

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori***Clinical proteomics identifies potential biomarkers in *Helicobacter pylori* for gastrointestinal diseases**

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Abstract

The development of gastrointestinal diseases has been found to be associated with *Helicobacter pylori* (*H. pylori*) infection and various biochemical stresses in stomach and intestine. These stresses, such as oxidative, osmotic and acid stresses, may bring about bi-directional effects on both hosts and *H. pylori*, leading to changes of protein expression in their proteomes. Therefore, proteins differentially expressed in *H. pylori* under various stresses not only reflect gastrointestinal environment but also provide useful biomarkers for disease diagnosis and prognosis. In this regard, proteomic technology is an ideal tool to identify potential biomarkers as it can systematically monitor proteins and protein variation on a large scale of cell's translational landscape, permitting in-depth analyses of host and pathogen interactions. By performing two-dimensional polyacrylamide gel electrophoresis (2-DE) followed by liquid chromatography-nanoESI-mass spectrometry (nanoLC-MS/MS), we have successfully

pinpointed alkylhydroperoxide reductase (AhpC), neutrophil-activating protein and non-heme iron-binding ferritin as three prospective biomarkers showing up-regulation in *H. pylori* under oxidative, osmotic and acid stresses, respectively. Further biochemical characterization reveals that various environmental stresses can induce protein structure change and functional conversion in the identified biomarkers. Especially salient is the antioxidant enzyme AhpC, an abundant antioxidant protein present in *H. pylori*. It switches from a peroxide reductase of low-molecular-weight (LMW) oligomers to a molecular chaperone of high-molecular-weight (HMW) complexes under oxidative stress. Different seropositivity responses against LMW or HMW AhpC in *H. pylori*-infected patients faithfully match the disease progression from disease-free healthy persons to patients with gastric ulcer and cancer. These results has established AhpC of *H. pylori* as a promising diagnostic marker for gastrointestinal maladies, and highlight the utility of clinical proteomics for identifying disease biomarkers that can be uniquely applied to disease-oriented translational medicine.

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Key words: Proteomics; Biomarker; *Helicobacter pylori*; Gastritis; Gastric ulcer; Gastric cancer; Alkylhydroperoxide reductase; Neutrophil-activating protein; Non-heme iron-binding ferritin

Core tip: This work aims to review the literature on the application of proteomics methodology for identifying proteins differentially expressed in *Helicobacter pylori* (*H. pylori*) under different environmental stresses, resulting in the identification of several biomarkers related to gastrointestinal disorders induced by *H. pylori* infection.

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INTRODUCTION

The discovery of *Helicobacter pylori* (*H. pylori*), a Gram-negative and microaerophilic bacterium has revolutionized the diagnosis and treatment of gastrointestinal diseases^[1,2]. Prolonged infection and inflammation because of persistent *H. pylori* colonization lead to chronic gastritis and severe gastric pathologies, such as peptic ulcer and gastric cancer^[3]. It is worth noting that variable clinical manifestations associated with *H. pylori* infection are common in the endoscopic examination of patients, about 80%-90% patients being asymptomatic gastritis (GA), 10%-15% gastric or duodenal ulcer (GU) and 1%-2% gastric cancers (GC)^[4]. Gastric cancers are generally believed to arise from sites of *H. pylori* infection accompanied by chronic irritation and inflammation^[5-7]. Such inflammatory ulcers of stomach are manifestly the result of severe immune response triggered by the host's immune system upon *H. pylori* infection^[8,9]. Therefore, to identify the mechanism underlying such pathogenesis and the capricious clinical outcomes in the gastric infections by *H. pylori* under varied environmental stresses *in vivo* is crucial to the development of efficient therapeutic approach against *H. pylori* infection and the associated gastrointestinal maladies^[10].

In this review, we have focused on the potential roles of three biomarkers identified in the course of our decade-long research in the pathogenesis of varied gastrointestinal diseases induced by *H. pylori* infection. The antioxidant enzyme alkylhydroperoxide reductase (AhpC) of *H. pylori* was found to be expressed with larger amounts in GC strains and exhibited a higher seropositivity for GC patients than gastritis (GA) ones. Based on the significant difference between AhpC in *H. pylori* isolated from patients with GA, GU and GC, it is conceivable that the antioxidant protein AhpC of *H. pylori* may be applied as a prognostic or diagnostic protein marker to monitor different stages of tissue damages from *H. pylori*-infected diseases. The relevance of up-regulated proteins NapA (neutrophil-activating protein) and non-heme iron-binding ferritin (Pfr) under osmotic and acid stresses, respectively is also explored regarding their corresponding regulation of cellular homeostasis. Finally, the potential diagnostic and therapeutic value of these novel biomarkers in *H. pylori*-related gastroenterological maladies is proposed for their translational medical applications.

ENVIRONMENTAL STRESSES AND GASTROINTESTINAL DISEASES

The variable clinical outcomes of *H. pylori* infection is

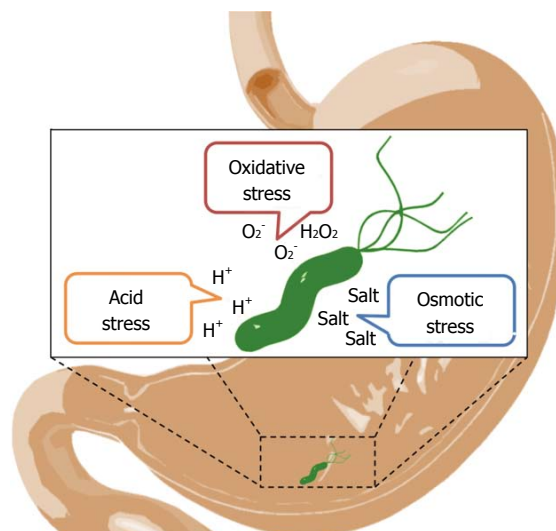


Figure 1 Diagram of *Helicobacter pylori* exposed to various environmental stresses in stomach and intestine. The development of gastrointestinal diseases is suggested to be associated with *Helicobacter pylori* (*H. pylori*) infection and environmental stresses in stomach and intestine. These stresses, such as oxidative, acid and osmotic stresses^[33-36], may bring detrimental effects on *H. pylori*.

suggested to result from different severity and distribution of *H. pylori*-induced gastric inflammation plus environmental stresses in stomach and intestine^[11]. Several studies have also shown the correlation between the protection of host cells against *H. pylori* infection and stress-induced protein factors^[12,13]. Figure 1 depicts *H. pylori* under various stresses *in vivo*, such as oxidative, osmotic and acid stresses, which may underlie the pathogenesis of the gastrointestinal infections and lay a firm basis for the development of efficient diagnosis, prognosis and therapeutic managements.

Oxidative stress

Oxidative stress was suggested to be associated with chronic inflammation and malignant transformation. It was also reported to be the causative factor for most cancers, including gastric cancer^[14]. Nevertheless, the pathogenic agent inducing the gastric inflammation was not known until the discovery of *H. pylori*^[15]. *H. pylori* infection can activate inflammatory responses and trigger immune system, leading to discharge of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide anion, and nitric oxide from phagocytes. The excessive ROS production may thereby trigger oxidative stress to cause inflammatory damage to host gastric mucosa, whereas *H. pylori* will also face the attack of oxygen-related free radicals released from phagocytes (Figure 1)^[16]. To survive under such ROS environment generated inside the host, *H. pylori* has developed some defensive mechanisms to protect against oxidative stress. Therefore, mapping proteome changes of *H. pylori* in response to oxidative stress can potentially identify indicators or markers for both the inflammation status and the progression of gastrointestinal diseases.

Osmotic stress

High dietary salt intake has been considered as a long-term risk factor for gastric carcinogenesis^[17,18]. A link between high salt consumption and gastric cancer has been shown in both animal model and human epidemiologic studies^[19-21]. High salt environment is thought to induce disturbance of osmotic homeostasis, which may potentially affect host cells and *H. pylori* in stomach and intestine (Figure 1)^[22]. Although aforementioned evidences suggest high-salt diet may mediate augmentation in the severity of *H. pylori*-induced disease outcomes, the molecular mechanisms underlying the purported cause-effect correlation between osmotic stress and *H. pylori* infection still remain poorly understood. Several studies have tried to use genetic approaches for identifying the key regulators in *H. pylori* in response to high concentration of NaCl. The change of gene-expression levels of two virulent factors, CagA and VacA, were reported by Loh *et al.*^[23] and Gancz *et al.*^[24], respectively. Both results not only support the synergistic effect between *H. pylori* infection and diets rich in sodium chloride but point out the potential of investigating whole-proteome change under osmotic stress to explore biomarker discovery.

Acid stress

H. pylori is unique for its ability to adapt to the extreme acidic environment of pH 1-2 in human stomach lumen as evident by the fact that *H. pylori* infection usually lasts for decades or more (Figure 1)^[25,26]. Survival of *H. pylori* under severe acid shock has been generally proposed to depend on its urease activity, which can release ammonia from urea, thereby neutralize gastric acid in stomach^[27,28]. However, other regulatory proteins and molecular mechanisms can also contribute to the acid adaptation of *H. pylori* in human stomach. To address this, several reports have analyzed differential gene expression in *H. pylori* under acidic conditions by employing DNA microarray and their findings supported that acidity is a signal modulating the expression of multiple genes related to metal ion homeostasis^[29-31]. Therefore, it is of interest and important to know whether proteomics could identify urease and its metal metabolism related proteins as indicators for acid stress in stomach, which may reflect the progression of gastrointestinal diseases.

CLINICAL PROTEOMICS FOR GASTROINTESTINAL DISEASE, BIOMARKER DISCOVERY AND VALIDATION

Proteomics is a robust methodology, which can depict snapshots of protein compositions of particular cells or tissues at defined time points. Clinician scientists have realized its potential and begun to apply innovative proteomic technology to clinical research especially on its application to translational medicine, which generally consists of several phases, that is (1) biomarker discov-

ery; (2) biomarker verification; and finally (3) biomarker validation and clinical trial^[32].

To this end, we have set up a proteomics-based platform to systematically identify and validate potential biomarkers in *H. pylori* for gastrointestinal diseases (Figure 2), which also represents in essence the application of translational medicine from clinics to lab research and then back to clinics. Firstly, *H. pylori* clinical isolates were obtained from gastric biopsy specimens from patients with different pathological manifestations, such as gastritis, duodenal ulcer, gastric ulcer and gastric cancer. All isolated strains were further confirmed for their urease activity and the helical morphology of these organisms observed by phase-contrast microscopy^[33]. Upon exposing to different stress conditions, *H. pylori* proteomes are compared and analyzed by two-dimensional polyacrylamide gel electrophoresis (2-DE) followed by liquid chromatography-nanoESI-mass spectrometry (nanoLC-MS/MS)^[33-36]. By making use of rapidly-evolved powerful bioinformatics analysis programs, highly up- or down-regulated proteins in *H. pylori* under various stresses could be identified as candidates of potential biomarkers. Further immunoblotting in *H. pylori* strains from gastrointestinal patients of varied clinical outcomes can then be used to validate whether these candidates are engendered to reflect environmental stresses in stomach and intestine^[37].

To translate the lab findings into clinics, enzyme-linked immunosorbent assay (ELISA) is an ideal tool to employ these *H. pylori* antigens for the detection of antibodies in sera from patients with different gastric diseases^[37]. For large-scale ELISA analysis, *H. pylori* recombinant antigens are produced by molecular cloning, overexpression in *Escherichia coli*, and followed by protein purification^[37,38]. Recombinant *H. pylori* antigens are further coated in 96-well plates for screening seropositivities of patient sera, useful for clinical diagnosis and prognosis of disease progression. In summary, our clinical proteomics pipeline provides a rapid and scalable platform for the identification of biomarkers in gastrointestinal diseases and can potentially be applied to other diseases as well, such as cataract and cancer^[39,40].

FUNCTIONAL SWITCH OF IDENTIFIED *H. PYLORI* BIOMARKERS IN RESPONSE TO ENVIRONMENTAL STRESSES

Our clinical proteomics platform has found out several stress-related biomarkers from *H. pylori* proteome (Table 1). Under oxidative stress induced by H₂O₂, several antioxidant (alkylhydroperoxide reductase AhpC, catalase KatA, serine protease HtrA) and virulent proteins (cytotoxin-associated protein CagA, vacuolating cytotoxin VacA, adhesin AlpA) are up-regulated in isolated *H. pylori* from patients with different gastrointestinal diseases^[35], revealing part of the mechanism concerning ROS-induced gastric carcinogenesis. In response to os-

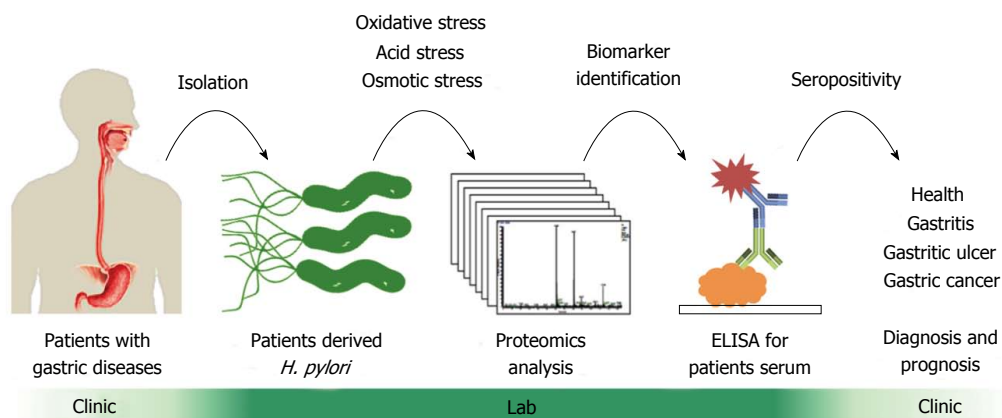


Figure 2 Flow chart of clinical proteomics platform designed for discovery and validation of *Helicobacter pylori* biomarkers for the diagnosis of gastrointestinal diseases. We have set up a proteomics-based platform^[32] to systematically identify and validate potential biomarkers in *Helicobacter pylori* (*H. pylori*) for gastrointestinal diseases, which can provide a major methodological support for translational medicine encompassing basic biomedical research and clinical therapeutic medicine. ELISA: Enzyme-linked immunosorbent assay.

Table 1 Proteomic analysis identifies upregulated proteins in *Helicobacter pylori* under different stresses

Stress	Source	Disease	Upregulated proteins (function)	Ref.
Oxidative stress	Reactive oxygen species (ROS): H ₂ O ₂ , O ₂ ⁻	Gastritis, duodenal ulcer, gastric ulcer, gastric cancer	AhpC, KatA, HtrA (antioxidant) CagA, VacA, AlpA (virulent)	Huang <i>et al</i> ^[35]
Osmotic stress	High salt: NaCl	Gastric cancer	NapA, Pfr (iron-binding) UreA, UreB (acid neutralizing)	Liao <i>et al</i> ^[36]
Acid stress	Gastric acid: H ⁺	Gastro-esophageal reflux disease, irritable bowel syndrome	Pfr, NapA (iron-binding) UreA, UreB, UreG (acid neutralizing)	Huang <i>et al</i> ^[34]

AhpC: Alkylhydroperoxide reductase; NapA: Neutrophil-activating protein; Pfr: Non-heme iron-binding ferritin.

motric and acid stresses, *H. pylori* overexpress both iron-binding (NapA, Pfr) and acid-neutralizing proteins (urease subunits UreA, UreB)^[34,36], indicating that these two stresses may share similar effects on *H. pylori*. To further understand the molecular mechanism, AhpC, NapA and Pfr are overexpressed and purified for biochemical characterization with or without stresses. Interestingly, various stresses lead to protein structure change and functional switch of these proteins (Figure 3), indicative of molecular and cellular connotation for *H. pylori* to adapt to different stress environments.

AhpC and oxidative stress

H. pylori AhpC, an important member of 2-Cys peroxidoredoxin (Prx) family^[41], is abundantly expressed for balancing the intracellular levels of ROS generated from metabolisms or mild environmental stresses^[42]. AhpC cooperates with thioredoxin (Trx) to decompose H₂O₂ and organic peroxides by its peroxidase activity^[43,44]. Previously, we found AhpC also displays dual-activities *in vivo*, which switches from a peroxide reductase of low-molecular-weight (LMW) oligomers under microaerobic conditions to a molecular chaperone of high-molecular-weight (HMW) complexes in the presence of Trx after long-term (16 h) oxidative stress^[38]. In brief, LMW-AhpC efficiently reduces toxic ROS by its peroxidase

or peroxidase activity through a canonical reaction cycle by coupling reactions involving Trx, thioredoxin reductase (TrxR) and NADPH. Upon facing severe and long-term oxidative stress, LMW-AhpC initially undergoes a peroxidative reaction cycle and becomes peroxidized, converting cysteine at its active site to cysteine sulfinic acid (-SO₂H) and sulfonic acid (-SO₃H). The oxidized LMW-AhpC would be converted to HMW-AhpC with chaperone activity for prevention of mis- or un-folded proteins from aggregation^[45]. In such a scenario, AhpC acts as a potent sensor for ROS and as chaperones for *H. pylori* to survive and persist in the oxidative environment of human stomachs.

NapA and osmotic stress

NapA, a 17-kDa protein, is known as a major antigen in the human immune response to *H. pylori* infection, which can stimulate neutrophils to generate oxygen radicals when the microorganism adheres to endothelial cells^[46]. In addition, *H. pylori* NapA is also reported as an iron-binding protein with dodecameric structure^[47], indicating that NapA may potentially work with dual functionalities under different stresses. Interestingly, NapA is found not only to be up-regulated in *H. pylori* under high salt stress but expose its hydrophobic surfaces to exterior environment^[36]. We also found the structure of the

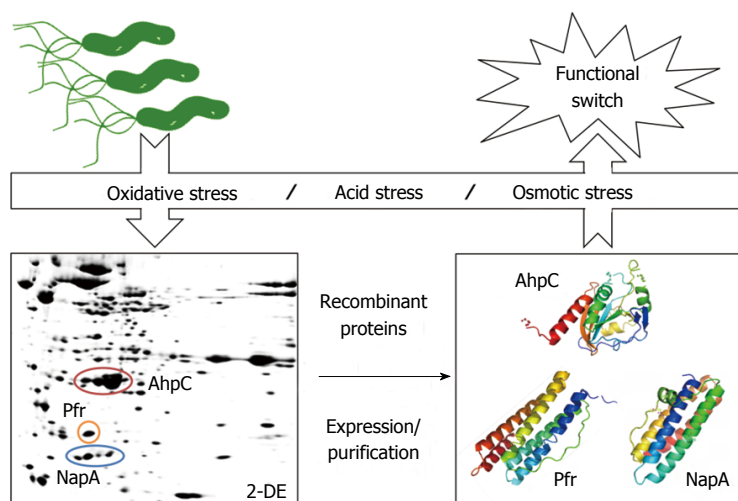


Figure 3 Functional switch of identified *Helicobacter pylori* biomarkers in response to environmental stresses. Alkylhydroperoxide reductase (AhpC), neutrophil-activating protein (NapA) and non-heme iron-binding ferritin (Pfr) are identified by clinical proteomics as potential biomarkers under different environmental stresses. Further biochemical characterization showed the protein structure change was accompanied by functional switch of these proteins under varied environmental conditions, such as oxidative, acid and osmotic stresses^[34,36,38]. The PDB ID of *Helicobacter pylori* (*H. pylori*) AhpC, NapA and Pfr are 1ZOF^[61], 1J14^[62] and 3EGM^[63], respectively.

NapA monomer was not affected by salt, but that the native oligomeric structure of NapA dissociated under high salt concentration, resulting in losing Fe²⁺-binding ability^[36]. The loss in iron binding of NapA under high-salt osmotic stress leads to release of Fe²⁺ ions, which would react with oxygen and produce H₂O₂^[48]. Elevation of H₂O₂ concentration would potentially increase the risk of oxidant-related cellular injury and DNA damage in the gastric lumen or mucosa^[49]. Therefore, a high dietary salt intake would aggravate the circumstances of *H. pylori*-associated infections, resulting in inflammation-related gastrointestinal diseases.

Pfr and acid stresses

Unlike animal ferritin as ferroxidase to convert active ferrous ions to the more innocuous ferric ions, ferritin Pfr (a special prokaryote class of ferritin family) of *H. pylori* was shown to be essential for iron storage in sub-cellular granules and enabled *H. pylori* to survive under severe iron starvation. Pfr can also protect *H. pylori* from acid-amplified iron toxicity^[50-52] caused by iron-mediated Fenton reaction^[53,54]. Similar to AhpC with dual function, we further found *H. pylori* ferritin (Pfr) can switch from an iron-storage protein with ferroxidase activity to a DNA-binding/protective function^[54]. The conformational change had occurred when ferritin was exposed to acid, leading to exposure of more hydrophobic region to exterior environment at pH 5 than at pH 7. Therefore, the discovery of the new function of ferritin indicates that anti-acid mechanism for the survival of *H. pylori* may involve several sequential steps. In the acidic environment of the gastric lumen or mucosa, the amplified metal-induced free radicals generated in the presence of ferrous ions and H₂O₂ may force *H. pylori* to exert some protection on its own vital DNA, triggering an increasing protein expression level of ferritin and a change in

the function from an iron-storage role with ferroxidase activity to that of DNA binding and protection^[34,50,54]. This lends support to the supposition that ferritin can act as an indicator or sensor reflecting the acid environment in stomach, and potentially as a biomarker to distinguish duodenal ulcer and gastric cancer due to their differences in acid secretion^[55].

H. PYLORI AHPC AS A BIOMARKER TO MONITOR DIFFERENT STAGES OF INFLAMMATION ASSOCIATED GASTROINTESTINAL DISEASES

H. pylori AhpC was identified by our clinical proteomics platform to be a potential biomarker for gastric diseases^[55]. As shown in Figure 4, we previously reported that none or low titers of serum antibody against LMW AhpC were detected in gastritis patients with weak inflammation in cases of *H. pylori* infection^[30]. However, higher titers of antibodies against HMW AhpC would be produced in gastric ulcer and cancer patients with more severe inflammation^[30]. Therefore, *H. pylori* AhpC can signal and monitor different stages of inflammation associated with *H. pylori* infection in gastrointestinal diseases. Also, both HMW AhpC and the serum level of antibody against AhpC are important to predict the risk of gastric cancer among *H. pylori*-positive patients (Figure 4).

Recently, the use of *H. pylori* AhpC as a potential vaccine against *H. pylori* infection^[56] or utilization of its monoclonal antibodies for detecting *H. pylori*^[57,58] were reported, again emphasizing its importance during the progression of inflammation-related gastrointestinal diseases. New technologies for producing *H. pylori* AhpC recombinant proteins, such as SDS-PAGE electroelu-

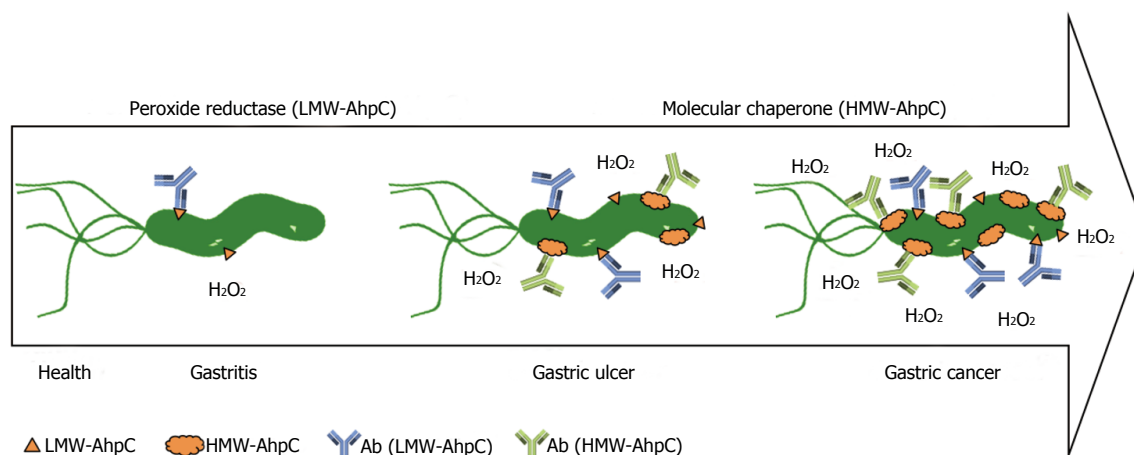


Figure 4 Alkylhydroperoxide reductase of *Helicobacter pylori*, a biomarker used to monitor different stages of inflammation associated with gastrointestinal diseases. In cases of *Helicobacter pylori* (*H. pylori*) infection, none or low titers of serum antibody against low-molecular-weight (LMW)-alkylhydroperoxide reductase (AhpC) were detected in patients of gastritis with weak inflammation. However, higher titers of antibodies against high-molecular-weight (HMW)-AhpC were found in gastric ulcer and cancer patients with severe inflammation. Therefore, *H. pylori* AhpC can signal and monitor different stages of inflammation associated with *H. pylori* infection in gastrointestinal diseases^[37].

tion-based rapid purification^[59] and different prokaryotic expression systems^[60], have also been developed. Taking all these findings and technologies into account, *H. pylori* AhpC is a potential biomarker for gastrointestinal diseases and it may be credible and promising to develop biomarkers kits of *H. pylori* for clinical application in the future, serving as a non-invasive detection method for diagnosis and prognosis. Most importantly, the successful identification of *H. pylori* AhpC as a biological marker highlights the utility of clinical proteomics for identifying disease biomarkers that can be translated from lab research into clinics.

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