Published in final edited form as:
Gastroenterol Clin North Am. 2013 June ; 42(2): 285–298. doi:10.1016/j.gtc.2013.01.006.

"*H. pylori* in gastric carcinogenesis-mechanisms"

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Keywords

Helicobacter pylori; gastric cancer; virulence factors

Introduction

Helicobacter pylori was definitively identified by culture in 1984 by Robin Warren and Barry Marshall,[1] and 10 years later this organism was recognized by the IARC (WHO) as a Type I carcinogen. *H. pylori* infection is the strongest known risk factor for gastric cancer, and epidemiological studies have estimated that, in the absence of *H. pylori* infection, 75% of gastric cancers would not exist.[2] *H. pylori* is considered to be the most common etiologic agent of infection-related cancers, and is estimated to be responsible for 5.5% of all cancers world-wide.[3] While it is clear that *H. pylori* is the strongest causative agent for gastric cancer, the precise mechanisms for gastric cancer development in response to *H. pylori* infection are less well defined, and a complex interplay of strain-specific bacterial constituents, inflammatory responses governed by host genetic diversity, and/or or environmental influences are involved in determining the fate of the host that is persistently colonized by *H. pylori*.[4] This review will focus on specific mechanisms utilized by *H. pylori* to drive gastric carcinogenesis.

H. pylori virulence factors that mediate carcinogenesis

The *H. pylori* type IV *cag* secretion system—The *cag* pathogenicity island (*cag* PAI) is a well-characterized and intensively studied *H. pylori* virulence determinant, and strains that harbor the *cag* PAI augment the risk for distal gastric cancer compared to strains that lack the *cag* island.[5] Genes within the *cag* island encode proteins that form a bacterial type IV secretion system (T4SS) that translocates proteins across the bacterial membrane into host gastric epithelial cells.[6-8] The terminal gene product of the *cag* island is CagA, and this is one of the substrates that is translocated into host cells by the T4SS.[9] CagA translocation occurs through the interaction of the *H. pylori* protein CagL, which is located on the distal tip of the T4SS pilus, with integrin $\alpha_5\beta_1$ on host epithelial cells.[10] CagI and CagY have also been shown to interact with β_1 integrin and mediate CagA translocation,[11] and CagL physically associates with CagI and CagH.[12] In addition, CagA facilitates its own

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translocation through specific binding to β_1 integrin.[11,13] CagA is also reported to be delivered into host epithelial cells by T4SS-induced externalization of phosphatidylserine from the inner leaflet of the cell membrane. The N-terminus of CagA then interacts with phosphatidylserine to gain entry into host epithelial cells.[14, 15] Once inside host cells, CagA is tyrosine phosphorylated by Src and Abl kinases at glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs located within the carboxyl-terminus of CagA.

There are four distinct CagA EPIYA motifs (EPIYA-A, -B, -C, or –D) and these are distinguished by different amino acid sequences surrounding the EPIYA motif.[16-18] In contrast to EPIYA-A and –B motifs, which are present in strains throughout the world, EPIYA-C is typically found only in strains from Western countries (Europe, North America and Australia), and in these strains, an increased number of CagA EPIYA-C sites confers a heightened risk for developing gastric cancer.[19, 20] The EPIYA-D motif is almost exclusively found in East Asian strains.[21] Tyrosine phosphorylation of CagA is tightly regulated, and upon injection into the host cell, CagA is immediately phosphorylated on EPIYA-C or EPIYA-D by Src kinase, followed later by phosphorylation on A-B-C or D motifs by Abl.[22]

Once phosphorylated by members of the Abl and Src family kinases, phospho-CagA targets and interacts with numerous intracellular effectors to lower the threshold for carcinogenesis. Phospho-CagA activates a eukaryotic tyrosine phosphatase (SHP-2), leading to sustained activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2), Crk adaptor, and C-terminal Src kinase, and induces morphological transformations similar to changes induced by growth factor stimulation.[23] Interactions of phospho-CagA with C-terminal Src kinase rapidly activates a negative feedback loop to down-regulate Src signaling and subsequently the generation of phospho-CagA.[24]

The quantity of phospho-CagA is tightly self-regulated; however, non-phosphorylated CagA also exerts effects within the cell that contribute to pathogenesis. Non-phosphorylated CagA interacts with the cell adhesion protein E-cadherin, the hepatocyte growth factor receptor c-Met, the phospholipase PLC-γ, the adaptor protein Grb2, and the kinase PAR1b/MARK2, and activates ß-catenin, [25-28] which culminate in pro-inflammatory and mitogenic responses, disruption of cell-cell junctions, and loss of cell polarity, all of which promote neoplastic progression. Non-phosphorylated CagA also associates with the epithelial tight-junction scaffolding protein ZO-1, and the transmembrane protein, junctional adhesion molecule (JAM)-A, leading to nascent but incomplete assembly of tight-junctions at sites of bacterial attachment distant from sites of cell-cell contacts.[29] CagA also directly binds PAR1b/MARK2, a central regulator of cell polarity, inhibits its kinase activity and promotes loss of cell polarity.[25, 30, 31] These events will be discussed in more detail (see Manipulation of the apical-junctional complex by *H. pylori*, and Figure 1).

Another pathway through which *H. pylori* CagA can increase the risk for gastric cancer is through manipulation of apoptosis, by increasing spermine oxidase (SMO) production in gastric epithelial cells. This generates oxidative damage and selects for a sub-population of DNA damaged cells that are resistant to apoptosis.[32] *H. pylori* also targets the tumor suppressor p53 to regulate apoptosis in a CagA dependent manner.[33, 34] CagA interacts with the apoptosis-stimulating protein of p53 (ASPP2) and prevents ASPP2 from inducing apoptosis through activation of p53. This results in proteasomal degradation of p53 and resistance to apoptosis.[33] Recent findings suggest that *H. pylori* induces specific p53 isoforms that inhibit p53 and p73 activities, induce NF-κB activity, and increase cell survival. [34]

CagA is not the only bacterial product delivered through the T4SS, as components of *H. pylori* peptidoglycan are also delivered into host cells and trigger signaling pathways that lower the threshold for carcinogenesis. Peptidoglycan interacts with the host intracellular pattern recognition molecule Nod1 which leads to activation of NF-κB-dependent pro-inflammatory responses such as secretion of IL-8[35] or β-defensin-2, as well as production of type I IFN.[36, 37] Translocated peptidoglycan can also activate PI3K-AKT signaling, leading to decreased apoptosis, increased proliferation and increased cell migration.[38, 39]

Vacuolating cytotoxin A (VacA)—Vacuolating cytotoxin A (VacA) is a toxin produced by *H. pylori* that is associated with increased disease risk.[40] VacA exerts multiple effects on epithelial cells including vacuolation as well as inducing apoptosis and suppressing T cell responses, which may contribute to the longevity of infection.[41-43]

The majority of *H. pylori* strains possess the *vacA* gene; however, there is considerable variation in v*acA* gene structures within the signal (s) region, the middle (m) region, and the intermediate (i) region.[44] The s-region and m-region are stratified into s1 or s2 and m1 or m2 alleles respectively. *vacA* s1/m1 strains induce greater vacuolation than s1/m2 strains, and there is typically no vacuolating activity in s2/m2 strains.[44-47] The *vacA* s1/m1 allele is strongly associated with duodenal and gastric ulcer disease, and gastric cancer.[4748, 49] There are two i region subtypes, i1 and i2; and the i region plays a functional role in vacuolating activity.[44] Colonization with *vacA* i1 strains is strongly associated with the presence of CagA, *vacA* s1, and gastric cancer.[44, 50]

Of great interest are recent reports suggesting that VacA and CagA are able to counter-regulate the effects of each other on the host, representing an effective mechanism to promote persistent colonization of *H. pylori*.[51, 52] Phospho-CagA is able to inhibit trafficking of VacA and in this way prevents VacA from reaching its intracellular targets and inducing vacuoles. Non-phosphorylated CagA is also able to oppose vacuolation by blocking VacA activity at the mitochondria.[51] Another example of the antagonistic effects of CagA and VacA is CagA activation of the NFAT (nuclear factor of activated T cells) family of transcription factors. CagA induces translocation of NFAT from the cytoplasm to the nucleus while VacA prevents the translocation of NFAT.[53] VacA is also able to counteract the effects of CagA by inactivating EGFR, and suppressing activation of ERK1/2 MAP kinase and preventing CagA-mediated cellular elongation.[52] Recent work has identified another mechanism for regulation of CagA by VacA, whereby VacA induces autophagy and degradation of CagA through specific binding of VacA m1 to low-density lipoprotein receptor-related preotein-1 (LRP1) on epithelial cells.[54] Interestingly, in cells expressing a marker of stem cells, CD44 variant 9 (CD44v9) VacA-induced degradation of CagA is circumvented due to resistance to reactive oxygen species.[54] These findings further highlight mechanisms through which *H. pylori* can avoid the induction of excess cellular damage and maintain long-term persistence in the gastric niche.

Although, CagA and VacA clearly counteract the effects of each other, one exception is facilitation of iron uptake from polarized host epithelial monolayers. In this situation, CagA and VacA act synergistically to create a replicative niche on the apical surface of host epithelial cells by inducing apical mislocalization of transferrin receptors to sites of bacterial attachment.[55]

Adhesins and outer membrane proteins—In order for *H. pylori* to colonize, deploy virulence factors, and persist within the gastric niche, adherence of *H. pylori* to gastric epithelium is required. Sequence analyses have revealed that an unusually high proportion of the *H. pylori* genome is predicted to encode outer membrane proteins (OMPs), and OMP

expression is associated with gastroduodenal ulceration and may heighten the risk for developing gastric cancer (Figure 1).[56]

Blood group antigen binding adhesin (BabA) is an OMP encoded by the *babA2* gene and binds to fucosylated Lewis^b antigen (Le^b) on the surface of gastric epithelial cells.[57-59] Le^b-mediated colonization may increase the pathogenic potential of *H. pylori*[60] and *H. pylori babA2*$^+$ strains are associated with an increased risk of developing gastric cancer, especially when found in conjunction with *cagA* and *vacA* s1 alleles.[57]

Another *H. pylori* adhesin is Sialic acid-binding adhesin (SabA). SabA binds to the carbohydrate structure sialyl-Lewis^x antigen expressed on gastric epithelium, and is associated with increased gastric cancer risk.[61] Sialyl-Lewis^x expression is induced during chronic gastric inflammation, suggesting that *H. pylori* modulates host cell glycosylation patterns to enhance attachment and colonization.[62]

Outer inflammatory protein (OipA) is an inflammation-related outer membrane protein,[63] and the presence of a functional *oipA* gene is associated with more severe disease outcome and gastric cancer.[61, 64] OipA expression is linked to increased production of pro-inflammatory cytokines including IL-8, IL-1, IL-17, and TNFα, [65, 66] as well as other host effector proteins such as upregulation of MMP-1, an MMP associated with gastric cancer, and induction of OipA can result in activation of β-catenin.[6764]

Effects of *H. pylori* on the host immune response that mediate carcinogenesis —Infection with *H. pylori* invariably results in chronic gastric inflammation, and this occurs through a variety of pathways.[68] As discussed above, bacterial factors play an important role in determining the severity of disease outcome; however, these alone are not sufficient to dictate the outcome of *H. pylori* infection. The immune response of the host is a key determinant of the development of gastric cancer. *H. pylori* upregulates several inflammatory molecules including IL-1β, IL-32, IL-10, and TNFα and this plays a key role in *H. pylori*-induced disease progression.[5]

IL-1ß is a Th1 cytokine that inhibits acid secretion and is increased within gastric mucosa of *H. pylori*–infected persons.[69] Polymorphisms in the IL-1ß gene cluster, specifically *IL-1ß*-31 and *IL-1ß*-511, are associated with increased IL-1ß production, and are associated with a significantly increased risk for hypochlorhydria, gastric atrophy, and distal gastric adenocarcinoma compared to persons with genotypes that limit IL-1ß expression, but only among persons infected with *H. pylori*.[70-72] Given that IL-1ß is a potent inhibitor of acid secretion, is profoundly pro-inflammatory, and is up-regulated by *H. pylori*, colonized individuals harboring high-expression IL-1ß polymorphisms are at increased risk for the development of gastric cancer.

Another cytokine that may increase the risk for gastric cancer is TNF-α. TNF-α is a pro-inflammatory, acid-suppressive cytokine that is increased within *H. pylori*-colonized human gastric mucosa.[73] TNF-α polymorphisms that increase TNF-α production are associated with an increased risk of gastric cancer and its precursors.[72, 74] Interestingly, TNF-α expression has been linked to increased ß-catenin signaling through inhibition of GSK3β through the use of transgenic mice that over-express the ß-catenin agonist Wnt1 and these mice develop gastric dysplasia. *In vitro* studies have revealed that supernatants from activated macrophages promote ß-catenin signaling in gastric epithelial cells, which is attenuated by inhibition of binding of TNF-α to its receptor on gastric epithelial cells, providing a potential mechanism through which enhanced levels of TNF-α may augment the risk for gastric cancer.[75]

In contrast to IL-1ß and TNF-α polymorphisms for which polymorphisms that increase cytokine production are associated with increased gastric cancer risk, polymorphisms that decrease the production of the anti-inflammatory cytokine IL-10 reciprocally increase the risk for distal gastric cancer.[74] Investigations into the combinatorial effects of IL-1ß, TNF-α, and IL-10 polymorphisms on the development of cancer have revealed that the risk of cancer increases progressively with an increasing number of pro-inflammatory polymorphisms and three high-risk polymorphisms increased the risk of cancer 27-fold over baseline.[74]

The role of IL-32, a recently described pro-inflammatory cytokine that is over-expressed in various inflammatory diseases and cancer has also been investigated in *H. pylori* infection.[76] Expression of IL-32 parallels the severity of gastric pathology, with elevated expression in gastritis and gastric cancer compared to uninfected gastric mucosa. *H. pylori*-induced IL-32 expression is *cag*PAI-dependent and requires activation of NFκB. Interestingly, within the context of *H. pylori* infection, IL-32 expression is linked with expression of the cytokines CXCL1, CXCL2, and IL-8, suggesting that IL-32 may function as a master regulatory protein that controls cytokine expression in *H. pylori* infection.[76]

Manipulation of the apical-junctional complex by *H. pylori*—Gastric mucosal barrier function is controlled by the apical-junctional complex and is essential for preventing potentially immunogenic elements present in the gastric lumen from gaining access to the gastric mucosa.[77] The apical-junctional complex is composed of tight junctions and adherens junctions, and studies have revealed that *H. pylori* targets many of the host molecules that form apical-junctional complexes with the resulting effect being a lowering of the threshold for carcinogenesis (Figure 1).

Studies focused on tight junction proteins have revealed that *H. pylori* recruits the tight junction proteins ZO-1 and JAM-A to the site of bacterial attachment,[29] and disrupts occludin localization at the tight junction.[78-80] *H. pylori* also induces redistribution of claudin-4 and claudin-5 and disrupts barrier function.[80]

One mechanism through which *H. pylori* can disrupt the tight junction is via the interaction of CagA with partitioning-defective 1b (PAR1b)/microtubule affinity-regulating kinase 2 (MARK2). PAR1b is a member of the PAR1 family of kinases, and has an essential role in maintaining epithelial cell polarity.[25, 81-83] The PAR1b-binding region of CagA is a 16-amino-acid sequence known as the CagA-multimerization (CM) sequence, which is involved in CagA dimerization.[84] The CM motif binds to the MARK2 kinase substrate binding site and mimics a host cell substrate that inactivates the kinase activity of PAR1, leading to defects in epithelial cell polarity and disruption of tight junctions.[8525] Dysregulation of tight junctions also permits *H. pylori* to gain access into sites previously deemed sanctuary sites, such as intercellular spaces and the lamina propria. *H. pylori* also targets specific components that comprise the adherens junction to promote progression towards gastric carcinogenesis (Figure 1). Adherens junctions are required for maintenance of adhesive cell-cell contacts, cell polarity, and for signal transduction to the nucleus to regulate transcription. E-cadherin is one adherens junction protein that *H. pylori* dysregulates via methylating the E-cadherin gene promoter, thereby reducing E-cadherin expression.[86-88] Loss of E-cadherin function is associated with gastric cancer,[86-88] and hypermethylation of the E-cadherin promoter can be reversed by eradication of *H. pylori*.[87-89]

H. pylori infection also disrupts the adherens junction through inducing translocation of membranous E-cadherin, β-catenin, and p120 to the cytoplasm of epithelial cells.[90-93] Specifically, non-phosphorylated CagA interacts with E-cadherin,[26, 94] leading to

destabilization of the E-cadherin/β-catenin complex, and accumulation of cytoplasmic and nuclear β-catenin, which subsequently transactivates β-catenin-dependent genes that may promote carcinogenesis.[26, 95] Through activation of PI3-K/Akt signaling by non-phosphorylated CagA, *H. pylori* inactivates GSK-3β which results in increased cytoplasmic expression of β-catenin.[96, 97] *H. pylori* closely regulates β-catenin activation within host cells through an inhibitory domain within the N-terminus of CagA.[98] Interestingly, the N-terminus of CagA counteracts effects exerted by the C-terminus of CagA to reduce host-cell responses by strengthening cell-cell contacts and decreasing CagA-induced β-catenin activity.[98]

H. pylori can also cleave E-cadherin through the actions of the secreted virulence factor high-temperature requirement A (HtrA).[99] Loss of E-cadherin from the adherens junction is associated with dissociation and movement of β-catenin and p120 from the adherens junction into the cytosol. Under normal physiological conditions, nuclear expression of p120 is low; however, in transformed cells, expression of p120 is elevated.[100-102] *H. pylori* is associated with mislocalization of p120 to the nucleus in human gastric epithelia and in infected murine primary gastric epithelial cells.[93, 103] Further analysis of downstream signaling pathways has determined that p120 mis-localized to the nucleus in response to *H. pylori* acts to relieve transcriptional repression of *mmp-7*, a matrix metalloproteinase implicated in gastric tumorogenesis, by an interaction with Kaiso.[93] Nagy *et al.* have also reported that a p120- and β-catenin target gene, PPARδ, regulates gastric epithelial proliferation via activation of cyclin E, representing another important mechanism through which *H. pylori* may lower the threshold for developing gastric cancer.[38]

The role of environmental influences—As discussed in this review, there are multiple ways in which *H. pylori* manipulates the host to lower the threshold for carcinogenesis and, conversely, the host can also signal to and alter the bacterium. Recent work has demonstrated that CagA expression is significantly upregulated when certain strains of *H. pylori* are cultured in a medium containing high salt concentrations, an epidemiologically defined risk factor for gastric cancer (Figure 1).[106] Using sequence analysis and site-directed mutagenesis, it was determined that salt-responsive strains of *H. pylori* are more likely to contain two copies of a TAATGA motif within the *cagA* promoter, while strains containing only a single copy of this motif are less likely to possess properties of salt-responsive CagA expression.[104]

Host iron levels have also been found to manipulate the virulence potential of *H. pylori*. *H. pylori* harvested from gerbils with low iron levels were found to assemble more T4SS pili per bacterium, translocate increased amounts of CagA, and augment IL-8 secretion compared to *H. pylori* strains isolated from gerbils with normal iron levels (Figure 1). Furthermore, strains isolated from patients with low ferritin levels induced significantly higher levels of IL-8 compared to strains isolated from patients with the highest ferritin levels, suggesting that iron deficiency in the host increases *H. pylori* virulence and the risk for developing gastric cancer.[105]

Summary—*H. pylori* infection induces chronic inflammation and is the strongest known risk factor for gastric cancer. The genomes of *H. pylori* are highly diverse and, therefore, bacterial virulence factors play an important role in determining the outcome of *H. pylori* infection, in combination with host-responses that are augmented by environmental and dietary risk factors. It is important to gain further understanding of the pathogenesis of *H. pylori* infection in order to develop more effective treatments for this common but deadly malignancy.

Acknowledgments

Grant support: NIH CA-116087, DK-58404, DK-58587, and CA-77955

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Key points

- Infection with *Helicobacter pylori* plays a central role in the development of gastric cancer.
- The pathogenicity of *H. pylori* infection is attributable to specific interactions between virulence components, variable host inflammatory responses, and environmental factors.
- Mechanisms for the carcinogenesis induced by *H. pylori* include changes in host gene expression, alterations in proliferation and apoptosis, and disruption of apical-junctional complexes

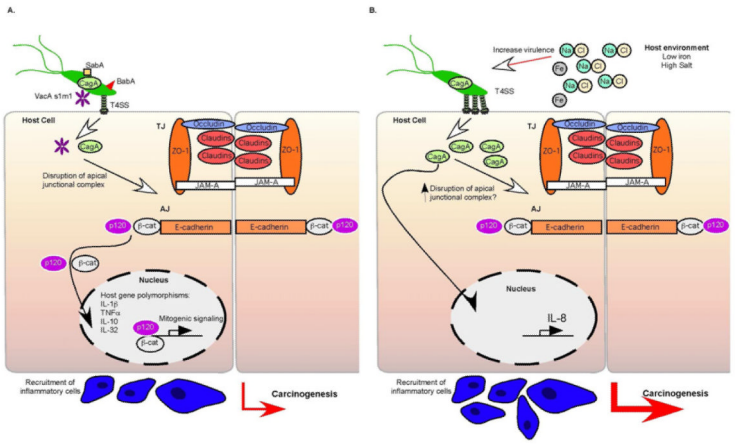

Figure 1.

Gastric cancer is a result of a complex interplay between bacterial virulence factors, host inflammatory responses, and environmental influences. A. *H. pylori* virulence factors including SabA, BabA, CagA, and VacA influence the outcome of *H. pylori* infection, with CagA and VacAs1m1 types associated with increased disease severity. *H. pylori* disrupts the apical-junctional complex at the level of the tight junction (TJ) and adherens junction (AJ), and disrupts cell polarity. Disruption of the adherens junction results in translocation of β-catenin and p120 to the nucleus, altering transcription of genes that promote disease progression. Host genetic diversity also contributes to gastric cancer, including polymorphisms within IL-1β, TNFα, IL-10 and IL-32. B. Host iron (Fe) levels and salt (NaCl) concentrations also impact the virulence of *H. pylori*. High salt increases CagA production and low iron levels augment assembly of T4SS pili, increase CagA translocation, and augment IL-8 secretion.