Helicobacter pylori CagA: The Bacterial Oncoprotein

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Abstract

Helicobacter pylori infects the human stomach, causing atrophic gastritis, peptic ulcer, and gastric cancer. Among the virulence factors of *H. pylori*, the *cag* pathogenicity island (*cag* PAI) has been identified in the *H. pylori* genome, coding for several proteins that constitute a type IV secretion system (T4SS), whose main function is to inject bacterial factors into the host cell. In particular, CagA protein, encoded by cytotoxin-associated gene A (*cagA*) that is part of the *cag* PAI, is injected into the host cell in a T4SS-dependent manner. CagA, once into the cell, can be phosphorylated by host enzymes. Both phosphorylated and non-phosphorylated CagA initiate a series of intracellular events, which may dramatically interfere with cell morphology, motility, polarity, proliferation, and differentiation, leading to invasive phenotypes of host cells. Thereby, CagA has earned the definition of "bacterial oncoprotein." Epidemiological studies in humans, as well as studies in animals infected with CagA-positive *H. pylori* strains, or in transgenic mice expressing CagA, indicated a clear link between CagA and the development of precancerous lesions and eventually gastric cancer. Although, besides CagA, other *H. pylori* factors have been linked to gastric cancer development, CagA appears to be the major agent responsible for the *H. pylori*-related carcinogenicity. The development of malignancy is also linked to host factors, such as proinflammatory genetic background. It might be expected that treatments or vaccines targeting CagA, even in the case they only partially affected the *H. pylori* burden, would decrease the risk of malignant outcome of the infection.

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Contents

Introduction

Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic, Gram-negative bacillus. It inhabits the human stomach with prevalence higher than 50% worldwide, varying according to the geographic area and generally increasing with the age of the subjects. *H. pylori* gastric colonization/infection is most commonly acquired at pediatric age, and it may persist for the entire life of the human host.

After the *H. pylori* presence in human stomach was reported for the first time (Marshall and Warren 1984), its relationship with gastritis and peptic ulcer in humans was evidenced (Marshall and Warren 1984; Goodwin et al. 1986). Initially the bacterium was named *Campylobacter pyloridis* and then *C. pylori*, before its definitive classification in the genus *Helicobacter*. The discovery of *H. pylori* and of its link to peptic ulcer led to award the 2005 Nobel Prize in Medicine to Barry Marshall and Robin Warren. It was subsequently found that *H. pylori*-infected subjects are at higher risk of gastric cancer (Correa et al. 1990; Forman et al. 1990): for this reason, and for the further evidences of the link between *H. pylori* infection and gastric cancer, the International Agency for Research on Cancer (IARC) has classified *H. pylori* as a group 1 carcinogen (IARC 1994, 2012). Moreover, *H. pylori* was found to be the causative effect of gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Wotherspoon et al. 1991). *H. pylori* is the only bacterium known to cause cancer in humans.

To establish effective and long-lasting colonization of the gastric mucosa and to get nutrients from the host's tissue, *H. pylori* has developed several mechanisms that generate an inflammatory status, at the same time allowing the bacterium to evade or alter the host immune response; these mechanisms in the majority of the infected

population lead to asymptomatic gastritis only, but in some subjects, they may be the origin of the immune pathogenesis of gastric inflammation and mucosal disease. Indeed, the pathological outcome of *H. pylori* infection results not only from direct bacterial action but also from host response and susceptibility, as indicated by several studies that, for instance, showed cytokine gene polymorphisms to be associated to resistance/susceptibility to *H. pylori* and different outcomes of *H. pylori* infection. Thus, when individuals with proinflammatory genetic background are infected by *H. pylori* strains expressing particularly dangerous factors, the immune response may initiate chronic inflammation causing corpus gastritis and hypochlorhydria, which in turn may evolve to gastric atrophy, to gastric ulcer, and eventually to malignancy.

Symptomatic patients that are diagnosed with *H. pylori* infection are usually subjected to antibiotic-based treatment. Successful eradication of *H. pylori* results in regression of peptic ulcer and MALT lymphoma. The efficacy of the current standard triple therapy based on proton pump inhibitor and two antibiotics has dropped below 80%, mainly due to antibiotic resistance: thus, modifications of the therapy composition and regimen are being actively investigated.

Among several *H. pylori* vaccines that had shown good efficacy in animal models, only few underwent clinical trials, generally giving disappointing results. More recently, instead, the encouraging results of a randomized, double-blind, placebo-controlled, phase 3 pediatric trial with a recombinant urease-based oral vaccine have been reported (Zeng et al. 2015). Urease is a major protein of *H. pylori*: it exerts an activity essential for the survival of the bacterium in the gastric niche, catalizing the conversion of urea to carbon dioxide and ammonia, which in turn neutralizes the gastric juice acidity. The vaccine afforded 71.8% protective efficacy against *H. pylori* infection 1 year after vaccination and still 65% efficacy 3 years after vaccination, with adverse events not significantly different from those that occurred in the placebo group, both in terms of quantity and quality (Zeng et al. 2015).

Among the virulence factors of *H. pylori*, the first to earn the definition of toxin was the vacuolating toxin A (VacA), whose cytotoxic activity was observed in vitro, and its direct action on damaging the gastric epithelial mucosa evidenced in vivo (see also ▶ Chap. 15, "Interaction of *Helicobacter pylor*i VacA Toxin with Its Target Cells," by V. Ricci, in the present book).

Then, another *H. pylori* protein was identified and characterized, whose presence was closely associated with that of VacA, encoded by cytotoxin-associated gene A (*cagA*) and thus named CagA (Covacci et al. 1993; Tummuru et al. 1993); the studies on CagA evidenced its relationship with the most severe outcomes of *H. pylori* infection, including gastric cancer (Blaser et al. 1995). For the numerous detrimental activities exerted by CagA on host cells, it deserves the definition of toxin, and moreover in particular it has been referred to as "bacterial oncoprotein" (Hatakeyama 2003).

The present chapter will focus on CagA and especially its role in the development of gastric carcinoma and the related mechanisms elucidated so far.

The *cag* Pathogenicity Island and the Type IV Secretion System

After the discovery of CagA, the studies on its gene *cagA* revealed that it is encompassed by a chromosomal DNA insertion element of about 40 kb, which exhibits typical characteristics of pathogenicity islands and thus was named *cag* pathogenicity island (*cag* PAI); it was proposed to represent a secretion system for exportation of bacterial factors (Censini et al. 1996). Indeed, this *H. pylori* structure was found to belong to the type IV secretion system (T4SS) family, which is harbored by many Gram-negative pathogens that use it to translocate virulence factors into host cells.

H. pylori strains were classified in two types, type I and type II, according to the presence or the absence of *cag* PAI. Type I strains, having the *cag* PAI in their genome, were found to be associated with the more severe gastroduodenal disease. Not only the presence but also the intactness of *cag* PAI was found to be associated with the severity of histopathological changes in the gastric tissue of patients infected with *H. pylori* (Ahmadzadeh et al. 2015). Interestingly, high *cag* PAI diversity was observed, as coexistence of variants of the same strain with different *cag* PAI genotypes was detected in a significant proportion of patients infected by *cag* PAI-positive *H. pylori* (Matteo et al. 2007). The relevance of this diversity to the *H. pylori* infection strategy deserves further investigation: it may be part of the genetic diversification that allows *H. pylori* to persist during chronic colonization/infection.

The *cag* PAI consists of about 30 genes, which encode as many Cag family proteins that constitute the *H. pylori* T4SS, or play a role in its biogenesis or functions, or whose function has not been identified yet (Backert et al. 2015). The *H. pylori* T4SS consists of inner and outer membrane-spanning complexes and a surface-located pilus, composed of the Cag family proteins. The major function of T4SS is to translocate CagA into the host cell, where it can be phosphorylated by the host enzymes and initiates a series of events that contribute to the gastric disease development and eventually to malignancy: these specific aspects will be treated in the subsequent paragraph dedicated to CagA and its activities on host cells. *H. pylori* utilizes T4SS also to deliver bacterial peptidoglycan into the host cell; internalized peptidoglycan has been suggested to contribute to the activation of proinflammatory signaling cascade through interaction with the cytosolic pathogen recognition molecule nucleotide-binding oligomerization domain containing 1 (NOD1), thus initiating a NOD1-mediated host defense against *cag* PAI-positive *H. pylori* strains (Viala et al. 2004). The *cag* PAI is the major factor responsible for the induction of transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and IL-8 (interleukin-8) by *H. pylori.*

Among the Cag proteins other than CagA, CagL deserves particular attention. It is surface-exposed on the tip of the T4SS and contains an RGD (Arg-Gly-Asp) motif that is commonly found in many integrin ligands; mutations in RGD site, even at the level of single amino acid substitution, abolish the type IV secretion and the subsequent CagA translocation and phosphorylation (Kwok et al. 2007). The RGD motif is able to directly bind the α5β1 integrin, a cell adhesion receptor that is located

in the basolateral host cell membrane (Kwok et al. 2007), and also the αVβ6 integrin, another receptor for viruses that expose an RGDLXXL (Arg-Gly-Asp-Leu-x-x-Leu) motif (Barden and Niemann 2015). Another surface-exposed motif of CagL, comprising FEANE (Phe-Glu-Ala-Asn-Glu) sequence, acts as an enhancer of the interaction of CagL with integrins, most likely in the early stage of T4SS-integrin interaction (Conradi et al. 2012). Thus, CagL was proposed to act as a specialized adhesive that allows T4SS to enter in contact with the host cells, necessary for the subsequent CagA delivery. It was also proposed that CagL, besides its role of establishing the contact with the host cell surface, concomitantly activates host tyrosine kinases to favor CagA phosphorylation at the site of injection (Kwok et al. 2007). Other Cag proteins have been described to bind host cell integrins, namely, CagA, CagY, and CagI, which are able to bind β1 integrin in an RGD-independent manner: this interaction of T4SS with the host cell causes a conformational switch that is necessary to initiate effector protein translocation (Jiménez-Soto et al. 2009). CagH (localized in the inner membrane), CagI (periplasmic, surface-associated, and secreted), and the already cited CagL deserve to be mentioned for their involvement in pilus biogenesis, and in particular CagI and CagL are essential to pilus formation, while CagH is relevant to the regulation of the pilus elongation (Shaffer et al. 2011). Moreover, the five components of the membrane-spanning core complex of *H. pylori* T4SS, namely, CagM, CagT, Cag3 (also known as Cagδ), CagX, and CagY, have been shown to be required for T4SS activity (Frick-Cheng et al. 2016). CagD (a dimer localized in the cytosol, inner membrane, periplasm, surface-associated, and released) has been shown to be essential for CagA translocation, but not for pilus assembly (Cendron et al. 2009).

H. pylori CagA Structure and Activities on Host Cells

CagA Structure

CagA was initially identified as a high-molecular-mass *H. pylori* antigen associated with peptic ulcer disease, as evaluated by the serum antibody levels against this protein found in infected patients. The first attempts of molecular characterization of this protein (1) did not reveal any significant homology with other known proteins; (2) identified a motif of five amino acids, EPIYA (Glu-Pro-Ile-Tyr-Ala), present in several repeats of 102 bp; (3) showed that *cagA* gene is not present in all *H. pylori* strains and, when present, it is strictly associated with the production of VacA, the *H. pylori* cytotoxin; and (4) evidenced that CagA is an immunodominant antigen and indicated that seropositivity to CagA associates with the more severe gastrointestinal disease and gastric cancer (Covacci et al. 1993; Crabtree et al. 1993; Tummuru et al. 1993). The molecular mass of CagA ranges from 128 to 144 kDa, depending on the number of repeats that are present.

The crystal structure of a large N-terminal portion of CagA, of about 100 kDa, has been resolved, corresponding to amino acids 1–876 (Hayashi et al. 2012) or 1–884 (Kaplan-Türköz et al. 2012), indicating a structured N-terminal region and an

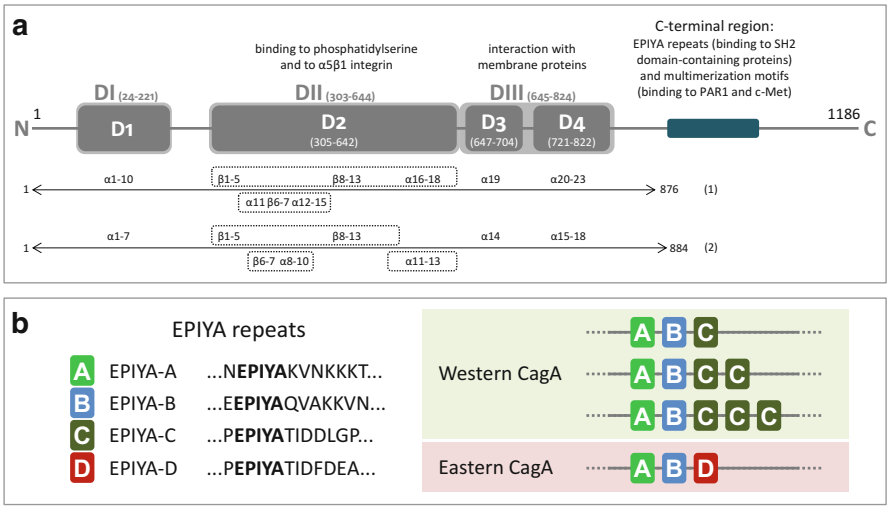

Fig. 1 CagA structure. (**a**) Structure indicating the protein domains (DI–DIII according to Hayashi et al. 2012; D1–D4 according to Kaplan-Türköz et al. (2012) with their main functions and the distribution of α-helices and β-strands according to (*1*) Hayashi et al. (2012) and (*2*) Kaplan-Türköz et al. (2012). The portions forming the subdomains of DII or D2 are surrounded by dashed boxes. (**b**) Families of EPIYA repeats characterizing Western and Eastern CagA (*left*) and scheme of the most frequent occurrences of the different repeats along the CagA sequence (*right*)

intrinsically disordered C-terminal region that directs versatile protein interactions (Fig. 1a).

According to the different reports, the N-terminal portion of CagA consists of three or four domains, DI, DII, and DIII (Hayashi et al. 2012) or D1, D2, D3 (Kaplan-Türköz et al. 2012) and D4, where DI corresponds to D1, DII to D2, and DIII to D3 plus D4.

According to Hayashi et al. (2012), DI consists of 10 α-helices, DII consists of 13 β-strands and 7 α-helices and is divided in two subdomains, and DIII comprises 5 α-helices. According to Kaplan-Türköz et al. (2012), D1 consists of the first 7 α-helices, D2 encompasses 13 β-strands and 6 α-helices (with helix 9 divided in 9a and 9b) and is divided in three subdomains, and D3 and D4 comprise 1 and 4 α-helices, respectively. The difference observed between DI and D1 in the number of α-helices is likely due to the fact that Kaplan-Türköz et al. reported poor quality of the electron density map in this area that prevented unambiguous model building.

D1 interacts only with D2 with a very small interaction surface, suggesting its mobility. Also, the other CagA domains contain loops that may confer flexibility, which may be necessary for the numerous interactions exerted by CagA with other molecules. D2 and D3/D4 together form a structural core of CagA made from 12 α-helices and a large β-sheet. D2 comprises 13 antiparallel β-strands, 11 of which form a single-layer β-sheet region, whose interactions with other tracts of the molecule suggest it is part of a rigid core of CagA. The single-layer β-sheet is

stabilized by two independent helical subdomains. The structure of D2 also revealed a basic amino acid cluster that mediates the interaction of CagA with host cell phosphatidylserine; moreover, it was shown that D2, and in particular the proximal part of the single-layer β-sheet, is involved in the β1 integrin binding (Hayashi et al. 2012; Kaplan-Türköz et al. 2012). D3/D4 structure, encompassing 5 α-helices (1 for D3 and 4 for D4), shows flexibility; D4 contains an N-terminal binding sequence that interacts with the disordered C-terminal binding sequence within the unstructured C-terminus of CagA: this intramolecular interaction induces a loop-like structure of the C-terminus.

The C-terminal portion of CagA contains the EPIYA segments and CagA multimerization (CM) sequence, which, respectively, act as binding sites for SH2 domain-containing protein tyrosine phosphatase (SHP2) and protease-activated receptor 1 (PAR1) (Higashi et al. 2002; Saadat et al. 2007): these sequences are exposed, thanks to the interaction of C-terminus with D3/D4.

The C-terminal CagA region shows sequence variability, in particular for the segment that can be found in several repeats, which includes the EPIYA motif, containing a tyrosine phosphorylation site. EPIYA motifs can be found in some variants, in particular with replacements for P, E, and A. Based on the flanking amino acids, four different EPIYA repeats have been identified, named EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D (Fig. 1b). While A and B variants are well conserved among *H. pylori* strains, marked difference in geographical distribution has been found for C and D variants. Based on such a difference, two CagA families have been identified, the so-called Western CagA and East Asian CagA. The Western CagA contains EPIYA-A and EPIYA-B followed by up to three C segments, while the East Asian CagA contains A and B followed by one D segment (Hatakeyama 2003; Higashi et al. 2002; Xia et al. 2009). The large majority of *H. pylori* isolates can be distributed among one of the above-described patterns of EPIYA repeats; however, occasionally, one or more of the repeats may be found absent or duplicated. It has been shown that the activity of CagA is influenced not only by the number but also by the flanking sequences of tyrosine phosphorylation sites; thus, the existence of distinct patterns of EPIYA repeats may explain why gastric carcinoma has higher prevalence in East Asia than in Western countries.

Binding of *H. pylori* to Host Cell and CagA Translocation

H. pylori adheres to the host cells in the proximity of the apical-junctional complex, which represents for the cell a barrier, adhesion site, and pathways network to control cell polarity, proliferation, and differentiation processes. Besides several *H. pylori* outer membrane proteins (OMPs) that mediate interactions between the bacterium and the host cells, T4SS, as mentioned before, is able to directly contact the host cell through binding of bacterial CagL to the host cell α5β1 integrin. Binding of T4SS is followed by CagA translocation into the host cell. However, CagA itself is able to interact with both α5β1 integrin (an interaction that enhances CagA translocation)

and plasma membrane phosphatidylserine. Phosphatidylserine is usually part of the inner leaflet of the cellular plasma membrane, but it is externalized to the outer leaflet in response to the contact of *H. pylori* with the cell, a prerequisite for CagA internalization (Murata-Kamiya et al. 2010). This suggests an alternative mechanism by which CagA, already exported to the bacterial surface, contributes to its translocation. However, CagA cannot enter autonomously inside the host cell in the absence of an intact T4SS.

Intracellular Events Triggered by CagA Internalization

Once into the host cell, CagA localizes on the inner surface of the plasma membrane and subsequently may undergo tyrosine phosphorylation at the EPIYA motifs by the host cell tyrosine-protein kinase Src (c-Src) (Stein et al. 2002) or by the nonreceptor tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (c-Abl) (Poppe et al. 2007): both of them are proto-oncogenes. Once internalized, CagA, either phosphorylated (p-CagA) or non-phosphorylated, is able to initiate a series of intracellular events, described in the subsequent paragraphs, which may dramatically interfere with cell morphology, motility, polarity, proliferation, and differentiation (Backert et al. 2015; Kaplan-Türköz et al. 2012; Stein et al. 2013), leading to invasive phenotypes of host cells. Most of the studies that analyzed the various activities exerted by CagA to alter the signal transduction of the host cell have been made possible by the use of CagA isogenic mutant *H. pylori* strains; moreover, the role of phosphorylated CagA was elucidated by using *H. pylori* strains having CagA mutated at the EPIYA phosphorylation sites.

Interaction with SHP-2

Phosphorylated EPIYA CagA motifs serve as scaffolds to recruit SH2 domain-containing proteins and specifically the Src homology phosphatase 2 (SHP-2) (Higashi et al. 2002). Consequently to the binding to p-CagA, SHP-2 is activated and in turn triggers the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascade (Higashi et al. 2004). This activates NF-kB and eventually results in both proinflammatory signaling and abnormal cell proliferation. The complex constituted of p-CagA, and SHP-2 can also block the focal adhesion kinase (FAK) (Tsutsumi et al. 2006), initiating an abnormal morphological transformation. Recently, it has been shown that duplication of EPIYA-C from one to two or more increases SHP-2 binding of Western CagA by more than 100-fold (Nagase et al. 2015), confirming that the number of EPIYA repeats influences the possible malignant outcome of *H. pylori* infection. This finding has been also proven in the animal model of *H. pylori* infection of Mongolian gerbil (Ferreira Júnior et al. 2015). Moreover, it has been proposed that a single SHP-2 is capable of binding two p-CagA proteins (Higashi et al. 2002) and that such a CagA dimerization markedly stabilizes the complex consisting of p-CagA and SHP-2, thereby potentiating SHP-2 deregulation (Nagase et al. 2011).

Interaction with Csk

Through binding to C-terminal Src kinase (Csk), p-CagA can inhibit the c-Src activity, i.e., it can block the CagA phosphorylation, thus constituting an interesting self-modulation by CagA of its own activity. This particular mechanism would deserve further investigation, as its higher or lower efficiency might contribute to explain why the *H. pylori* infection with CagA-positive strains evolves to malignancy only in a relatively low number of subjects. Moreover, it has been reported that c-Src inactivation leads to tyrosine dephosphorylation of the actin-binding protein cortactin and concomitant cortactin redistribution to actin-rich cellular protrusions. c-Src inactivation and cortactin dephosphorylation are required for rearrangements of the actin cytoskeleton (Selbach et al. 2003).

Interaction with ASPP2

In the cytoplasm, p-CagA interacts with the apoptosis-stimulating protein p53-2 (ASPP2), causing relocalization of ASPP2 from cytoplasm to the inner surface of the plasma membrane (Buti et al. 2011). The interaction involves the N-terminal region of CagA and a proline-rich sequence of ASPP2 plus numerous ASPP2 regions distributed throughout the protein sequence (Nešić et al. 2014; Reingewertz et al. 2015). ASPP2 is normally activated by DNA damage or oncogenic stimuli to initiate the apoptotic pathway. Instead, the relocalization of ASPP2 upon interaction with p-CagA leads it to interact with p53, causing abnormal p53 degradation, which in turn determines the block of the apoptotic signaling that otherwise, in the absence of CagA, p53 would have induced. Compromised apoptosis would permit the survival of damaged cells; thus, it could favor cancer development. Moreover, the degradation of p53 can interfere with terminal cell differentiation.

Interaction with PAR1b/MARK2

H. pylori causes recruitment of the polarity-associated PAR1b/MARK2 serine/threonine kinase, a member of the partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK), from the cytosol to the plasma membrane. PAR1 family has been shown to possess a CagA-binding sequence. The interaction of CagA with PAR1b/MARK2, with the consequent inhibition of the latter, not only causes disruption of apical junctions and polarity defects but also prevents lumen formation and tubulogenesis, which are important hallmarks of epithelial differentiation. Given the role exerted by MARK kinase in phosphorylating microtubule-associated proteins, it may be hypothesized that CagA may inhibit PAR1-dependent microtubule-associated protein phosphorylation and thereby may elicit junctional and polarity defects through impaired microtubule-based transport (Saadat et al. 2007; Zeaiter et al. 2008).

Interaction with PRK2

Similar to PAR1b/MARK2, the serine/threonine kinase PRK2 has been shown to be recruited to the plasma membrane in presence of CagA, upon direct interaction between CagA and PRK2; however, such interaction appears to involve different

domains from those involved in the binding to PAR1b/MARK2 (Mishra et al. 2015). The interaction between CagA and PRK2 inhibits PRK2 kinase activity, which eventually may influence cytoskeletal rearrangements and translocation of β-catenin to the nucleus (see the paragraph "Interaction with E-Cadherin and β-Catenin") leading to disruption of cellular polarity, with consequent destabilization of cellular junctions and/or cell adhesion.

Interaction with c-Met

CagA has been reported to bind and activate the hepatocyte growth factor receptor (c-Met) (Churin et al. 2003), which is implicated in invasive growth of tumor cells. Binding of CagA to c-Met promotes cellular processes leading to a forceful motogenic response; this invasive phenotype can be suppressed in the presence of E-cadherin. However, the interaction of CagA with c-Met and the subsequent events are still controversial (Pachathundikandi et al. 2013). Binding of CagA to c-Met has been also proposed to influence the nuclear accumulation and transcriptional activity of β-catenin, as described in the following paragraph. Recently, in human- and mouse-derived gastric organoids, CagA was found to interact also with CD44, which acts as a co-receptor for c-Met; such interaction was found to play a functional role in *H. pylori*-induced epithelial cell proliferation that indeed was lost in infected organoids derived from CD44-deficient mouse stomachs (Bertaux-Skeirik et al. 2015).

Interaction with E-Cadherin and β-Catenin

E-Cadherin binds β-catenin, in the complex that anchors the cytoplasmic domain of E-cadherin to actin cytoskeleton, forming adherens junctions between epithelial cells. In normal cells, this regulates the epithelial barrier formation, the paracellular pathway, and the polarity. CagA, independently of its phosphorylation, is able to bind E-cadherin, thereby destabilizing the E-cadherin/β-catenin complex and causing cytoplasmic/nuclear accumulation of β-catenin, which in turn activates proinflammatory, proliferative, and anti-apoptotic signaling (Murata-Kamiya et al. 2007). Moreover, CagA directly associates with β-catenin and indirectly through the binding to mucin-1 (MUC1), whose cytoplasmic region is known to bind β-catenin; interestingly, the increase of MUC1 expression in the gastric mucosa was found to be able to counteract *H. pylori*-induced IL-8 production, likely by binding β-catenin and thereby impeding its accumulation (Guang et al. 2012). More recently, CagA-dependent, mediated by c-Met and/or PI3K/Akt (phosphatidylinositol-3-kinase), phosphorylation of β-catenin has been reported, which may contribute to nuclear accumulation and transcriptional activation of β-catenin and eventually lead to induction of cancer stem cell-like properties (Yong et al. 2016). The proposed mechanism includes from one side CagA binding to glycogen synthase kinase 3β (GSK-3β) and depletion of its activity, inhibiting β-catenin degradation; from the other side, the already mentioned CagA interaction with E-cadherin makes β-catenin available for phosphorylation, which is mediated by Akt upon interaction of CagA with c-Met. This phosphorylation increases nuclear accumulation and transcriptional activity of β-catenin, resulting in increased Wnt

(Wingless-related integration site)/β-catenin signaling. This activation upregulates the expression of octamer-binding transcription factor 4 (Oct-4) and Nanog; since these transcription factors have the role of maintaining the pluripotency and self-renewal of embryonic stem cells, their upregulation may promote the emergence of cancer stem cell-like properties in gastric cancer cells (Yong et al. 2016).

Interaction with Grb-2 and Activation of Ras-ERK Pathway

It has been reported that ERK can be activated by non-phosphorylated CagA and that such an activation may occur independently of both SHP-2 and c-Met, through a Ras-Raf-MEK-ERK-NF-kB signaling pathway, with consequent induction of IL-8 production. It has been hypothesized that the event initiating this signaling might be the binding of CagA to the adaptor protein growth factor receptor-bound protein 2 (Grb-2), which has been shown to activate Ras (Brandt et al. 2005).

Tumor Suppressor Gene Hypermethylation

Epigenetic changes are involved in the development of many cancers, and, in particular, aberrant hypermethylation of promoter region CpG islands of tumor suppressor gene is largely involved in carcinogenesis in the stomach. *H. pylori* infection has been shown to be associated to high levels of hypermethylation in gastric epithelium. It has been found that O^6-methylguanine DNA methyltransferase (MGMT) gene methylation, already known to be related to gastric carcinogenesis, is also significantly associated with infection with CagA-positive *H. pylori* strains (Sepulveda et al. 2010). This has been more recently confirmed by the observation that CagA downregulates the MGMT expression by inducing hypermethylation in its promoter region, suggesting that CagA might induce gastric carcinogenesis by causing hypermethylation of tumor suppressor genes, with the MGMT as a representative (Zhang et al. 2016). The mechanism involves CagA-enhanced interaction between 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt, increasing Akt phosphorylation; p-AKT activates NF-kB, which then binds the DNA methyltransferase 1 (DNMT1) promoter and increases its expression. Finally, the upregulated DNMT1 promotes tumor suppressor genes hypermethylation (Zhang et al. 2016).

Evidence of the Role of *H. pylori* CagA in Gastric Cancer

The Evidence that *H. pylori* Infection Causes Gastric Cancer

The relationship between *H. pylori* infection and increased risk of gastric cancer was initially established by epidemiological investigation. Indeed, the first studies that assessed the prevalence of seropositivity to *H. pylori* in relation with the gastric cancer found significant correlation (Correa et al. 1990; Forman et al. 1990) and estimated for the subjects infected by *H. pylori* an increased risk of developing gastric cancer, as compared with the risk observed for noninfected subjects (odd ratio = 2.77) (Forman et al. 1990). As already stated in the "Introduction," the risk

of developing gastric cancer upon *H. pylori* infection may also increase depending on host factors, such as a proinflammatory genetic background. The link between *H. pylori* infection and gastric cancer was confirmed by several subsequent studies (IARC 2012). A recent meta-analysis that included 24 studies, corresponding to 715 incident gastric cancers among a total of 48,064 individuals, showed that patients who underwent eradication of *H. pylori* infection had a lower incidence of gastric cancer than those who did not receive eradication therapy; eradication provided significant benefit for asymptomatic infected individuals and for those after endoscopic resection of gastric cancers (Lee et al. 2016), confirming previous observations.

The availability of animal models of *H. pylori* infection made possible to obtain formal evidence that *H. pylori* infection causes gastric cancer. Atrophic changes were initially observed in a mouse model of *H. pylori* infection after long-term observation (Lee et al. 1993): since atrophic gastritis is considered to be a premalignant condition that favors the development of gastric cancer, further studies attempted to evaluate the possible malignant outcome of *H. pylori* infection in animal models. It was found that mice do not develop gastric cancer upon *H. pylori* infection alone, but coadministration of carcinogenic substances such as *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) allowed observing significantly higher development of gastric cancer in the *H. pylori*-infected group as compared with control, noninfected mice (Han et al. 2002). While wild-type mice do not develop gastric cancer upon *H. pylori* infection, some transgenic mice do, such as those deficient for TGF-β, p27, or trefoil factor 2 (TFF2) or those overexpressing gastrin or IL-1β (reviewed in IARC 2012): these studies, besides confirming the relevance of *H. pylori* infection to gastric cancer development, provided insights in the role of the host factors targeted by the mutations. Recently, also trefoil factor 1 (TFF1) knockout mice demonstrated increases in chronic inflammation and frequency of invasive gastric adenocarcinoma upon *H. pylori* infection (Soutto et al. 2015): both TFF1 and TFF2 belong to the trefoil factor family secretory peptides, which exert protective and healing effects after mucosal damage. Also, mice knocked out for osteopontin, which is overexpressed in various types of cancer, showed decreased *H. pylori*-induced gastric carcinogenesis upon MNU administration, mainly due to suppression of a proinflammatory immune response (Lee et al. 2015).

A carcinogenicity study was performed in a rhesus monkey model of *H. pylori* infection in combination with the oral carcinogen N-ethyl-N-nitrosoguanidine (ENNG), which is similar to nitrosamines found in foods such as smoked fish and pickled vegetables: transcriptional analysis of biopsy specimens 5 years post-infection revealed striking changes in monkeys receiving both *H. pylori* and ENNG, showing a neoplasia-specific expression profile characterized by changes in multiple cancer-associated genes. Monkeys receiving *H. pylori* + ENNG developed gastritis, intestinal metaplasia, and neoplasia, while those receiving *H. pylori* alone developed gastritis only. Based on these results, a synergistic effect of *H. pylori* and the carcinogen in inducing gastric neoplasia in primates was proposed (Liu et al. 2009).

Another suitable animal model to study *H. pylori*-related carcinogenesis seems to be Mongolian gerbil. Indeed, an initial study demonstrated that gerbils spontaneously develop gastric adenocarcinoma upon long-term *H. pylori* infection, without the need of treatment with carcinogen substances (Watanabe et al. 1998). However, subsequent studies showed high variability of the gastric cancer development rates; thus, also in this model the additional use of MNU or MNNG was introduced, obtaining not only high and reproducible gastric cancer development rates but also the possibility of shortening the period of observation (Tokieda et al. 1999).

The Evidence that CagA Plays a Central Role in *H. pylori*-Related Gastric Cancer

The analysis of CagA seropositivity in a population including 103 *H. pylori*-infected subjects that developed gastric cancer, and 103 *H. pylori*-infected subjects that did not develop gastric cancer, revealed an association between the infection with CagA-positive strains and the increased risk of developing adenocarcinoma of the stomach (odd ratio = 1.9) (Blaser et al. 1995). Such an association was confirmed by several subsequent reports (IARC 2012). This observation stimulated the investigation on the role of CagA in determining the malignant outcome of the *H. pylori* infection in appropriate in vitro and in vivo models.

All of the CagA activities described in the previous paragraph "Intracellular events triggered by CagA internalization" demonstrated a crucial role of CagA, once delivered into the host cell, in triggering abnormal intracellular signaling that may eventually lead to invasive phenotype.

In the gastric carcinogenicity model of Mongolian gerbil, the essential role of T4SS in inducing the most severe disease was demonstrated, as the *cagE* isogenic mutant, in which the translocation of CagA is impaired, induced very mild histopathological changes as compared with both wild-type strain and *vacA* isogenic mutant (Ogura et al. 2000). However, another study in gerbils did not found different changes at the level of gastric epithelium between wild-type and an isogenic *cagA* mutant *H. pylori* strain (Peek et al. 2000), then focusing the attention on the role of the intact *cag* PAI and consequently of the functional T4SS rather than CagA only (Israel et al. 2001). A further study evidenced that both in gerbils and mice, upon infection with a wild-type *H. pylori* strain, but not with the *cagA* isogenic mutant, the levels of spermine oxidase (SMO) increased in gastric epithelial cells, with generation of oxidative stress and consequent H_2O_2 production, apoptosis, and DNA damage (Chaturvedi et al. 2011); notably, while it was found that *H. pylori* caused apoptosis in gastric epithelial cells, it was also observed that a substantial fraction of cells infected with CagA-positive strains were protected from apoptosis, thus at high risk for malignant transformation.

A formal demonstration of the role of CagA as a bacterial oncoprotein and of the importance of CagA tyrosine phosphorylation in the development of *H. pylori*-associated neoplasms was provided by transgenic mice expressing CagA. In fact,

transgenic mice expressing wild-type CagA, but not those expressing a CagA phosphorylation-resistant mutant, showed gastric epithelial hyperplasia, and some of the mice developed gastric polyps and adenocarcinomas of the stomach and small intestine (Ohnishi et al. 2008). A recent work, done with transgenic mice systemically expressing CagA and treated with a colitis inducer, revealed that CagA worsens the inflammation, whereas inflammation strengthens the oncogenic potential of CagA, thus evidencing that CagA and inflammation may reinforce each other in creating a downward spiral toward neoplastic transformation (Suzuki et al. 2015).

Conclusion and Future Directions

A large body of evidence indicates that the risk of gastric cancer or premalignant lesions is higher in subjects infected with CagA-positive *H. pylori* strains versus those infected with CagA-negative strains. CagA tyrosine phosphorylation plays a central role in CagA activity (Cover 2016), even though non-phosphorylated CagA is also able to trigger very dangerous intracellular signalings. It appears that the progression toward malignancy derives from the effects that CagA exerts on the host cells combined with an enhanced inflammatory response at the level of the gastric mucosa, which can reinforce each other (Cover 2016; Suzuki et al. 2015). It must be said that several other *H. pylori* factors have been proposed to be involved in increasing the risk of gastric cancer development: the already mentioned VacA and in particular some of its isoforms, several OMPs such as outer inflammatory protein A (OipA) and the adhesins BabA and SabA (blood group antigen-binding adhesion and sialic acid-binding adhesion, respectively), DupA (duodenal ulcer-promoting gene), etc. (Cover 2016); however, the most virulent alleles of the genes coding for these proteins are often associated with CagA-positive strains, an observation that reinforces the idea that CagA plays a major role in the malignant outcomes of *H. pylori* infection.

It has been suggested that, in spite of the association of *H. pylori* infection with gastric cancer, it was able to avoid negative selection in that it did not damage severely the premodern human societies (Atherton and Blaser 2009). Indeed, gastric cancer is developed by elderly people, scarcely affecting the population if the life expectancy is below 50 years and, very important from the point of view of evolutive pressure, not influencing the population at the age of reproduction; conversely, it represents a threat in the modern society, in which the life expectancy is much higher. On the other hand, it has been proposed that *H. pylori* provides some benefits on early life, in particular in reducing the risk of acid-related esophageal diseases and asthma (Atherton and Blaser 2009). However, these data appear weak or controversial, when considering large-controlled studies (Graham 2015; IARC 2012; Wang et al. 2013), most probably reflecting the diversity of infecting strain types, host genetic background, geographic areas, socioeconomic status, etc., differences already observed more in general to influence the outcome of *H. pylori* infection. The idea that *H. pylori* infection might provide some benefits to the host may deserve further scientific investigation, but at the same time, if misinterpreted, it might orient

the public opinion against the eradication of *H. pylori*, increasing the risk of peptic ulcer and gastric cancer. In this frame, a vaccine or a treatment effective against the disease rather than the infection could be the balanced solution. In other words, for instance, a treatment or a vaccine specifically targeting CagA might only partially influence the *H. pylori* burden in the stomach, but it could be expected to prevent the severe outcomes of the infection. To date, while the therapeutic treatment against *H. pylori* has been shown to be efficacious in counteracting peptic ulcer and MALT lymphoma, results from randomized studies have not had sufficient power to evaluate the effect of the impact of *H. pylori* eradication on gastric carcinoma risk (IARC 2012). This is conceivably due also to the fact that, at the stage in which the treatment is administered, it might be too late to induce regression of the mechanisms of carcinogenesis, that once initiated, may reach a point of no return and thus progress even in the absence of the bacteria. From this point of view, prophylactic vaccination appears more promising in terms of reduction of risk of gastric carcinoma.

Cross-References

► Interaction of *Helicobacter pylor*i VacA Toxin with Its Target Cells

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