that hypermethylation was associated with *H. pylori* infection in the case of the MGMT promoter[97].

**Genes linked to inflammation**: TFF-2 gene encodes the Trefoil factor 2 protein, involved in wound healing and modulating the inflammatory response in the stomach[98,99]. Chronic *H. pylori* infection was shown to enhance promoter methylation of TFF-2 gene in the gastric mucosa. Furthermore, methylation of the TFF2 promoter is an early event that increases throughout gastric tumor progression[100]. On the other hand, the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) has been described to be hypermethylated in gastritis associated with *H. pylori* infection and bacterial eradication reverts completely the methylation[93]. COX enzymes are important to maintain tissue homeostasis and wound healing in the gastric mucosa. Therefore, methylation-mediated silencing of these genes might tend to favor the appearance of pre-neoplastic lesions.

**Genes encoding transcriptional factors**: RUNX3 is a transcription factor that is thought to function as a tumor suppressor by regulating the expression of several cancer-related genes, including p53, p27 and caspase-3, among others[101,102]. The RUNX3 promoter is hypermethylated in pre-neoplastic gastric lesions, increasing progressively from chronic atrophic gastritis to gastric cancer[103]. Additionally, *H. pylori* infection contributed to inactivation of the RUNX3 gene in gastric epithelial cells by augmenting promoter hypermethylation[103]. Moreover, an in vitro study confirmed that *H. pylori* promotes RUNX3 promoter methylation via NO produced during coinubcation with macrophages[24].

FOXD3 is a member of the forkhead box (Fox) transcription factor family, whose promoter is hypermethylated in gastric mucosa infected with *H. pylori*. The evidence provided indicates that silencing of FOXD3 could be important during gastric carcinogenesis given that ectopic expression of FOXD3 in cancer cell lines reduces cell proliferation and their invasive capacity[104].

The upstream stimulatory factors, USF1 and USF2, are pleiotropic transcriptional factors that regulate the expression of genes linked to immune responses, cell cycle control and cell proliferation[105]. Bussière and colleagues demonstrated in vitro that *H. pylori* decreases the expression of USF1 and USF2 genes by hypermethylation of their promoters regions. These observations were confirmed in gastric tissue samples from infected mice, where they observed decreased expression of USF1 and USF2 and hypermethylation of USF1 and USF2 promoters in *H. pylori*-associated gastritis[106]. These factors control cell growth and have been shown to block cMyc/Ras-mediated transformation of primary rat cells[107].

For the transcription factors GATA-4 and GATA-5, both tumor suppressors, a higher methylation status was detected in gastric samples from *H. pylori* infected patients than in those from non-infected patients. Changes in methylation were particularly notable for the GATA-4 promoter. In addition, among gastric tissues with or without chronic gastritis, those with GATA-4 methylation were from *H. pylori* infected patients and no GATA-4 methylation was observed in biopsies from individuals without *H. pylori* infection[107].

**Other tumor suppressor genes**: The LOX and HRASLS genes encode a lysyl oxidase and HRAS-like suppressor proteins, respectively. The promoters of these genes are highly methylated in *H. pylori*-infected patients as compared to those without infection. In the same study, higher levels of methylation were observed for the CpG islands present in the promoter regions of the genes THBD, HAND1 and FLN in *H. pylori*-positive than in *H. pylori*-negative individuals[90]. On the other hand, Perri *et al*[93] determined that APC is hypermethylated in gastritis associated with *H. pylori* infection and that bacterial eradication was sufficient to reduce methylation of the promoter. p41ARC is a protein that regulates Arp2/3 complex formation and is necessary for cell migration[108]. For p41ARC, a higher degree of methylation was observed in an exonic CpG island in *H. pylori*-positive individuals than in non-infected individuals[90]. The tumor suppressor gene WWOX encodes the WW-domain containing oxidoreductase, a protein frequently down-regulated in several cancers. An in vitro study demonstrated that in gastric cancer cell lines infected with *H. pylori*, methylation of WWOX is elevated and, furthermore, in this model methylation was associated with *H. pylori*-enhanced expression of DNMT1 and DNMT3[109].

**MICRORNAS IN GASTRIC CANCER, THE PRECANCEROUS CASCADE AND *H. PYLORI* INFECTION**

**MicroRNA biology**

MiRNAs are 20-24 nucleotides single-strand RNAs that alter the stability and translation of target mRNAs and, in doing so, modulate gene expression at the post-transcriptional level[83,110]. A considerable number of reports in the literature suggest that miRNAs control several processes during carcinogenesis that favor tumor development and progression[83]. Reduced translation and stability of messenger RNAs (mRNAs) can be triggered by miRNA binding to the 3’ untranslated region (3’UTR) of messenger RNAs (mRNAs). Most miRNAs are found in introns and intergenic regions and possess their own promoter and regulatory elements[111]. miRNAs have many physiological functions and control a variety of biological processes, including cellular differentiation, development, proliferation, metabolism, apoptosis and immune responses[112]. Not surprisingly, deregulation of miRNA levels is observed in...
many pathological conditions beyond cancer, including neurodegenerative and cardiovascular diseases\(^{113,114}\). Notably, miRNA genes are frequently found in regions of chromosomal instability (amplification, translocation or deletion) or near chromosomal breakpoints\(^{115}\). In tumorigenesis, the target mRNAs affected by miRNAs include those involved in inflammation, cell cycle regulation, stress responses (autophagy), differentiation (epithelial-mesenchymal transition, EMT), apoptosis and invasion\(^{116-118}\). Several of the relevant target genes include onco genes or tumor suppressors that are upregulated or downregulated, respectively, in tumor progression and metastasis\(^{112}\). In addition to their function in physiological and pathological processes, miRNAs play an important role during microbial infection caused by viruses, bacteria, parasites and fungi\(^{119}\).

**MiRNA signatures in gastric cancer**

Tong et al\(^{120}\) reported on frequent aberrant expression of miRNAs in gastric cancer. An association between miRNA expression and alterations in cell proliferation (miR-139, by targeting the CXCR4 gene; and miR-1-34a and -504 by targeting the FOXP1 gene), cell cycle progression (miR-221, -222, -160b, -93 and -25, by targeting the p57, p21 and p27 genes) and invasion/metastasis (miR-21, by targeting the RECK gene) were detected. Ueda et al\(^{121}\) analyzed 160 paired tumor and non-tumor mucosa samples and detected 22 miRNAs that were upregulated and 13 that were downregulated in gastric cancer. Interestingly, for intestinal and diffuse types of gastric cancer two different miRNA signatures were identified. Importantly, the miRNAs (miR-105, miR-100, miR-125b, miR-199a, miR-99a, miR-143, miR-145 and miR-133a) and (miR-373*, miR-498, miR-202*, and miR-494) were upregulated in diffuse-type and intestinal-type gastric cancer, respectively. Differential analysis of miRNA expression also revealed that progression in TNM staging correlated with higher expression levels of miR-125b, miR-199a, and miR-100. Also, reduced expression of let-7g and miR-433 and elevated expression of miR-214 were identified as independent predictors of poor survival that were not related to depth of invasion, lymph-node metastasis or TNM stage. These findings show that miRNAs are differentially expressed in histological gastric cancer subtypes and that these are characterized by specific miRNA signatures.

Moreover, the expression of particular miRNAs is also linked to gastric cancer progression and prognosis. Ueda et al\(^{121}\) identified miRNA expression profiles for gastric cancer by RT-PCR in 100 patients and identified a group of seven miRNAs as independent predictors of overall survival and relapse-free survival. In this study, hazard ratios (HRs) assessed by Cox regression analysis, demonstrated that miRNA expression levels correlated directly or indirectly with death probability: 3 miRNAs (let-7a, miR-126, miR-30a-5p) were protective (HR < 1) and 4 miRNAs (miR-10b, miR-21, miR-223, miR-338) were associated with higher risk of relapse and poorer survival (HR > 1).

The role of miRNAs in the precancerous cascade of gastric cancer was evaluated by Wang et al\(^{122}\). Using an in silico approach with Gene Expression Omnibus (GEO) datasets, these authors identified 20 differentially expressed miRNAs between *H. pylori*-related gastritis and intestinal metaplasia samples. These miRNAs modulated a variety of cell functions, including signal transduction, cell proliferation and death, as well as metabolite transport and catabolism. Among the target genes, RAB22A, SOX4, IKZF2, PLAG1 and BTBD7 were found to be simultaneously regulated by several differentially expressed miRNAs. On the other hand, miR-204 was decreased in *H. pylori*-associated atrophic gastritis\(^{123}\). Knockdown of this miRNA enhanced gastric cancer cell proliferation and invasion in vitro. Alternatively, miR-204 down-regulation and over-expression of SOX4 promoted the EMT process\(^{123}\). Additionally, the potential of microRNAs as biomarkers for early gastric detection was recently assessed\(^{124-126}\).

In a population-based study in Linqu, a high-risk area of gastric cancer in China, Song et al\(^{124}\) investigated the relevance of serum miRNAs as biomarkers. Serum pools from GC control and validated gastric cancer and dysplasia subjects were correctly identified by several differentially expressed miRNAs. A signature of 16 miRNAs that were upregulated in gastric cancer patients was identified. miR-221, miR-744, and miR-376c were subsequently validated as non-invasive biomarkers for gastric cancer detection with 82.4% sensitivity and 58.8% specificity. Fu et al\(^{127}\) identified an additional miRNA, miR-222, for which circulating plasma levels increased in atrophic gastritis patients compared to healthy controls (P < 0.001) with a diagnostic accuracy of 0.850 and 66.1% sensitivity as well as 88.3% specificity. Also, the expression patterns of serum let-7 microRNA (miRNA) and its target gene pepsinogen C (PGC) were correlated with different stages of the multistage cascade of gastric cancer\(^{126}\). Finally, the feasibility of using gastric juice as test material was examined by Yu et al\(^{125}\). Significantly lower levels of gastric juice miR-129-1-3p and miR-129-2-3p with AUC values of 0.639 and 0.65 to distinguish gastric cancer from healthy controls.

**Deregulation of miRNAs expression during *H. pylori* infection**

*H. pylori* infection in vivo and in vitro alters the expression of many miRNAs\(^{128}\). Several of these miRNAs were also found to be deregulated in gastric cancer. On the one hand, down-regulation of let7a, mir-31, mir-101, miR-141, miR-203, miR-210, miR-218, miR-375, miR-449 is observed, while miR-17, miR-20a, miR-21, miR-146a, miR-155 and miR-223 are upregulated\(^{129}\). Interestingly, it has been suggested that these miRNAs may also be relevant to understanding *H. pylori* induced inflammation and carcinogenesis\(^{129}\). These miRNAs target miRNAs involved in biological processes, such as invasion and metastasis, pro-
further studies are required.

H. pylori
Again, as for miR-155, silencing of miR-210 following the DNA repair system (promoting genetic instability), stem cell survival under hypoxic conditions, stalling growth arrest, repression of mitochondrial metabolism, the target mRNAs are implicated in the regulation of cell factor (HIF1α) is an hypoxia responsive multifunctional miRNA, whose potential role in the presence of two relevant NF-κB binding sequences present in the BIC/miR-155 promoter at -178 and -1150.[131]. Similar to other pathogens, H. pylori infection elevates miR-155 levels in vitro and in vivo.[132]. Several studies have focused on hematological and inflammation-associated malignances linked to this miRNA. For instance, gastric MALT lymphoma can be reverted by 60%-80% following antibiotic eradication of bacteria; however, resistant MALT lymphomas maintain miR-155 overexpression even after bacterial eradication.[133]. Other studies show that miR-155 is a negative regulator of pro-inflammatory responses by targeting the receptor-associated adapter MyD88, thereby reducing NF-κB activation and IL-8 secretion.[134]. In another study, H. pylori-dependent miR-155 induction reduced DNA-damage and induced apoptosis in macrophages, in a manner dependent on the cagPAI secretory system and TLRs, but independent of CagA.[135]. In summary, miR-155 represents a multifunctional miRNA, whose potential role in H. pylori-associated pathologies is apparent, but still poorly understood.

As mentioned previously, several miRNAs are known to be down-regulated during gastric cancer progression and, similar to tumor suppressor genes, they can be also silenced by promoter hypermethylation. For instance, the expression of several microRNAs, such as miR-10a, -10b, -127, -148a, -181c, -195, -219-2-3p, -224, -338-3p, -340, -378, -433 and -452 among others was demonstrated that is induced after X-ray-irradiation in a p53-dependent manner. Ectopic p53 expression also results in the induction of the expression of RPM. In both scenarios, RPM causes G2 arrest of the cell cycle in association with the inhibition of nuclear translocation of cyclin B1 and Cdc2 activity.[147]. An inverse association between RPM gene expression and promoter methylation (Spearman rank R = -1; P = 0.042) was recently identified. Additionally, RPM overexpression robustly inhibited colony formation and anchorage-independent growth. Furthermore, in primary gastric cancer cases, RPM protein was not detected in tumor tissues but was found to be present in non-tumor adjacent mucosa (P = 0.001). Furthermore, the loss of expression is associated with invasive stages GC (stage I to II–IV, P = 0.006). Interestingly, these findings correlated with p73 (P < 0.0001 and kappa value = 0.363) but not p53 expression, suggesting a potential link to other members of the p53 family.[148]. In a quantitative analysis of RPM DNA promoter methylation, the H. pylori virulence factors cagA (including segments of the 3 end, encoding EPIYA polymorphisms) and vacA s1 and m1 regions were associated with increased RPM promoter methylation.[149]. These results favor the notion that H. pylori regulates RPM gene expression.[149]. Bernal et al.[150] reported that methylated RPM promoter sequences are not only a common finding in primary tissues of gastric cancer patients, but also that they can be detected in the plasma of these patients and rarely in healthy donors. A translational potentiality of this observation is the possibility that methylated circulating cell-free DNA

Taken together, the data presented here suggest that miRNAs play essential roles in gastric cancer by modulating cell proliferation, cell cycle and invasion/metastasis. In the gastric precancerous condition, miRNAs are linked to H. pylori-related gastritis and intestinal metaplasia. From a biomarker point of view, miRNAs have a strong potential as novel molecular tools for non-invasive risk assessment of gastric cancer and precancerous lesions.

REPRIMO AS A MODEL FOR EPIGENETIC CHANGES IN TUMOR SUPPRESSOR GENES IN GASTRIC CANCER

The Reprimo gene (official symbol RPRM, MIM 612171, GeneID 56475) is located in chromosome 2q23.3.[146]. Sequence analysis indicates that RPRM is 303 bp long and does not have introns. The protein sequence includes 109 aa and has two glycosylation sites (AA 7-10 and 18-21), one phosphorylation site (AA 95-98) and one myristylation site (AA 13-18) (http://hits.isb-sib.ch/cgi-bin/motif_scan). Computational analysis shows a possible transmembrane domain between aa 56-78 (www.ensembl.org). According to Ohki and coworkers, RPM is an intracellular cytoplasmic protein[147] that is induced after X-ray-irradiation in a p53-dependent manner. Ectopic p53 expression also results in the induction of the expression of RPM.

As mentioned previously, several miRNAs are known to be down-regulated during gastric cancer progression and, similar to tumor suppressor genes, they can be also silenced by promoter hypermethylation. For instance, the expression of several microRNAs, such as miR-10a, -10b, -127, -148a, -181c, -195, -219-2-3p, -224, -338-3p, -340, -378, -433 and -452 among others was recently reported to be silenced by aberrant methylation in gastric cancer.[136–138]. Interestingly, Kiga et al.[144] demonstrated that H. pylori-associated inflammation promotes DNA methylation of the miR-210 locus and enhanced proliferation of gastric cells as a consequence of augmented STMN1 and DMT1 expression. MIR-210 (Figure 1) is an hypoxia responsive multifunctional microRNA whose promoter contains Hypoxia Inducible factor (HIF1α and 2a) response elements (HRE) and the target mRNAs are implicated in the regulation of cell growth arrest, repression of mitochondrial metabolism, stem cell survival under hypoxic conditions, stalling the DNA repair system (promoting genetic instability), angiogenesis induction and cellular differentiation.[145]. Again, as for miR-155, silencing of miR-210 following H. pylori infection is likely to contribute to disease, but further studies are required.
may be employed as a non-invasive biomarker for detection of gastric cancer and precancerous lesions.

**THE H. PYLORI γ-GLUTAMYL TRANSPEPTIDASE CONNECTION TO ADDITIONAL MODES OF REGULATION IN GASTRIC CANCER: SURVIVIN AS A MODEL**

GGT is an enzyme that catalyzes the transpeptidation and hydrolysis of the γ-glutamyl moiety from glutathione and glutathione-conjugated compounds, to amino acids[151]. *H. pylori* γ-glutamyl transpeptidase (HpGGT) is constitutively expressed and detectable in all *H. pylori* strains[152], suggesting it is relevant to bacterial physiology. Among the many effects observed in gastric cells, GGT induces apoptosis[153], cell cycle arrest[154], and generates ROS, in particular H₂O₂, that potentially damages DNA[155]. Importantly, significantly higher GGT activity has been observed in *H. pylori* isolates obtained from patients with peptic ulcer disease than in those from patients with non-ulcer dyspepsia[156]. Gastric ulcers are associated with an elevated risk of developing gastric cancer[156] and these observations implicate HpGGT as being clinically relevant to the progression of precancerous gastric lesions.

Beyond the indicated alterations in gastric cells induced by GGT, recent evidence suggests that this virulence factor may also enhance proteasome-mediated degradation of proteins required for cell viability, as is the case for survivin, a member of the inhibitor of apoptosis (IAP) family of proteins. Survivin expression levels are controlled at the transcriptional level by several transcription factors and at the post-transcriptional level by phosphorylation and proteasome-mediated degradation[157]. Survivin is generally not expressed in normal differentiated tissues, except in proliferating stem cell populations, but is strongly upregulated in most human cancer cells where it enhances proliferation, viability, metastasis and angiogenesis[157-159]. Interestingly, the gastric mucosa apparently represents an exception to this general rule, because normal mucosa cells express high levels of survivin and it has been suggested that presence of the protein may favor mucosa cell survival in the adverse gastric environment. Importantly in biopsies from gastritis patients with *H. pylori* infection, survivin protein levels are decreased as compared to control samples from gastritis patients without infection[160]. In the same study, infection in vitro of cells of the gastrointestinal lineage with *H. pylori* was shown to trigger the loss of survivin protein and as a consequence reduces cell viability. Subsequently, this ability of *H. pylori* was linked to the secretion of HpGGT and enhanced turnover of the protein via the proteasome pathway, possibly via a mechanism involving the generation of ROS[161]. It is interesting to note here that this mechanism specifically upregulates survivin but not other anti-apoptotic proteins, like Bcl2 or Bcl-xL, and one may speculate that related events might control turnover of a number of yet to be identified components in infected cells. Finally, this GGT-proteasome connection may explain how early in disease development *H. pylori* damages the gastric tissue in zones with high levels of bacterial infection, favoring the genesis of a pro-inflammatory environment and potentially subsequent invasion by bone marrow-derived cells and intestinal metaplasia[162]. Further experimentation is required to substantiate this intriguing hypothesis that could also open up new avenues for the treatment of *H. pylori* positive gastritis patients.

**CONCLUSION**

In summary, this review highlights how in addition to *H. pylori*-associated virulence factors, epigenetic changes in infected host cells, such as DNA methylation and miRNAs, are likely to play a significant role in gastric cancer development and the progression of the precancerous cascade. Moreover, recent results revealing how HpGGT modulates the turnover of specific protective host proteins underscores the tremendous variability and selectivity of changes that may be attributed to *H. pylori* infection. Importantly, from the clinical perspective, some of the epigenetic factors discussed can be determined by analyzing blood and other body fluid samples, thus opening up a new avenue for non-invasive risk assessment of gastric cancer. In doing so, we may anticipate that earlier diagnosis and, as a consequence, more effective treatment of this still very deadly cancer will become possible.

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