The role of the putative virulence markers (cagA and vacA) of *Helicobacter pylori* in peptic ulcer disease

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ABSTRACT

Helicobacter pylori is genetically diverse and certain strains are more virulent and cause more severe diseases than others and such diversity is reflected on the clinical outcome. The cytotoxin-associated gene (cagA) and vacuolating cytotoxin (vacA) gene are 2 putative markers that were associated with peptic ulcer disease. The basis for the epidemiological association between the cagA and vacA genes is not known. In this review, the molecular characteristics of these markers, and its role in the pathogenesis of peptic ulcer disease and gastric cancer are discussed.

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Helicobacter pylori (*H. pylori*) is the causative agent of chronic gastritis (CG) and peptic ulcer disease (PUD) and a major risk factor for the development of gastric cancer (GC) and mucosal associated lymphoid tissue (MALT) lymphoma. Although most infected individuals remain asymptomatic, approximately 10% suffer from overt disease and still very few progress to gastric cancer.[1] The clinical outcome of *H. pylori* infection is likely to be determined by a combination of factors including strain virulence, host responses and other environmental factors. The virulence markers of the infecting strains such as urease, flagella, adhesins, oxidase, catalase, and vacuolating cytotoxin (VacA) allow *H. pylori* to persist and stimulate an intense inflammatory response for years.[2] Genes in the cag pathogenicity island (PAI) such as the cytotoxin-associated gene (cagA) also, contribute to the inflammatory process.[1,2] Variations in the clinical outcome were found potentially due to high levels of genotypic diversity among each *H. pylori* strain that is expressed in the production of the vacuolating cytotoxin encoded by the vacA gene and the cytotoxin-associated protein encoded by the cagA gene, while many strains from asymptomatic carriers do not produce either the vacuolating toxin or the CagA protein.[3] Much interest was focused on these putative markers since their description in the early 1990's.

Molecular characteristics. a) Cag pathogenicity island (PAI). The PAI is a 40-kb locus inserted in the chromosomal glutamate racemase gene that enables the bacterium to cause cellular damage. The locus has a G-C content of 35% that is different from the rest of the genome (39%) suggesting an acquisition from another organism by site-specific or generalized recombination.[4] The region is flanked by 31bp direct repeats; insertion sequences (that may be present in multiple copies) and a high density of genes packed in this region.[5] The cag PAI can be found either as a single uninterrupted unit, divided into 2 regions (cagI and cagII), by an insertion sequence called IS605 or by a large piece of chromosomal DNA, or it can be partially or totally deleted. Studies from France, Japan and Italy had shown that 54-94% of the isolates had an entire cag PAI, 8% had the cag PAI split in 2, 6-25% contained partial deletions and 32% had no cag PAI.[5-9]

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b) Cytotoxin associated gene. The cagA is one of 30 genes located in the right half of the cag PAI. It encodes a highly immunodominant protein that induces specific antibodies in sera of virtually all infected individuals and affects host cell physiology after being delivered to gastric epithelial cells.[10] The protein varies in molecular weight between 120-140 kDa, which was attributed to the presence of a variable number of repeated sequences in the 3' region of the gene.[5,10] Sequencing of the cagA gene revealed a region of internal duplications that may be responsible for size heterogeneity.[7] The biological importance of the repeat regions and its influence on the clinical outcome with respect to diversity at the cagA gene level is still undetermined.[10] Yamaoka et al[5] specifically examined this 3' variable region and found that there were 4 categories of repeating sequences named A, B, C and D in which 86% of type C variants were found in patients with gastric cancer. This allelic variation in the cagA gene and the distinct *H. pylori* sub-genotypes that circulate in different regions may provide a marker for differences in virulence among cagA-positive strains.[5,11] The sequence variability of the cagA gene in isolates from different geographic origins may lead to underestimation of the true prevalence of cagA-positive *H. pylori* strains. Although the proportion of *H. pylori* isolates, which are cagA-positive varies from one geographic region to another, cagA gene is possessed by approximately 50-60% of the strains.[11]

c) Vacuolating cytotoxin A. The vacA gene is present in almost all strains of *H. pylori*, but approximately 50% of those strains produce detectable amounts of the cytotoxin. This may be due to the allelic differences in the signal sequence and middle region of the gene.[12] The vacuolating cytotoxin of 87kDa molecular weight is the major toxin secreted by *H. pylori* that induces vacuolation in the human epithelial cells.[13] It is thought that this toxin works as an AB-type toxin, where the c-terminus of the polypeptide is the B sub-unit that mediates binding to gastric epithelial cells and the A portion is the enzymatic component.[14]

Earlier molecular studies indicated that the vacA gene comes in one of 2 different forms (type 1 and type 2). Strains that produce the vacuolating toxin had a type 1 vacA, while strains that do not produce the toxin possess the type 2 vacA version of the gene.[15] At the DNA level, most non-toxigenic strains carry a complete but non-toxigenic allele of vacA.[4] Analysis of *H. pylori* isolates from diverse geographic locations permitted a comprehensive description of the vacA sequence (s) and middle (m) regions.[16,17] The nucleotide sequence of vacA is approximately 70% homologous in both toxin-positive and toxin-negative *H. pylori* strains. The main divergences were found in the region encoding the signal sequence and the middle regions.[3] The vacA s region (which encodes the signal peptide) exists as s1 or s2 allelic types. Among the s1 type strains, subtypes s1a, s1b, and s1c have been identified. The m region exists as m1 or m2 allelic type. Two subtypes have been identified within the m2, designated m2a and m2b.[18] All possible combinations of these regions have been detected, although s2/m1 alleles occur quite rarely. In general, type s1m1 and type s1m2 strains produce high and moderate levels of toxin, respectively, while s2m2 strains show little or no vacuolating toxin activity.[19]

Pathogenesis. *Helicobacter pylori* inflammation is characterized by active CG with continuous recruitment and invasion of polymorphonuclear as well as mononuclear cells, which leads to increased epithelial permeability and acute mucosal damage. The mechanisms behind this unusual inflammation pattern are still unknown, but endothelial cell function and activation are probably key components in this process.[20] Secretion of interleukin-8 (IL-8) and other interleukins attract neutrophils that migrate from the capillaries through the lamina propria and emerge between the epithelial cells. At the site they release their digestive products such as proteases and reactive oxygen species that result in tissue damage. The neutrophil infiltration in gastric mucosa was significantly milder in patients infected with partially or totally deleted cag PAI strains than in those with intact cag PAI strains.[21] In the antrum and similarly in the corpus, the degree of inflammation was associated with cagA-vacA s1 genotypes, and the degree of neutrophil activity was associated with the CagA-positive genotype. Whereas the degree of atrophy was associated with cagA-vacA s1m1 genotypes.[20] The cag PAI is one of the major *H. pylori* virulence factors that is found more frequently in isolates patients with severe gastroduodenal diseases, including peptic ulcers and gastric adenocarcinomas.[1] The activation of the gastric epithelial cell, mitogen-activated protein (MAP) kinases system, by *H. pylori* cag PAI-positive strains may be crucial in inducing gastroduodenal inflammation, ulceration, and carcinoma since MAP kinases regulate cell proliferation, cell differentiation, programmed cell death, reactions to stress and inflammatory responses.[6] The type IV secretion machinery encoded by the cag PAI of *H. pylori* has been implicated in a series of host responses during infection. The phenotypic changes and phosphorylation of cagA depended on the genes in both cagI and cagII loci including the complete virB/D complex, however the induction of IL-8 (a neutrophil-attracting cytokine) secretion depended largely on the same set of genes but was independent of cagA and VirD4.[22] The identification

of 2 genes named picA (cagC and cagD) and picB (more recently renamed cagE) in cagA-positive strains has been associated with a pathogenic role in duodenal ulcer disease.[7] The CagE gene (located upstream of cagA in the right half of the cag PAI), encoding a protein involved in the process of IL-8 expression in gastric epithelial cells; and the virB11 gene (located in the left half of the cag island) encoding, together with other genes, a type IV secretion system, which allows the delivery of the cagA protein to gastric epithelial cells.[7]

Attachment of *H. pylori* to the cell membrane induces tyrosine phosphorylation of host cell proteins, which following a series of degradation and activating steps, regulates IL-8 expression.[6] Genes of the cag PAI in cagA-positive, but not cagA-negative strains provoke potentially damaging inflammatory responses in infected host tissue and induce synthesis of the proinflammatory cytokine IL-8 in gastric biopsies and cultured cells even though cagA itself is not needed for the induction process. Chromosome walking and mutational experiments identified several genes upstream of cagA present in cagA-positive but absent from cagA-negative strains are needed for IL-8 induction.[4] The induction of IL-8 was decreased or suppressed when the cag PAI genes (CagE, G, H, I, L and M) but not (cagA, F or N) were disrupted.[1] Audibert et al[6] indicated that the cag PAI, or at least part of it is not the only element required for IL-8 induction. Earlier, Yamaoka et al[5] found that the cag PAI-negative strains containing a functional HP0638 gene, encoding one of the 32 outer membrane inflammatory proteins detected in the genome sequence, produced 3-fold more IL-8 than cag-negative strains containing a non-functional HP0638 gene. Similarly 89 (96%) of 93 cagA-positive strains had HP0638 in frame, versus none of 16 cagA-negative strains.[23] These studies indicate that the existence of strains inducing IL-8 secretion regardless of the cag PAI structure suggests that this region is not the only requirement for IL-8 secretion.

The VacA toxin target several different cell components, including endocytic vesicles, mitochondria, cytoskeleton and epithelial cell-cell junctions.[24] The cellular effects induced by VacA toxin include vacuolation, alteration of endolysosomal function, pore-formation in the plasma membrane, apoptosis and epithelial monolayer permeability. The exact mechanism of how the toxin exerts its effect is still unclear. Mature toxin molecules are released into the extracellular space, and also it may be retained on the surface of the bacterium. It remains unclear whether VacA should be classified as an A/B type toxin, a channel-forming toxin, or both.[14]

From the above-mentioned studies, bacterial virulence factors and environmental influences contribute to the pathogenesis process, but do not explain the divergent outcomes. There is emerging evidence that host genetic factors also play a key role in determining the clinical outcome of *H. pylori* infection. In particular, proinflammatory genotypes of the interleukin-1 beta (IL-1b) gene, which is associated with an increased risk of gastric cancer and its precursors. The effects are most likely mediated through the induction of hypochlorhydria and severe corpus gastritis with the subsequent development of gastric atrophy.[24] Patients with duodenal ulcer with antrum-dominant *H. pylori* gastritis and had only a very low-grade corpus *H. pylori* gastritis retain normal (or even high) acid secretion, and they are very rarely to develop gastric carcinoma, whereas individuals with extensive corpus gastritis develop hypochlorhydria and gastric atrophy.[25,26] Thus, host factors seem to exert a crucial effect since IL-1 gene cluster polymorphisms suspected of enhancing production of IL-1b (a powerful inhibitor of gastric acid secretion) are associated with an increased risk of both hypochlorhydria induced by *H. pylori* and gastric cancer. This might suggest why some individuals infected with *H. pylori* develop gastric cancer and others do not.[26]

Role in peptic ulcer disease. It is generally accepted that genetic diversity in *H. pylori* strains may affect the function and antigenicity of virulence factors associated with bacterial infection and, ultimately, disease outcome. Audibert et al[6] reported that an entire cag PAI was statistically correlated with the ability to induce IL-8 secretion but not with clinical presentation of the infection. Although the presence of a functional cag PAI increases the proinflammatory power of a strain, it may have no predictive value for the presence or the future development of a clinically significant outcome, as other factors influence the evolution of the disease. Nevertheless, it is likely that strains with functional cag PAI are more often involved in severe outcomes.[6] The link between the expression of cagA and PUD in symptomatic subjects has been studied in both developed and developing countries and the results were in discordance. Several studies carried out on subjects from Portugal, Netherlands, Italy and Germany indicated that individuals colonized with *H. pylori* cagA-positive strains are at increased risk for developing peptic ulceration as compared to subjects harboring cagA-negative strains.[24,27,28] In contrast, the risk for PUD might be significantly decreased in those patients who are infected with *H. pylori* strains lacking the cagA gene.[11,29,30] These epidemiological observations are supported by studies in the mouse model. Sonic extracts of cag-positive strains, but not those from cag-negative strains, induce gastric damage with histological lesions (epithelial vacuolation, mucosal erosion, necrosis, and ulceration) that are similar to those found in biopsies from patients infected with *H. pylori*.[31]

On the other hand, studies from other countries indicated no significant association between these markers and PUD. The high prevalence of cagA-positive strains (98%) in Taiwanese subjects suggests that cagA-positive phenotype could not be used as a single marker in high-risk patients in Taiwan. Sequence analysis of the cagA gene indicated that Taiwanese *H. pylori* strains contain different gene sequences from those in other geographic regions.[31] Similar findings were also reported from Korean, Chinese, Japanese, Nigerian and Jamaicans subjects.[5,32-34] The cagA status as determined by molecular methods was also evaluated by detecting serum anti-CagA antibodies. Studies have indicated that *H. pylori* expressing cagA are better able to colonize the stomach.[35] Increased prevalence of anti-CagA antibodies in both PUD and gastric cancer patients was reported.[27,36] In Japan, Ando et al[37] found that anti-CagA responses associated with neutrophil infiltration, intestinal metaplasia, *H. pylori* density, and IL-8 levels, suggesting that the absolute levels of these antibodies may be markers for gastric inflammation and pre-malignant changes. Results from several other studies indicated no such association.[38-41] It appears to be major geographic differences in the prevalence of cagA-positive strains. In Australia, anti-CagA antibodies in duodenal ulcer group were of higher titers than those of the asymptomatic group, while in China these antibodies were prevalent in both asymptomatic and gastric cancer group.[38] In our study on symptomatic and asymptomatic Turkish subjects, we detected anti-CagA antibodies in both groups, regardless of the presence of gastroduodenal disease.[42] A similar correlation was also described for the VacA gene and the disease outcome. Strains expressing the VacA gene have been detected more frequently among patients with PUD than those with CG.[39] According to Papini et al[14] early studies noted that a higher proportion of *H. pylori* strains cultured from patients with PUD exhibited cytotoxic activity than strains did from patients without the disease. Subsequently, other studies have shown that patients with PUD are usually infected with strains containing vacA s1 alleles, whereas strains containing s2 alleles are found predominantly in patients without ulcers.[3,43] These data suggest that the vacA gene contributes to the pathogenesis of PUD. The linkage between the cagA and vacA gene expression is not yet clear since they are located more than 300 kb apart on the chromosome of *H. pylori*.[31] Moreover, it has been shown that inactivation of the cagA gene has no consequence on expression of the vacA gene or on the ability to induce IL-8. As noted before, the associations of *H. pylori* genotypes with disease differ between Western countries and Asia. Earlier studies in Italy and the United States of America (USA) suggested that *H. pylori* expressing the CagA and VacA antigens are more frequently associated with PUD.[19,31] While studies from India, Estonia and Sri Lanka showed no such association.[44-46]

Several investigators looked at other *H. pylori* virulence markers and tried to correlate their expression with the disease outcome. It was shown that strains of *H. pylori* co-expressing cagA, vacA s1m1, and babA2 virulence genes worsened inflammation significantly.[47] Mizushima et al[48] indicated that in Western countries, the gene encoding blood group antigen-binding adhesin (babA2) of *H. pylori*, is of high clinical relevance and is a useful marker to identify patients who are at a higher risk for peptic ulceration and gastric adenocarcinoma, as are vacA and cagA. However, their study in Japan revealed no correlation between the babA2 and cagA genotypes, and the clinical outcome. This was in agreement with a recent study reported from Taiwan.[49] These results indicate that babA2 status is not of high clinical relevance in Japan and Taiwan and that these strains are different from those infecting western populations. Other studies showed that the elements that appeared to be the best markers for the presence of cag PAI were the cagE and the virD4 genes. Ikenoue et al[21] indicated that the cagE gene was a more accurate marker of an intact cag PAI than the cagA gene, and cagE seemed to be more useful in discriminating between *H. pylori* strains causing different rates of disease progression. However, because the cag PAI may be partially deleted or diversely organized, it is likely that the presence of one or even several genes is not sufficient to assess the presence of this region.[6] Thus, McGee and Mobley[1] suggested that the use of cagA or the cag PAI to correlate clinical outcome of infection might not be reliable in all geographic locations.

Role in gastric cancer. According to Hohenberger et al[50] gastric cancer is the second largest cause of cancer-related death worldwide. The disease is most common in Japan and China; in Europe the annual incidence is 12-15 per 100000. In the USA, the incidence is low and the death rate for gastric cancer is 52 deaths per 100000 compared with 90 per 100000 in Japan. During the past 50 years, incidence and mortality have decreased, especially in developed countries due to the improvement in the standards of living and dietary consumption. There is mounting evidence that *H. pylori* infection plays an important role in the pathogenesis of gastric cancer.[51,52] Recently, the World Health Organization (WHO) declared *H. pylori* as a class I carcinogen due to its association with gastric malignancies.[52] *Helicobacter pylori* is known to cause chronic active inflammation of the gastric mucosa in the majority of colonized subjects and in a considerable number, this will eventually lead to a loss of gastric glands (atrophy) that is

associated with the development of intestinal metaplasia.[53] An 8-fold increased risk for both conditions occurs in the presence of infection that is influenced by the age at which infection occurred and the presence of cagA. Approximately 40-50% of infected subjects develop these conditions, but they are rare in non-infected subjects.[53] Gastric cancers such as other cancers, mainly occur due to its multiple genetic changes that stop cell size and division regulation. At least 7 different mutations were described in *H. pylori* isolated from gastric cancer patients. In adenocarcinoma of the stomach, mutations present are unlike adenocarcinoma of other tissues. This could suggest that other factors contributed by *H. pylori* are involved.[54]

Earlier reports regarding the relationship between *H. pylori* infection and gastric cancer were conflicting. Huang et al[55] did a meta-analysis study and found that the heterogeneity in the reported results was caused by differences in the selection of controls, patient age, and the site and stage of gastric cancer. The strains of *H. pylori* that express the CagA protein are associated with a 3-fold increased gastric cancer risk as compared to strains that do not express cagA.[56,57] In Germany, Miehlke et al[58] found a significant association between the *H. pylori* cagA, vacA s1m1 genotype, cytotoxic activity and gastric cancer. Other studies also reported similar results.[51,52,59] In a population-based case-control study, immunoglobulin anti-*H. pylori* antibodies were more prevalent in patients with gastric adenocarcinomas than in controls.[50] More evidence came from studies on experimental animals were Mongolian gerbils infected with *H. pylori* showed that 37% of the animals developed adenocarcinoma compared to none in the controls.[60] The epidemiological association between *H. pylori* and gastric carcinoma does not prove a causal relationship. There are some contradictory data available including the fact that only a small population of infected patients develops carcinoma and occasionally carcinoma arising in patients who are not infected.[53,61] In addition, the prevalence of *H. pylori* in Africa is high but the incidence of gastric carcinoma is low.[53] A high prevalence of cagA-positive strains in Brazil was also observed in patients with gastric carcinoma, but no significant difference was observed when compared with peptic ulcer or gastritis patients.[62] It is clear that other co-factors must play a role in carcinogenesis. These include age of onset of *H. pylori* infection, dietary factors, salt intake and altered immune status. The age of onset of *H. pylori* infection seems to be very important. Studies have found that more than half of the children are to be infected in high-risk geographic regions compared to other lower risk regions.[53] *Helicobacter pylori*-infected younger patients have a higher relative risk for gastric cancer than older patients with odds ratios decreasing from 9.29 at age <29-70 years.[55] The persistence of an inflammatory response over many years results in the exposure of dividing epithelial stem cells to inflammatory products. This can lead to mutations and malignant transformation. The risk is amplified by dietary factors such as nitrates, carbohydrates, and excessive salt consumption and also by deficiencies of antioxidants, which may limit the inflammatory related oxidative damage.[53] It has been shown that *H. pylori* substantially impair the bio-availability of vitamin C that result in lowering the plasma level. The reduced circulating concentrations of vitamin C in people infected with *H. pylori* may be a causative factor in gastric cancer, as well as other diseases associated with antioxidant deficiency.[63] Results of a randomized, placebo-controlled trial in a Columbian population of people at a high risk of developing gastric cancer showed that vitamin C, beta-carotene, and *H. pylori* eradication were statistically significant in promoting regression of precursor lesions.[64]

The applicability of these findings is uncertain, but they may provide more support that *H. pylori* induces gastric cancer. The most significant evidence linking *H. pylori* to gastric cancer is that the eradication of this bacterium causes regression of MALT lymphomas. It was found that MALT lymphoma is antigenically stimulated by *H. pylori* and early eradication of the bacteria can result in 60-92% tumor regression.[65] Studies on the efficiency of screening and eradication of *H. pylori* are underway to determine if this leads to a decrease in the incidence of gastric cancer. These investigations may provide real evidence supporting the theory that *H. pylori* has a role in causing gastric cancer, however despite recent advances in knowledge on differences in carcinogenesis of gastric cancer subtypes, genetic alterations between *H. pylori* positive and non-*H. pylori* associated cancers could not be verified.

In conclusion, although *H. pylori* infection occurs worldwide, there are significant differences in its prevalence both within and between countries. The data suggest that there is a major geographic difference in the prevalence of cagA-positive strains and that VacA contributes to the pathogenesis of PUD. The use of cagA or the cag PAI to correlate clinical outcome of infection might rely on the geographic locations. Factors that influence the risk for cancer in *H. pylori* infected patients may be related to the onset of infection and to the genetic makeup of the bacterial strain and host factors.

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