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***Helicobacter pylori* virulence factors in gastric carcinogenesis**

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Abstract

Helicobacter pylori infection is the most important risk factor in the development of non-cardia gastric adenocarcinoma; host genetic variability and dietary co-factors also modulate risk. Because most *H. pylori* infections do not cause cancer, *H. pylori* heterogeneity has been investigated to identify possible virulence factors. The strongest candidates are genes within the *cag* (cytotoxin associated antigen) pathogenicity island, including the gene encoding the CagA protein, as well as polymorphic variation in the VacA vacuolating exotoxin and the blood group antigen binding adhesin BabA. Improved understanding of the pathogenesis of *H. pylori*-associated gastric cancer may improve risk stratification for prevention and therapy.

Keywords

Gastric cancer; *H. pylori*; virulence factors; CagA; VacA

1. Introduction

The worldwide mortality from gastric cancer remains very high, especially in Asia and much of the developing world [1]. Although the incidence of this disease continues to slowly decline in the West, gastric cancer is currently the second most common cause of cancer death in the world [2] and the fifth most common cancer and the fourth leading cause of cancer-related death in Europe [3].

Helicobacter pylori (*H. pylori*) is a micro-aerophilic spiral-shaped Gram-negative bacterium that colonizes the stomach for almost the entire lifetime of the host. *H. pylori* infects more than half of the world's human population, and produces gastroduodenal diseases, such as peptic ulcer disease in about 10% and gastric adenocarcinoma in 1–2% of those that it infects. It has been evident for over 20 years that *H. pylori* is involved in the development of gastric adenocarcinoma; in 1994, the WHO concluded that *H. pylori* is a definite or class I carcinogen in humans. Prospective studies reveal that the risk for development of gastric carcinoma is much greater in *H. pylori*-infected populations than in uninfected populations [4]. In 2002, an estimated 1.9 million cases, or 17.8% of the worldwide incidence of cancer, were considered

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Conflicts of interest Statement
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to be attributable to infectious diseases, with *H. pylori* infection as the leading cause (5.5% of all cancers), followed by human papilloma viruses, hepatitis B and C viruses, Epstein-Barr virus, human immunodeficiency virus, and human herpesvirus-8 [5]. *H. pylori* is responsible for about 75% of all noncardia gastric cancers and 63.4% of all stomach cancers worldwide [5].

Despite the close association of *H. pylori* infection with non-cardia gastric cancer, most infected persons do not develop the disease. The clinical outcome of *H. pylori* infection is determined by multiple factors, including host genetic predisposition (especially certain cytokine polymorphisms [6]), *H. pylori* strain heterogeneity and environmental factors such as dietary salt intake [7]. *H. pylori* is a highly heterogeneous bacterial species, both genotypically and phenotypically, and is highly adapted for survival in the gastric niche. The genomic diversity of *H. pylori* parallels that of its host species, consistent with colonization of the earliest humans and co-migration out of East Africa at least 60,000 years ago [8]. *H. pylori* heterogeneity and the association of certain *H. pylori* products with specific diseases (virulence factors) has been intensively investigated over the past two decades since *H. pylori* was first cultured. In an attempt to bring order to a sometimes chaotic field, it has been proposed that for designation as virulence factor the following criteria should be met (1) the *H. pylori* determinant should have a disease or other in-vivo correlation, (2) there should be epidemiologic consistence across populations and regions, (3) there should be biologic plausibility, and (4) the biologic activity should be reduced or eliminated by gene deletion and restored by complementation [9]. With respect to gastric cancer, the major *H. pylori* candidate virulence factors include the *cag* pathogenicity island (PAI), the CagA protein, the vacuolating toxin VacA, the blood group antigen binding adhesin (BabA) and possibly the duodenal ulcer producing gene (*dupA*) (Figure 1). We shall review the association of these virulence factors with gastric cancer and discuss the possible underlying molecular mechanisms.

2. Cytotoxin associated antigen (CagA)/*cag* pathogenicity island (*cag* PAI)

The *cag* (cytotoxin-associated gene) pathogenicity island (*cag* PAI) is a 40 kilobase segment of DNA, containing 31 genes, many of which encode components of a type 4 bacterial secretion system [10;11;12]. The secretion system acts as a molecular syringe for delivery of bacterial products, including the *cag* gene product CagA and peptidoglycan component into eukaryotic cells [13;14]. The *cag* PAI plays an important role in *H. pylori* pathogenesis, and is not expressed in all strains. Approximately 60% of *H. pylori* strains isolated in Western countries carry the *cag* PAI, whereas almost all of the East Asian strains isolated are *cag* PAI-positive [15]. CagA is a 121–145 kDa immuno-dominant protein, encoded by one of the genes (*cagA*) within the *cag* PAI. It is commonly used as a marker for the entire *cag* locus in epidemiological studies. In Western populations, *cagA*-positive strains are more commonly associated with peptic ulceration atrophic gastritis and gastric adenocarcinoma than *cagA* negative strains, [16;17;18;19] but this relationship is not observed in many high gastric cancer populations including East Asia where almost all strains are *cag*-positive.

More recent studies in animal and cell culture models indicate the likely role of CagA and the *cag* PAI in human gastric cancer associated with *H. pylori* infection [20;21;22;23]. Infection of gerbils with partially or completely disrupted *cag* PAI strains results in significantly less gastric inflammation and fewer gastric ulcers compared with wild-type infections [20;24]. CagA deletion also prevents gastric carcinogenesis associated with *H. pylori* in the gerbil model [23]. Ohnishi et al recently provided the first direct evidence of the potential oncogenicity of CagA in vivo [25]. Transgenic expression of CagA in mice led to the development of gastric epithelial hyperplasia and adenocarcinomas of the stomach and small intestine. Interestingly, these effects were not seen with a transgene expressing a phosphorylation-resistant form of CagA (see section 2.3 below). However, the development of gastric cancer in the absence of

inflammation and also its occurrence in non-gastric organs raises questions about the applicability of this model to humans. Nevertheless, these results provide strong evidence for CagA acting as a bacterium-derived oncoprotein in the development of *H. pylori*-associated neoplasms in an animal model.

2.1 Delivery of CagA into gastric epithelial cells

How does the translocation of the CagA protein into gastric epithelial cells by the type 4 secretion system promote cancer? Recent studies have shown that CagF, a chaperone-like protein which interacts with the C-terminal secretion signal of CagA, is involved in the early steps of CagA recognition and is crucial for CagA delivery [26;27]. CagL, another product of a *cag PAI* protein-encoded gene, utilizes host integrin $\alpha_5\beta_1$ as a cell surface receptor. This CagL-integrin interaction then triggers CagA delivery into target cells and subsequently activates focal adhesion kinases (FAKs) and SRC kinases [28]. However, vector-expressed CagA alone undergoes efficient tyrosine phosphorylation when expressed in gastric epithelial cells [29], strongly suggesting that the CagL-integrin interaction is not an essential prerequisite for the tyrosine phosphorylation of CagA by host cell tyrosine kinases.

2.2 Phosphorylated CagA and CagA phosphorylation-dependent effects

Once injected into gastric epithelial cells, CagA can be tyrosine phosphorylated at its five amino-acid Glu-Pro-Ile-Tyr-Ala (EPIYA) repeat region [29]. This phosphorylation is initially mediated by SRC family tyrosine kinases (SFKs) and then continuously by the c-ABL kinase [30;31;32;33]. Tyrosine-phosphorylated CagA subsequently interacts with SRC homology (SH2) domain-containing host cell proteins, including the tyrosine phosphatase SHP-2, the C-terminal Src tyrosine kinase (CSK) and the adaptor protein CRK. This results in cytoskeletal reorganization and cell elongation - a phenotype that leads to cell scattering and the so-called "hummingbird" morphological change [29;34;35].

The hummingbird phenotype is a result of CagA-activated SHP-2 dephosphorylating FAK, thereby down-regulating FAK kinase activity and activation of ERK MAP kinases [36;37]. MAP kinase activation also promotes cell-cycle progression and this, together with the phenotypic changes induced, has been taken as evidence that CagA-activated SHP-2 may play an important role in cell transformation and gastric cancer promotion. Activation of CRK by CagA induces several downstream signaling pathways, such as SoS1/H-RAS/RAF1 and C3G/RAP1/B-Raf, suggesting that CagA/CRK signaling is required for both *H. pylori*-induced cell scattering and cell-cell dissociation [35]. While most phosphorylated CagA interacts with SHP-2, some also interacts with CSK, through a tyrosine phosphorylation-dependent mechanism leading to the inhibition of SFK activity. This blocks CagA phosphorylation and subsequently attenuates excess CagA-SHP2 signaling [34] through negative feedback.

In addition to the presence or absence of the *cag PAI*, variations in the EPIYA repeat region of CagA have also been associated with *H. pylori* pathogenicity [38]. Four distinct types of EPIYA motif have been identified, termed, EPIYA-A through EPIYA-D [39;40]. CSK binds specifically to tyrosine phosphorylated EPIYA-A or EPIYA-B, whereas SHP2 specifically binds to tyrosine phosphorylated EPIYA-C or EPIYA-D [29;34]. CagA from *H. pylori* strains found in the Western world typically contain EPIYA-A, EPIYA-B and EPIYA-C in the C-terminal EPIYA repeat region. In contrast, East Asian forms of CagA also possess EPIYA-A and EPIYA-B but not the EPIYA-C variant; instead, they contain an East Asian CagA-specific EPIYA-D sequence. The EPIYA-D region of East Asian CagA shows stronger SHP2 binding activity and induces the hummingbird phenotype in cultivated gastric epithelial cells to a greater extent than that induced by the EPIYA-C segment of Western CagA [40]. The number of EPIYA-C or EPIYA-D segments region also directly correlates with higher levels of tyrosine phosphorylation, SHP2 binding activity and more induction of the hummingbird morphology,

which may therefore explain why populations infected with East Asian CagA-positive *H. pylori* are at a greater risk for gastric cancer than those infected with Western-type CagA-positive strains [41;42].

2.3 Effects of CagA independent of CagA phosphorylation

In addition to phosphorylation-dependent activities, CagA also perturbs cell functions in a tyrosine phosphorylation-independent manner. Nonphosphorylated CagA interacts with certain host cell proteins such as the epithelial tight junction-scaffolding protein zonulin (ZO-1) [43], the cell adhesion protein E-cadherin [22], the hepatocyte growth factor receptor c-Met [44], the cadherin-associated protein β -catenin [21], the adaptor protein GRB-2 [45] and the kinase PAR1 [46;47]. These CagA-host protein interactions disrupt tight and adherent junctions, leading to a loss of cell polarity, and inducing pro-inflammatory and mitogenic responses-effects that may be important in the development of gastric carcinoma.

Tight junctions play an important role in maintaining paracellular permeability and cell polarity, and are also involved in cell motility, cell-cell adhesion and cell proliferation. Independent of tyrosine phosphorylation, CagA interacts with and mediates recruitment of the scaffolding protein ZO-1 and the tight-junction protein JAM to sites of bacterial attachment on host cell membranes, causing disruption of the assembly and function of both tight and adherent junction adhesion [43]. CagA also directly interacts with PAR1, a key regulator of cell polarity, and in turn inhibits PAR1 activity resulting in the loss of epithelial cell polarity [46;47]. As a result, the loss of cell polarity and disruption of cell adhesion induces transition from a polarized to an invasive phenotype in cultured epithelial cells [48]. CagA also interacts with E-cadherin independently of CagA tyrosine phosphorylation, leading to impairment of E-cadherin/ β -catenin complex and to cytoplasmic and nuclear accumulation of β -catenin. Downstream events include transcription of genes involved in intestinal differentiation such as *cdx1*, and the *muc2* mucin gene, causing transdifferentiation from gastric to intestinal type epithelial cells [22]. These results suggest that the ability of CagA to induce destabilization of E-cadherin/ β -catenin complexes may contribute to the development of intestinal metaplasia, a precursor lesion in the histological progression from a normal to a neoplastic gastric mucosa.

In vitro, intracellular nonphosphorylated CagA also interacts with growth factor receptor GRB2, causing activation of the RAS/MEK/ERK pathway, resulting in increased cell scattering and proliferation [45]. CagA can also target the c-Met hepatocyte growth factor receptor and deregulate c-Met receptor signaling leading to a mitogenic response, again independent of CagA tyrosine phosphorylation [44].

In vivo, *H. pylori* infection is associated with the production of pro-inflammatory cytokines and chemokines, such as IL-8, that stimulate neutrophil infiltration into the gastric mucosa. Most studies demonstrate that the *cag PAI*, but not CagA itself, is essential for *H. pylori*-induced IL-8 expression [12;49;50]. However, some recent studies suggest that CagA may promote IL-8 release via a RAS→RAF→MEK→ERK→NF- κ B signaling pathway independent of SHP-2- and c-Met [51]. CagA can also translocate nuclear factor of activated T cells (NFAT) from the cytoplasm to the nucleus in gastric epithelial cells. Translocation and activation of NFAT requires the EPIYA-containing region of CagA but is also independent of CagA phosphorylation [52]. Thus, CagA activates transcription factors such as NF- κ B, NFAT and TCF by MEK/ERK and Wnt/ β -catenin signaling pathway in a CagA tyrosine phosphorylation independent manner, resulting in the deregulation of many downstream genes including those encoding cytokines, anti-apoptotic proteins and metalloproteases [22;51;52].

The data from in vivo co-cultures indicate that following CagA injection into gastric epithelial cells by the type 4 secretory system, phosphorylated or nonphosphorylated CagA physically interacts with host cell proteins to trigger distinct signaling pathways. As a result, cells elongate,

scatter and exhibit disturbances of cell junctions with loss of polarity. Other downstream events include pro-inflammatory gene expression and the deregulation of proliferation and apoptosis. These phenomena are consistent with an oncogenic role for CagA translocation in gastric carcinogenesis, but their significance remains uncertain until they can be confirmed to be relevant *in vivo*.

3. Vacuolating cytotoxin (VacA)

Another major *H. pylori* virulence determinant is the vacuolating cytotoxin, VacA, which induces cytoplasmic vacuolation in cultured epithelial cells. Unlike the *cag PAI*, the *vacA* gene is present in all strains. *vacA* encodes a large (~140-kilodalton) preprotoxin, which includes an amino-terminal signal peptide and a carboxy-terminal domain. Following cleavage during secretion, toxin monomers of 88 kilodalton result [53;54]. The mature toxin subunits are released as soluble proteins into the extracellular space. They may also be retained on the surface of the bacterium [55], and they can aggregate into oligomeric complexes for insertion into lipid bilayers to form anion-selective channels [56].

Polymorphisms among the VacA alleles result in different levels of cytotoxicity. Variations in the signal (s) region and the mid (m) region greatly influence the effects of VacA [57]. Specifically, s region variation is associated with the vacuolating activity of VacA, [58] whereas the m region determines the cell specificity of vacuolation by affecting toxin binding to host cells [59;60]. All possible combinations from these regions have been identified and among them, vacuolating activity is highest in s1/m1 strains, less in s1/m2 strains and is absent in *H. pylori* expressing s2/m2 forms [57]. VacA polymorphisms are correlated with gastric diseases, particularly peptic ulcer disease, in studies of Western populations. For example, VacA s1/m1 may be strongly associated with duodenal and gastric ulcer disease and with gastric cancer [57;61;62]. However, East Asian strains are almost universally s1/m1 and are not associated with any specific clinical outcome.

Rhead et al recently identified a third polymorphic determinant of vacuolating activity in strains from Western populations that are located between the signal region and mid region, which they termed the intermediate (i) region [63]. In that report, two allelic variants of this region were denoted i1 (vacuolating) and i2 (nonvacuolating). All s1/m1 *vacA* alleles were of type i1, all s2/m2 alleles were of type i2 and s1/m2 alleles could be i1 or i2. In an Iranian population i1-type strains were strongly associated with gastric adenocarcinoma and of the three VacA polymorphic sites, the i1 genotype appeared to be a better predictor of carcinoma-associated *H. pylori* strains than s or m genotype. The same group have also reported VacA type i1 to be associated with duodenal ulcer disease, not just gastric cancer in a larger sample, though the association with ulcer disease was weaker than with cancer [42]. In contrast, Ogiwasa and colleagues were unable to confirm the *vacA* i as a virulence determinant in 314 strains isolated from East Asia and Southeast Asia where gastric cancer is highly prevalent. [64]

VacA has been reported to produce multiple structural and functional alterations in epithelial cells. VacA disrupts endosomal maturation resulting in vacuolation [65;66], selectively increase the permeability of polarized epithelial cell monolayers leading to barrier dysfunction at tight junctions [67] and also induces mitochondrial damage, cytochrome c release and gastric epithelial cell apoptosis [68;69;70]. Recent studies have also demonstrated multiple effects of VacA on the immune system. For example, VacA can interfere with phagocytosis and antigen presentation [71;72;73] and decrease the activation of Jurkat T cells through inhibiting the activation of NFAT, a key transcription factor required for the expression of genes involved in T cell activation [74]. VacA can also inhibit T cell proliferation through mechanisms independent of NFAT activation or IL-2 expression [75;76]. This effect requires an intact N-terminal hydrophobic region of VacA [75] and the T cell by β 2 integrin [77]. These effects of

VacA on the immune system might explain how *H. pylori* can evade the adaptive immune response to establish persistent infection. However, as for much of the CagA literature, it remains to be determined whether the above effects of VacA that have been so carefully investigated in vitro also occur during chronic *H. pylori* infection in humans in vivo, and therefore whether they might be clinically relevant.

4. Adherence

Adherence is important for *H. pylori* virulence. The intimate attachment observed between *H. pylori* and gastric epithelial cells may facilitate *H. pylori* colonization and efficient delivery of virulence factors such as VacA or CagA into host cells. Functional receptors for *H. pylori* adherence include fucosylated ABO blood group Lewis b antigens [78;79] and sialyl-Lewis x/a antigens [80]. The blood group antigen binding adhesin (BabA), a 78-kDa outer membrane protein, encoded by the *babA2* gene, binds to Lewis b antigens and ABO antigen [78;81;82], and the sialic acid binding adhesin (SabA) binds to the sialyl-Lewis x/a antigen [80]. There are two distinct *babA* alleles (*babA1* and *babA2*) and one highly homologous gene, *babB* [81], but only the *babA2* allele is functionally active. The only difference between *babA1* and *babA2* is that the former lacks a 10-bp insertion, encoding a signal peptide, that results in the *babA1* gene being silent [81]. The *babB* gene is highly homologous to *babA* at its 5' and 3' ends but not at the central region that (in *babA*) determines the specificity of receptor binding. *H. pylori* alters expression of its adhesins during infection. The expression of the *babA* gene can be modulated (switched from “on to off” or from “off to on”) through a recombination event (gene conversion) between *babA* and *babB* or by slipped strand mispairing (phase variation) based on the number of CT dinucleotide repeats in the 5' region during infection [79;83;84; 85]. SabA expression is regulated similarly.

The adherence of *H. pylori* to gastric epithelial cells mediated by BabA facilitates colonization, induces mucosal inflammation and promotes expression of sialyl-Lewis x/a [86;87]. The presence of *babA*, *cagA* and *vacAs1* together (“triple-positive strains”) is associated with duodenal ulcer and gastric adenocarcinoma in Western populations [17].

The intimate adherence mediated by SabA may enhance inflammatory response and facilitate the utilization of nutrients exudated from damaged host cells. Once the host inflammatory responses become too strong, the expression of SabA may be switched off, allowing the bacteria to escape from intimate contact with the inflamed epithelium, ensuring long-term persistence of the infection [80].

In addition, SabA was found to mediate binding of *H. pylori* to sialylated structures on neutrophils (which is a prerequisite for their nonopsonic activation [88]) and also to erythrocytes [89]. However, the pathophysiological importance of these latter findings is uncertain and clinicoepidemiological evidence linking SabA to gastric cancer is currently lacking.

5. Duodenal ulcer promoting gene (DupA)

Recent studies have reported that a duodenal ulcer-promoting gene (*dupA*), located in the “plasticity region” of the *H. pylori* genome, may be a novel virulence marker. Analyzing 500 *H. pylori* strains isolated from patients in Colombia, South Korea, and Japan, Lu et al reported that infection with *dupA*-positive strains was significantly associated with duodenal ulceration but negatively associated with gastric cancer [90]. Although others have not confirmed these findings regarding *dupA* these findings are particularly intriguing given that other proposed *H. pylori* virulence genes have been associated with both ulcer disease and gastric cancer, whereas patients with duodenal ulcers are paradoxically at *decreased* risk of gastric cancer [91].

6. Conclusions

The hunt for *H. pylori* factors that are correlated with specific disease status has identified several positive virulence-associated genetic loci and resulted in the extensive investigation of their effects, largely using co-culture models in vitro. Clinical studies and animal models have demonstrated that some of these factors, especially the cag PAI and the VacA polymorphisms, may be determinants of gastric cancer development in vivo. However, there remains a considerable gap in knowledge between the detailed dissection of the events observed in co-culture and clinico-epidemiological confirmation of their role in the pathogenesis of human diseases. Analysis of the association of *H. pylori* virulence factors with *H. pylori*-related outcome, particularly gastric cancer, has highlighted the need to consider the geographical contributions to disease outcome. For example, in Western countries, the presence of CagA is associated with an increased risk of severe disease outcome [17;19]. However, in East Asian populations, even though East Asian CagA is more potent biologically its presence is not indicative of increased risk of *H. pylori*-related diseases [92;93]. Further studies will need to consider other environmental cofactors as well as the genetic diversity of specific Eastern and Western populations and how this might relate to the different outcomes of *H. pylori* infection. For example, host polymorphisms in several genes that regulate inflammatory responses, such as the genes encoding interleukin-1 beta and the interleukin-1 receptor-antagonist, have been found to be associated with the development of gastric cancer [94;95] in Western populations though they appear less important in other regions of the world [96]. While the mechanisms of *H. pylori*-associated gastric carcinogenesis are still relatively poorly defined, their future elucidation may provide opportunities to develop effective strategies for gastric cancer prevention and therapy.

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The pathogenesis of *H. pylori* infection. Virulence factors such as CagA, genes within the *cag* PAI, VacA, BabA and SabA are involved in numerous host cell effects. *H. pylori* adherence to gastric epithelial cells is mediated by BabA and SabA binding Lewis^b, and Lewis^{x/a} respectively, thus facilitating *H. pylori* colonization and the efficient delivery of virulence factors to host cells. Following the attachment of *H. pylori* to gastric epithelial cell, the CagA protein is delivered into gastric epithelial cells through the *H. pylori* type 4 secretion system (T4SS). There, CagA may be tyrosine phosphorylated at EPIYA sites initially by SRC and in more sustained fashion by ABL kinases. CagA can interact with intracellular proteins and deregulate distinct signaling pathways through both tyrosine phosphorylation-dependent or independent mechanisms. The pathobiological effects including cell elongation (producing cell scattering and the “hummingbird” phenotype), disruption of intercellular junctions, loss of cell polarity, the promotion of inflammation and dysregulation of proliferation and apoptosis. The effects of VacA on host cells include induction of cytoplasmic vacuolation, mitochondrial damage, cytochrome *c* release, apoptosis and immune evasion. Many of these events may contribute to gastric carcinoma development.