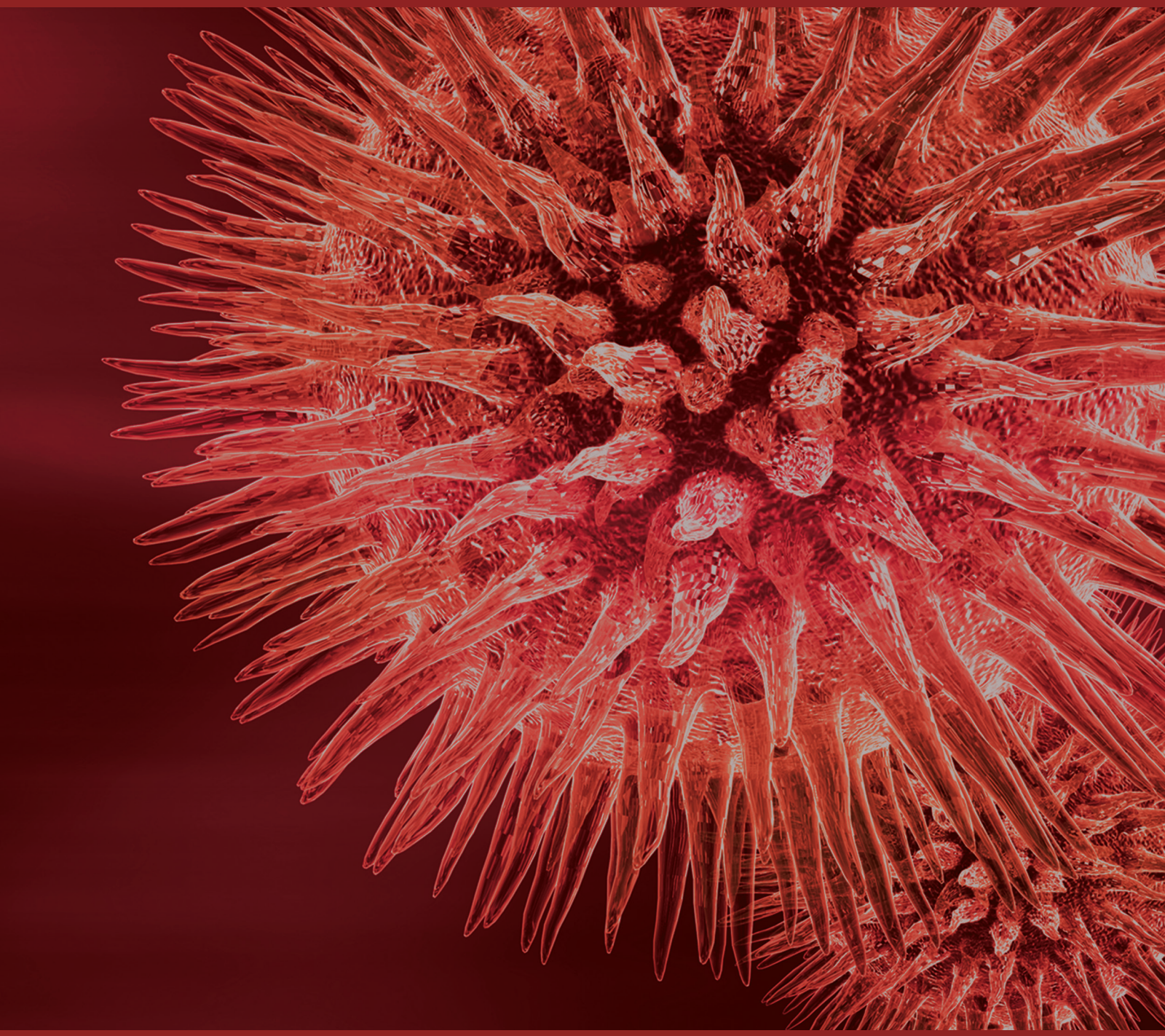
BioMed Research International

Helicobacter pylori and Pathogenesis

Guest Editors: Akio Tomoda, Shigeru Kamiya, and Hidekazu Suzuki



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Contents

***Helicobacter pylori* and Pathogenesis**, Akio Tomoda, Shigeru Kamiya, and Hidekazu Suzuki
Volume 2015, Article ID 304768, 2 pages

Gastric Carcinogenesis and Underlying Molecular Mechanisms: *Helicobacter pylori* and Novel Targeted Therapy, Toshihiro Nishizawa and Hidekazu Suzuki
Volume 2015, Article ID 794378, 7 pages

Interplay of the Gastric Pathogen *Helicobacter pylori* with Toll-Like Receptors,
Suneesh Kumar Pachathundikandi, Judith Lind, Nicole Tegtmeyer, Emad M. El-Omar, and Steffen Backert
Volume 2015, Article ID 192420, 12 pages

Comment on "*Helicobacter pylori* Outer Membrane Protein 18 (Hp1125) Is Involved in Persistent Colonization by Evading Interferon-γ Signaling", Amin Talebi Bezmin Abadi and Enzo Ierardi
Volume 2015, Article ID 354519, 2 pages

***Helicobacter pylori* Outer Membrane Protein 18 (Hp1125) Is Involved in Persistent Colonization by Evading Interferon-γ Signaling**, Yuqun Shan, Xingxiao Lu, Yingnan Han, Xinpeng Li, Xiao Wang, Chunhong Shao, Lixiang Wang, Zhifang Liu, Wei Tang, Yundong Sun, and Jihui Jia
Volume 2015, Article ID 571280, 12 pages

Nordihydroguaiaretic Acid Disrupts the Antioxidant Ability of *Helicobacter pylori* through the Repression of SodB Activity *In Vitro*, Hitoshi Tsugawa, Hideki Mori, Juntaro Matsuzaki, Tatsuhiro Masaoka, Tasuku Hirayama, Hideko Nagasawa, Yasubumi Sakakibara, Makoto Suematsu, and Hidekazu Suzuki
Volume 2015, Article ID 734548, 8 pages

Prevalence of *Helicobacter pylori vacA*, *cagA*, and *iceA* Genotypes in Cuban Patients with Upper Gastrointestinal Diseases, Onelkis Feliciano, Oderay Gutierrez, Lidunka Valdés, Trini Fragoso, Ana Maria Calderin, Antonio Eduardo Valdes, and Rafael Llanes
Volume 2015, Article ID 753710, 6 pages

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 304768, 2 pages
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Editorial
Helicobacter pylori and Pathogenesis

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By calling for manuscripts for this special issue, many manuscripts were submitted to the editorial office. After careful reviewing by expert referees, highly qualified papers concerning the topics were accepted as review and research articles for publication in the journal. From the accepted articles, some interesting ones are introduced as follows.

S. K. Pachathundikandi et al. reviewed an interplay of *H. pylori* with toll-like receptors (TLRs). TRL2 is able to recognize various different pathogen associated molecular patterns (PAMPs) including lipoproteins, lipoteichoic acid, and peptidoglycan. *H. pylori* activated NF-κB primarily through TLR2 and induced chemokine expression. Lipopolysaccharide (LPS) of *H. pylori* was identified as the ligand for TLR4, and *H. pylori* induced the secretion of IL-12 and IL-10 in mouse macrophages through TLR4/MyD88. It was also shown that *H. pylori* LPS can promote proliferation and progression of gastric cancer cells via a TLR4-dependent pathway. Flagellin from *H. pylori* is the ligand for TLR5, and the involvement of TLR5 in the recognition and further inflammatory processes is important for establishing a persistent infection of *H. pylori* at the mucosal surface. A chimeric flagellin composed of terminal regions from *Escherichia coli* and the middle region from *H. pylori* was reported to activate TLR5, suggesting that the chimeric flagellin might be a vaccine candidate with significant protection against *H. pylori* infection. Correlation between TLR8/9 sensing nucleic acids and *H. pylori* infection is also discussed in the review article.

T. Nishizawa and H. Suzuki reviewed recent findings on gastric carcinogenesis and underlying molecular mechanisms. Reactive oxygen species (ROS) induced by *H. pylori* can bind with nucleic acids, turning them into mutated forms that play a role in multistep carcinogenesis. Correlation of CD44 variant, cell-surface marker of cancer stem-like cells with ROS defense system was reported. The important roles of CagA and activation-induced cytidine deaminase (AID) in carcinogenesis are also reviewed. *H. pylori* infection up- or downregulates expression of microRNAs that is linked to gastric tumorigenesis. Activation of epidermal growth factor receptor (EGFR) and erythroblastic leukemia-associated viral oncogene B (ErbB2) induced by *H. pylori* infection results in survival of gastric epithelial cells with DNA damage. In addition, recent advances in molecular targeting therapies by anti-EGRF are introduced.

H. Tsugawa et al. identified novel FecA1-binding compounds *in silico* and examined the effect of NDGA (nordihydroguaiaretic acid) that is one of the above compounds, on SodB activity, metronidazole (Mtz) susceptibility, and H_2O_2 sensitivity of *H. pylori*. NDGA reduced SodB activity and increased both H_2O_2 sensitivity and Mtz susceptibility. These results suggest that NDGA might be effective for the development of a novel eradication therapy.

Y. Shan et al. reported that outer membrane protein 18 (Hp1125) of *H. pylori* is involved in persistent colonization by evading interferon- (IFN-) gamma signaling. It was shown that IFN-gamma induced higher expression of *H. pylori* Omp18 and reduced the expression of CagA and NapA. By mouse infection model, isogenic omp18 mutant strain showed defective colonization and increased inflammatory changes in gastric mucosa. It was also shown that the isogenic mutant strain induced more production of cytokine, chemokine, and NO, indicating that Omp18 is involved in bacterial survival against oxidative stress and phagocytosis by

macrophages. Comment on this paper was sent from A. T. B. Abadi and E. Ierardi. They hypothesize that more factors except Omp18 are contributing to long term infection of *H. pylori* in gastric mucosa as the connection of a unique factor to the drive of the final pattern of this phenomenon could be too speculative.

O. Feliciano et al. reported the prevalence of *vacA*, *cagA*, and *iceA* genotypes of *H. pylori* strains isolated from Cuban patients with upper gastrointestinal diseases. It was shown that the *vacA* s1 allele, *cagA* gene, and *iceA2* allele were the most prevalent (72.0%, 56.0%, and 57.3%, resp.). Significant statistical association was observed between *iceA2* allele and patients with nonpeptic ulcer dyspepsia as well as virulence genotypes (*s1, s1m2*) and patients over 40 years old. Although the total number ($n = 75$) of the isolates was not enough to conclude clearly, it was indicated that a high prevalence of main virulence factors was detected in Cuban isolates similar to that observed in other Western populations.

Since the discovery of *H. pylori* in 1983 (first isolation in 1982), many research studies were performed to clarify the mechanisms by which this microorganism causes not only gastroduodenal diseases including gastric cancer but also extragastric diseases such as idiopathic thrombocytopenic purpura and iron-deficiency anemia. However, the details on the correlation between *H. pylori* infection and gastric/extragastric pathogenesis in human remain to be determined. The review and research articles published in this special issue may give us a hint to resolve the above question, but further studies on pathogenesis of *H. pylori* infection need to continue to be done.

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Review Article

Gastric Carcinogenesis and Underlying Molecular Mechanisms: *Helicobacter pylori* and Novel Targeted Therapy

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The oxygen-derived free radicals that are released from activated neutrophils are one of the cytotoxic factors of *Helicobacter pylori*-induced gastric mucosal injury. Increased cytidine deaminase activity in *H. pylori*-infected gastric tissues promotes the accumulation of various mutations and might promote gastric carcinogenesis. Cytotoxin-associated gene A (CagA) is delivered into gastric epithelial cells via bacterial type IV secretion system, and it causes inflammation and activation of oncogenic pathways. *H. pylori* infection induces epigenetic transformations, such as aberrant promoter methylation in tumor-suppressor genes. Aberrant expression of microRNAs is also reportedly linked to gastric tumorogenesis. Moreover, recent advances in molecular targeting therapies provided a new interesting weapon to treat advanced gastric cancer through anti-human epidermal growth factor receptor 2 (HER-2) therapies. This updated review article highlights possible mechanisms of gastric carcinogenesis including *H. pylori*-associated factors.

1. Introduction

Helicobacter pylori infection is one of the most prevalent infectious diseases worldwide and 40–50% of the global human population is estimated to be infected. *H. pylori* has been identified as a group 1 carcinogen by the World Health Organization International Agency for Research on Cancer (WHO/IARC, 69372 Lyon CEDEX 08, France) and is associated with the development of noncardia gastric cancer. Eradication of *H. pylori* infection has been reported as an effective strategy for both the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue (MALT) lymphoma as well as prevention of gastric cancer [1–3].

A randomized controlled trial (RCT) in Japan demonstrated significant prophylactic effects of *H. pylori* eradication on the development of metachronous gastric carcinoma after endoscopic resection [1]. According to a systemic review of 15 papers, *H. pylori* eradication significantly reduces the prevalence of gastric cancer by approximately one-third [4]. A recent meta-analysis of RCTs also shows that *H. pylori* eradication seems to reduce gastric cancer risk [relative risk (RR): 0.65, 95% confidence interval (CI): 0.43–0.98] [5]. In 2009, the committee of the Japanese Society for Helicobacter Research (JSHR) revised the guidelines for diagnosis and treatment of *H. pylori* infection [6]. *H. pylori* eradication therapy achieved a strong recommendation, because it is useful for the treatment of gastric or duodenal ulcers, treatment and prevention of *H. pylori*-associated diseases such as gastric cancer, and inhibition of the spread of *H. pylori* infection.

Bacterial virulence factors, such as, cytotoxin-associated gene A (CagA), cause inflammation and activate oncogenic pathways. Activated neutrophils are the main source of reactive oxygen species (ROS) and reactive nitrogen species production in *H. pylori*-infected stomachs. Excessive oxidative stress can damage DNA in gastric epithelial cells, indicating its possible involvement in gastric carcinogenesis [7]. Gastric cancer arises from multiple genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell cycle regulators, cell-adhesion molecules, and DNA repair genes.

Recent studies provided evidence that expression of an aberrant DNA/RNA editing enzyme, activation-induced cytidine deaminase (AID), might be a mechanism of mutation accumulation in the gastric mucosa during *H. pylori*-associated gastric carcinogenesis. The roles of microRNAs are increasingly apparent, and aberrant expression of microRNAs may contribute to the development and progression of gastric cancer. Moreover, recent advances in molecular therapies provided a new interesting weapon to treat advanced gastric cancer through anti-human epidermal growth factor receptor 2 (HER-2) therapies.

Consequently, this paper summarizes the molecular mechanism of *H. pylori*-associated gastric carcinogenesis.

2. Oxidative Stress

Oxygen-derived free radicals that are released from activated neutrophils are considered potential toxic factors that contribute to *H. pylori*-induced gastric mucosal injury. Neutrophils express myeloperoxidase, which produces the hypochlorous anion (OCl^-). OCl^- reacts with the ammonia (NH_3), which is produced from urea by *H. pylori*-associated urease, to yield a lipophilic, highly cytotoxic oxidant, monochloramine (NH_2Cl), that freely penetrates biological membranes to oxidize intracellular components [8]. Free radicals, including ROS and reactive nitrogen species, can bind with nucleic acids, turning them into mutated forms that play a role in multistep carcinogenesis [9].

The CD44 variant (CD44v), which is a cell-surface marker, that is associated with cancer stem-like cells, interacts with a glutamate-cystine transporter and controls the intracellular level of reduced glutathione (GSH) [10]. Human gastrointestinal cancer cells with a high level of CD44 expression revealed an enhanced capacity for glutathione synthesis and defense against ROS. These findings indicate that cancer stem-like cells with CD44v expression could have an ROS defense system that results from glutathione synthesis.

H. pylori upregulates spermine oxidase (SMOX) in gastric epithelial cells. SMOX metabolizes the polyamine spermine into spermidine and generates H_2O_2, which causes apoptosis and DNA damage. However, a subpopulation of $SMOX^{high}$ cells is resistant to apoptosis, despite their high levels of DNA damage. Chaturvedi et al. reported that activation of epidermal growth factor receptor (EGFR) and erythroblastic leukemia-associated viral oncogene B (ErbB2) by *H. pylori* results in survival of gastric epithelial cells with DNA damage [11].

3. CagA

Epidemiological evidence indicates that *H. pylori* strains containing CagA are more virulent. CagA-positive *H. pylori* increases the risk of both intestinal and diffuse types of noncardia gastric cancer [12]. The CagA protein of *H. pylori*, which is delivered into gastric epithelial cells via bacterial type IV secretion system, is an oncoprotein that can induce malignant neoplasms. After the CagA protein is injected into the host cell cytoplasm, the EPIYA (Glu-Pro-Ile-Tyr-Ala) motif of CagA is tyrosine-phosphorylated by host Src kinases and subsequently changes the gastric epithelial morphology [13]. CagA binding to the protooncogene Src homology 2-containing protein tyrosine phosphatase (SHP-2) causes aberrant activation of SHP-2 and consequently of the ERK-MAPK (mitogen-activated protein kinase) pathway, which has been reported to play a role in carcinogenesis by inducing mitogenic responses [14]. East Asian CagA that contains the EPIYA-D motif demonstrates higher affinity for SHP-2 than Western CagA. These findings are consistent with the fact that East Asian strains dominate in countries with the highest rates of gastric cancer [15].

On the other hand, CagA interacts with many signaling molecules (e.g., Per-1, c-Abl, Crk proteins, Grb proteins, and c-Met) that are important for the regulation of cell proliferation, scattering, and morphology (Figure 1). CagA, that is translocated into the host cell, is degraded by autophagy and short-lived. However, CagA, that is translocated into CD44v9-positive gastric cancer stem-like cells, which are characterized by ROS resistance that results from their rich GSH content, is thought to escape ROS-dependent autophagy, resulting in gastric carcinogenesis [16].

4. AID

The DNA/RNA editing enzyme, AID, is a 198 amino acid protein that directly converts cytosine into uracil (Figure 2). AID is specifically induced in germinal center B cells to carry out somatic hypermutation and class-switch recombination, which are two processes that are responsible for antibody diversification. Because of its mutagenic potential, AID expression and activity are tightly regulated to minimize unwanted DNA damage. Surprisingly, AID is also induced by inflammation and microbial infections in nonimmune cells.

H. pylori infection mediates aberrant AID expression in gastric mucosal epithelial cells. Additionally, AID expression was shown to be triggered by proinflammatory cytokines, such as TNF-α or IL-1, and correlated with mononuclear cell infiltration and intestinal metaplasia. After eradication of *H. pylori*, the level of AID expression decreases [17]. Strong evidence indicates that CagA-positive *H. pylori*-mediated upregulation of AID resulted in the accumulation of nucleotide alterations in the TP53 tumor suppressor gene in gastric cells [18]. Whole-exome sequencing revealed that somatic mutations accumulated in various genes in inflamed gastric tissues. The mutations that accumulated in gastric tumors as well as gastric mucosal tissues with *H. pylori*-induced gastritis were predominantly C : G > T : A transitions in GpCpX motifs, which are markers of cytidine deamination that are induced by AID [19]. Increased cytidine deaminase activity in *H. pylori*-infected gastric tissues appears to promote the accumulation of various mutations and might promote gastric carcinogenesis.

5. Oncogenes

Mutational activation and/or amplification of several oncogenes such as ErbB, KRAS, PIK3CA, MET, and MYC has been documented in gastric cancer.

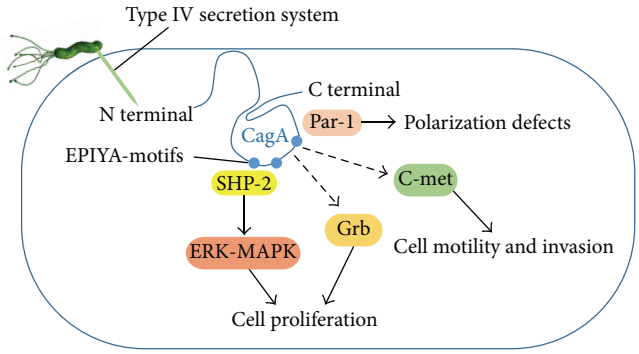

Figure 1: CagA and major functions. CagA is delivered into gastric epithelial cells via bacterial type IV secretion system. CagA interacts with many signaling molecules that are important for the regulation of cell proliferation, polarity, and motility.

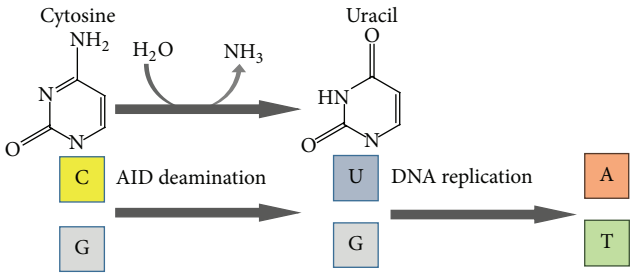

Figure 2: Activation-induced cytidine deaminase (AID) as the DNA/RNA editing enzyme. AID is induced by *H. pylori* infection and promotes the accumulation of various mutations.

Epidermal growth factor receptor (EGFR), a member of ErbB receptor family, is involved in the regulation of gastric mucosal cell proliferation and progression of gastric cancer. Overexpression of ErbB1 (EGFR) and ErbB2 (HER-2) is found in 27–64% and 6–34% of gastric cancer, respectively [20]. Activation triggers a cascade of events that involves autophosphorylation and activation of tyrosine kinase domain, Ras/Raf/MAPK pathway, phospholipase C-γ, and phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR). Ras, an oncogenic small GTPase, has three isoforms, namely, K-Ras, H-ras, and N-ras. Constitutive activation of Ras and Ras-related proteins promotes cell proliferation and increase invasion and metastasis while inhibiting apoptotic cell death. *K-RAS* gene was found to be mutated (codon-12) in intestinal-type cancer, but not in diffuse-type cancer [21]. *K-RAS* gene mutations in *H. pylori*-associated chronic gastritis are more frequent in gastric cancer patients than in cancer-free patients, suggesting that K-RAS mutation may be involved in the early stage of gastric carcinogenesis [22].

Trastuzumab is a monoclonal antibody that binds to extracellular domain of the receptor, acting by blockage of the HER-2 receptor cleavage, inhibition of dimerization, and the induction of antibody-dependent cellular cytotoxicity (ADCC). In phase III ToGA trial, the addition of trastuzumab to standard cisplatin and 5-fluorouracil improved overall survival from 11.1 to 13.8 months in patients with HER-2 amplified gastric adenocarcinomas [23]. In phase III EXPAND trial, the addition of cetuximab (chimeric monoclonal anti-EGFR antibody) to standard cisplatin and capecitabine did not improve progression free survival in patients with advanced gastric cancer [24]. Similarly, in phase III REAL-3 trial, the addition of panitumumab (fully humanized monoclonal anti-EGFR antibody) to epirubicin, oxaliplatin, and capecitabine did not improve overall survival in patients with advanced gastric adenocarcinoma [25]. In addition, retrospective biomarker analysis has failed to identify a clear patient subset that would derive benefit from EGFR-directed therapies. Lapatinib is dual tyrosine kinase inhibitor active on EGFR and HER-2. In phase III TYTAN trial, the addition of lapatinib to weekly paclitaxel in second-line setting improved overall response rate in patients with HER-2-positive gastric adenocarcinomas (odds ratio, 3.85; 95% CI, 1.80–8.87, $P < 0.001$) but did not significantly improve overall survival (lapatinib+paclitaxel, 11.0 versus paclitaxel, 8.9 months; $P = 0.104$) [26].

Phase III GRANITE-1 study evaluated the efficacy of mTOR inhibitor everolimus compared to the best supportive care in patients with advanced gastric cancer that progressed after initial chemotherapy. In this study, median survival was reportedly 5.39 months with everolimus versus 4.34 months with placebo (HR = 0.90; 95% CI, 0.75–1.08) [27]. Patients in this study were not preselected with respect to PI3K/AKT/mTOR pathway alteration.

6. Tumor-Suppressor Genes

The *p53* tumor-suppressor gene, the guardian of human genome, is frequently inactivated in the tissue of gastric cancer as well as in preneoplastic lesions, by loss of heterozygosity (LOH), missense mutation, or frame-shift deletions [12]. The p53, 53 kDa phosphoprotein, is a transcription factor for including DNA repair genes in response to DNA damage. Activation of p53 also arrests the cell cycle to allow enough time for fixation of DNA damage. However, if DNA damage is beyond repair, p53 induces apoptotic cell death. The mutations of *p53* have also been identified in gastric adenoma and intestinal metaplasia. We have recently reported that p53 downregulation due to increased MDM2-phosphorylation induces autophagy, which causes CagA oncoprotein degradation translocated from the bacterial body of *H. pylori* to gastric epithelial cells and then inhibits mTOR [16].

APC is a multidomain protein with binding sites for numerous proteins including Wnt signaling pathway. APC plays major role in cell adhesion, cell migration, spindle formation, and chromosome segregation [28]. *APC* mutations are the second most frequent mutations in gastric cancer and have been observed in 30–40% of intestinal type cancer and in less than 2% of diffuse type cancer [29]. The mutations of *APC* have also been identified in gastric adenoma and intestinal metaplasia, indicating that they occur during preneoplastic stage of gastric cancer development. LOH and mutations of phosphatase and tensin homolog (*PTEN*) were observed in gastric cancers as well as in precancerous lesions [30].

7. DNA Methylation

Methylation of CpG islands in a promoter region inhibits gene transcription by interfering with transcriptional initiation and serves as an alternative mechanism of inactivating tumor suppressor genes without gene mutation. Among factors known to cause aberrant DNA methylation, aging and chronic inflammation are known to promote the accumulation of DNA methylation. *H. pylori* infection induces aberrant promoter methylation in tumor-suppressor genes, including *p16*, *LOX*, and *CDH1*. Furthermore, a number of tumor suppressor genes, including *hMLM1*, *p14*, *p15*, *GSTP1*, *RASSF1*, *COX-2*, *APC*, *CDH4*, *DAP-K*, *THBS1*, *TIMP-3*, *RARβ*, *MGMT*, *CHFR*, *DCC*, *RUNX3*, and *TSLC1*, are known to be silenced by hypermethylation in gastric cancer [31] (Figure 3).

Recent meta-analysis revealed that the frequencies of *p16* promoter methylation in gastric cancer tissue were higher than those of normal and adjacent tissues [Normal: odds ratio (OR) = 23.04, 95% CI = 13.55–39.15, $P < 0.001$; Adjacent: OR = 4.42, 95% CI = 1.66–11.76, $P = 0.003$]. Furthermore, significant associations of *p16* promoter methylation with TNM stage, histologic grade, invasive grade, and lymph node metastasis are shown (TNM stage: OR = 3.60, 95% CI: 2.17–5.98, $P < 0.001$; Histologic grade: OR = 2.63, 95% CI: 1.55–4.45, $P < 0.001$; Invasive grade: OR = 3.44, 95% CI: 1.68–7.06, $P = 0.001$; Lymph node metastasis: OR = 2.68, 95% CI: 1.66–4.32, $P < 0.001$) [32].

Forkhead box (Fox) proteins comprise an evolutionarily conserved family of transcriptional regulators. In particular, FOXD3 bound directly to the promoters and activated transcription of genes that encode the cell death regulators CYFIP2 and RARB. Recent methylation profile analyses revealed that FOXD3-mediated transcriptional control of tumor suppressors is deregulated by *H. pylori* infection-induced hypermethylation [33]. Alternatively, CagA enhanced DNA methyltransferase 3B and enhancer of zeste homologue 2 expression, which resulted in the attenuation of *let-7* expression by histone and DNA methylation [34]. Aberrant epigenetic silencing of *let-7* expression leads to Ras upregulation.

DNA methylation levels of specific CpG islands are associated with risk of gastric cancer. Nanjo et al. identified seven novel gastric cancer risk markers that reflect epigenomic damage, that is induced by *H. pylori* infection, and the hypermethylated CpG islands had high ORs (12.7–36.0) in an analysis [35]. Moreover, 5-aza-2′-deoxycytidine (5-aza-dC) is a DNA demethylating agent. Niwa et al. showed that 5-aza-dC treatment can prevent *H. pylori*-induced gastric cancers in the Mongolian gerbil model [36]. Removal of induced DNA methylation and/or suppression of DNA methylation induction could become a target for prevention of chronic inflammation-associated cancers.

8. Angiogenesis

Elevated concentrations of vascular endothelial growth factor (VEGF) have been described in patients with advanced gastric cancer and correlated with decreased survival. VEGF and VEGF receptors are overexpressed in 36–40% of gastric cancer. Phase III AVAGAST and AVATAR studies have evaluated the addition of bevacizumab (anti-VEGF-A monoclonal antibody) to first-line platinum-fluoropyrimidine chemotherapy in advanced gastric cancer patients but failed to demonstrate improved survival [37, 38].

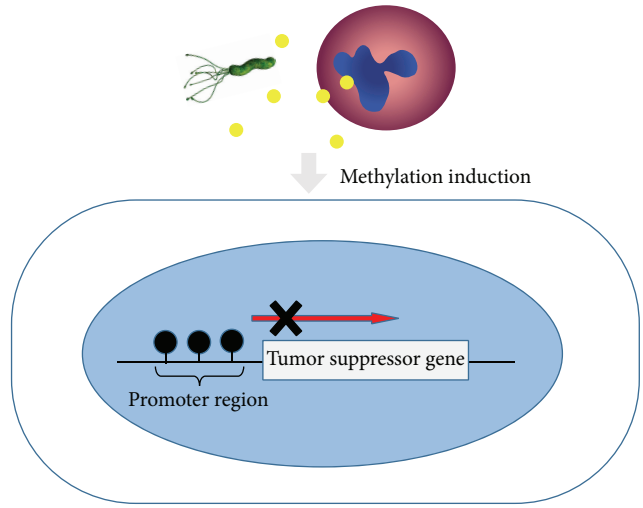

Figure 3: DNA methylation inactivates tumor suppressor genes without gene mutation. Chronic inflammation by *H. pylori* infection induces aberrant promoter methylation in tumor-suppressor genes.

Phase III REGARD trial evaluated the efficacy of ramucirumab, a monoclonal antibody VEGFR-2 antagonist, compared to the best supportive care (BSC) in patients with advanced gastric cancer that progressed after previous chemotherapy. Median survival was 5.2 months with ramucirumab versus 3.8 months with placebo (HR = 0.776; 95% CI 0.603–0.998; $P = 0.047$) [39]. Based on data from this REGARD trial, the Food and Drug Administration (FDA) in United States approved ramucirumab as second-line therapy for patients with advanced gastric cancer.

Apatinib, inhibitor of VEGF-2, showed improved progression free survival and overall survival in heavily pretreated patients with metastatic gastric cancer in phase II trial [40]. Phase III trial of apatinib is ongoing.

9. MicroRNAs

MicroRNAs are noncoding RNAs that are comprised of 18–24 nucleotides that can posttranscriptionally downregulate various target genes. Recent studies have shown that a considerable number of microRNAs are altered following infection with *H. pylori*, and specific microRNA deregulation was found to contribute to host inflammation, cell-cycle progression, inhibition of apoptosis, cell invasion, and metastasis [41]. MicroRNAs that function as oncogenes, including *miR-17*, *miR-21*, and *miR-106a*, were upregulated, whereas microRNAs that function as tumor suppressors, such as *miR-101*, *miR-181*, *miR-449*, *miR-486*, and *let-7a*, were downregulated in gastric cancer [42, 43].

Shiotani et al. reported that the expression of oncogenic microRNAs (*miR-17/92* and the *miR-106b-93-25* cluster, *miR-21*, *miR-194*, and *miR-196*) is significantly higher in the intestinal than nonintestinal metaplasia. *H. pylori* eradication improves microRNA deregulation, but not in the intestinal metaplasia. MicroRNA deregulation is not completely reversible by eradication alone in long-term *H. pylori* colonization [44].

Runt domain transcription factor 3 (RUNX3) is a tumor suppressor, that is silenced in cancer via hypermethylation of its promoter. Previously, we reported that *H. pylori* eradication significantly increases RUNX3 expression in gastric epithelial cells [45]. Lai et al. reported that *miR-130b* expression is upregulated in gastric cancer, and this is inversely associated with *Runx3* hypermethylation [46]. *miR-130b* overexpression increases cell viability and reduces cell death following the downregulation of RUNX3 protein expression. Recently, Wang et al. reported that *miR-301a* is upregulated in gastric cancer and directly downregulates RUNX3 expression [47].

Furthermore, *microR-34b/c* is considered a tumor suppressor and transcriptional target of p53. Based on a scheduled follow-up study of endoscopic biopsy from noncancerous mucosa in the gastric body from 129 patients after curative endoscopic resection of gastric cancer, the cumulative incidence of metachronous gastric cancer was significantly higher among patients with elevated miR-34b/c methylation, indicating that methylation of *miR-34b/c* in the gastric mucosa may be a useful biomarker for predicting the risk of metachronous gastric cancer [48].

10. Cancer Stem Cell

Cancer stem cells have been defined as a unique subpopulation in tumors that possess the ability to initiate tumor growth and sustain tumor self-renewal. Gastric cancer stem cells were first isolated and identified in 2009. Takaishi et al. identified CD44 as a gastric cancer stem cell marker as CD44+ cells were able to form into spheroid colonies in serum-free media *in vitro* and gastric tumors after xenografts in nude mice *in vivo*, whereas CD44-negative sorted cells were not [49]. These cells have the ability for self-regeneration and resistance for chemotherapy- or radiation-induced cell death. Cancer stem cells-targeted therapy is a novel direction for treating and preventing gastric cancer. In our recent study, translocated CagA from *H. pylori* to the epithelial cells could accumulate in CD44v9-expressing gastric cancer stem-like cells by escaping the autophagic degradation pathway due to their characteristics of xCT-dependent ROS resistance [16].

The high expression of CD44 is positively correlated with malignant transformation, metastasis, and relapse of gastric cancer [50]. We previously reported that the recurrence rate of early gastric cancer was significantly higher in the CD44v9-positive than the CD44v9-negative cohorts (hazard ratio, 21.8; 95% CI, 5.71–83.1) [42]. CD44v9 expression in the tissue of primary gastric cancer represents a potential predictive marker for recurrence.

Additionally, there is another hypothesis. It is thought that gastric cancer stem cells are derived from bone-marrow-derived mesenchymal stem cells. When there is injury, the bone-marrow-derived mesenchymal stem cells can mobilize from the bone marrow and participate in tissue repair. Varon et al. labeled bone-marrow-derived cells with green fluorescent protein in mice model of infection with *H. pylori*. After 1 year, gastric glands that contained green fluorescent protein-positive cells were detected in 90% of mice infected with *H. pylori*. Almost 25% of high-grade dysplastic lesions included cells originating from the bone marrow [51]. The results suggest that bone-marrow-derived mesenchymal stem cells are the source of gastric cancer.

11. Conclusion

As shown in this review, molecular mechanisms of gastric carcinogenesis have been extensively studied. Alterations in multiple genes and complex copy number and gene expression profiles have been identified in gastric cancer over the two decades. New strategies had been developed for advanced gastric cancer treatment. However, the response rates remain in the 25–40% range across published trials, and novel molecularly directed approaches are needed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Interplay of the Gastric Pathogen *Helicobacter pylori* with Toll-Like Receptors

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Toll-like receptors (TLRs) are crucial for pathogen recognition and downstream signaling to induce effective immunity. The gastric pathogen *Helicobacter pylori* is a paradigm of persistent bacterial infections and chronic inflammation in humans. The chronicity of inflammation during *H. pylori* infection is related to the manipulation of regulatory cytokines. In general, the early detection of *H. pylori* by TLRs and other pattern recognition receptors (PRRs) is believed to induce a regulatory cytokine or chemokine profile that eventually blocks the resolution of inflammation. *H. pylori* factors such as LPS, HSP-60, NapA, DNA, and RNA are reported in various studies to be recognized by specific TLRs. However, *H. pylori* flagellin evades the recognition of TLR5 by possessing a conserved N-terminal motif. Activation of TLRs and resulting signal transduction events lead to the production of pro- and anti-inflammatory mediators through activation of NF-κB, MAP kinases, and IRF signaling pathways. The genetic polymorphisms of these important PRRs are also implicated in the varied outcome and disease progression. Hence, the interplay of TLRs and bacterial factors highlight the complexity of innate immune recognition and immune evasion as well as regulated processes in the progression of associated pathologies. Here we will review this important aspect of *H. pylori* infection.

1. Introduction

The Gram-negative bacterium *H. pylori* is an extracellular pathogen infecting about 50% of the human world population. Infections with *H. pylori* can persist lifelong and are associated with chronic, often asymptomatic gastritis in all infected individuals, while some individuals can develop more severe gastric diseases such as peptic ulceration, MALT lymphoma, and gastric cancer [1, 2]. Disease progression is controlled by multiple factors such as genetic predisposition of the host, bacterial genotype, and environmental constraints [2–5]. Clinical *H. pylori* isolates are highly diverse both in their genetic polymorphisms and potential to induce pathogenicity. Myriads of bacterial factors have been associated with *H. pylori* pathogenesis. Well-known pathogenicity-associated mechanisms include urease-mediated neutralization of pH, flagella-driven bacterial motility, shedding of outer-membrane vesicles, secretion of proteases (such as HtrA) in the extracellular space, and peptidoglycan-dependent immune responses [6–10]. However, the two major *H. pylori* virulence factors are the vacuolating cytotoxin (VacA) and the CagA protein encoded by the cytotoxin-associated genes pathogenicity island (*cag*PAI). VacA is secreted in the culture medium and can induce multiple responses such as pore formation in the host cell membrane, modification of endolysosomal trafficking, cellular vacuolation, immune cell inhibition, and apoptosis, whereas the *cag*PAI represents a type IV secretion system for delivery of CagA into the host cell [11–14]. In addition, *H. pylori* express various typical surface adhesins,

which permit the tight interaction of the bacteria with host target cells. The *Helicobacter* outer membrane porin (Hop) class of factors plays an important role in this context, comprising several well-described proteins including BabA, SabA, AlpA/B, OipA, HopI, HopQ, and HopZ [4, 15, 16]. Interestingly, *H. pylori* targets various host surface structures such as carbohydrates, phosphatidylserine, heparin sulfate, cholesterol, sphingomyelin, and other lipids as well as a broad range of host protein receptors [15, 17, 18]. Here we review the various molecular strategies of *H. pylori* to hijack a specific class of host protein receptors, the toll-like receptors (TLRs). We focus on the identified TLR family members and bacterial factors but also discuss several downstream signaling cascades, which are a crucial part of the host immune system.

2. Composition and Function of TLRs

TLRs constitute a group of cell surface and subcellular transmembrane proteins, which are expressed on cells of the host immune system including macrophages and dendritic cells (DCs) but are also present on the gastrointestinal epithelium and other nonimmune cells [19–21]. TLRs belong to the class of pattern recognition receptors (PRR) of the host innate immune system. These germ line encoded type I transmembrane glycosylated protein receptors are composed of an ectodomain with leucine-rich repeats (LRR), transmembrane region, and intracytoplasmic Toll/IL-1 receptor (TIR) domain [20–22]. TLRs act as sensors where they recognize microbial pathogens of bacterial, viral, fungal, and protozoan origin. A wide variety of ligands can bind to different TLRs to induce downstream signaling. The ligands are called pathogen associated molecular patterns (PAMPs) and include a broad array of microbial molecules build-up of proteins, nucleic acids, lipids, or synthetic chemicals. Upon activation by microbial factors, TLRs trigger the coordinated expression of host genes involved in specific signaling cascades for the regulation of innate and adaptive immunity, tissue repair, and regeneration processes [19, 20]. By binding of microbial ligands to a given TLR, there is activation of signaling transduction pathways involving the TIR domain and binding to cytoplasmic adaptor molecules including myeloid differentiation factor 88 (MyD88), TIR domain-containing protein (TIRAP), TIR domain-containing adaptor inducing interferon-beta (TRIF), and TIR domain-containing adaptor inducing interferon-β-related adaptor molecule (TRAM). This complex has been described to activate two main signaling pathways, the MyD88-dependent (applied by most TLRs except TLR3) and the MyD88-independent TRAM/TRIF cascade (applied by TLR3 and some signals of TLR4) [20–22]. The MyD88-dependent pathway signals through a cascade of interleukin-1 receptor associated kinase (IRAK), tumor necrosis factor receptor associated factor 6 (TRAF6), and transforming growth factor-beta-activated kinase 1 (TAK1) and activates transcription factor nuclear factor kappa B (NF-κB) and its translocation from the cytoplasm to the nucleus as well as c-jun N-terminal kinase (JNK) and p38-mediated activator protein 1 (AP-1) stimulation [19–21]. NF-κB and AP-1 can then bind to the promoter region of a variety of immune and inflammatory genes resulting in the transcription of proinflammatory and anti-inflammatory cytokines, including tumor necrosis factor- (TNF-) α and interleukin-6 (IL-6). The MyD88-independent pathway uses TRAM/TRIF adaptor proteins for initiating signaling, which leads to the cascade involving TRAF3, inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε), and TANK binding kinase 1 (TBK-1) to the production of type-1 interferons [19–21]. In addition, the TRIF pathway was reported to activate NF-κB through a cascade involving receptor interacting protein 1 (RIP1) and TAK1. In this manner, TLRs regulate the production of cytokines, opsonization, coagulation cascades, complement activation, and upregulation of costimulatory molecules on antigen presenting cells. However, exact role of TLRs in *H. pylori* infection is highly controversial in the literature and needs to be reviewed. The various reported interactions with TLR2, TLR4, TLR5, TLR8, and TLR9 as well as some identified bacterial interaction partners and resulting downstream targets are summarized in Table 1. An overall model for major TLR activities and proposed signaling strategies exploited by *H. pylori* is presented in Figure 1.

3. Interaction of *H. pylori* with TLRs and Downstream Signaling

3.1. TLR2 in H. pylori Infection. The actual *H. pylori* ligand(s) of TLR2 are so far elusive and high efforts are made to detect the relevant contributors in activation. In general, TLR2 is able to recognize various different PAMPs, most importantly bacterial lipoproteins, lipoteichoic acid, or peptidoglycan. TLR2 forms heterodimers with either TLR1 or TLR6 to build a specific PRR able to recognize these different PAMPs [21, 25]. The surface expressed TLR2 receptor has so far been detected in intestinal and gastric epithelial cells and is known to be important in activating the inflammation system [26–28]. An important model cell system is cultured human embryonic kidney 293 (HEK293), endogenously not expressing most of the TLRs. These cells are therefore often used to study TLR involvement in infections by stably transfecting them with different reporter systems such as luciferase, SEAP (secreted embryonic alkaline phosphatase), and others. For example, Smith and coworkers investigated the expression of proinflammatory signals of various *H. pylori* strains on HEK293 cells that were cotransfected with TLR2, TLR5, or TLR4 and an NF-κB luciferase reporter [28]. *H. pylori* activated NF-κB primarily through TLR2, but not TLR4, in stably transfected HEK293 and MKN45 gastric epithelial cell lines and induced chemokine expression (IL-8, MIP3α, and GROα) through these TLRs. In these cells, TLR2 induction resulted in highly enhanced expression of chemokines as compared to TLR5. As control, expression of dominant-negative TLR2 mutants of gastric epithelial cell lines showed reduced activation by *H. pylori* and suggested that *H. pylori* lipopolysaccharide (LPS) is a TLR2 ligand [28]. However, it was reported that whole *H. pylori* cells activated TLR2 in transfected HEK293 cells, while LPS recognition

Table 1: Targeted TLR receptors with proposed role during infection with *H. pylori*.

TLR receptor	Host cells and mouse lines used	*H. pylori* strain(s) used	Applied methods	Proposed role during infection	References
TLR2	AGS, HEK293, MKN45, CHO	26695, LC11, 98, 99	RT-PCR, LRGA	NF-κB activation and chemokine expression	[28]
	HEK293	26695	ELISA, IP, WB, SPIA	MAPK activation and chemokine expression	[39]
	HEK293, PBMCs, primary human monocytes and macrophages, PECs	SS1, 43504, Astra 244	ELISA, RT-PCR, cDNA-MA	Intact bacteria activate TLR-2, while LPS activates TLR-4	[29]
	mBMDCs	SS1, J99, TX30, B128	ELISA, RT-PCR, cDNA-MA, FACS, LRGA, KD	Activates TLRs to induce production of pro- and anti-inflammatory cytokines	[30]
	MKN28, MKN45, HEK293, T24, THP1	Clinical strains	ELISA, FACS, RT-PCR, LRGA, WB	Highly purified LPS is a weak agonist, activates NF-κB through TLR-2/1 heterodimer	[35]
	KATO III	43504	ELISA, ABB, RT-PCR, NB, WB, TFA, ICC, RPT	HpHSP60 is a ligand, activates NF-κB and chemokine expression in epithelial cells	[36]
	NOMO1, U937	43504	ELISA, ABB, TFA, RT-PCR, siRNA, SPIA, FACS, WB, RPT	HpHSP60 is a ligand, activates MAPK and chemokine expression in monocytes	[37]
	PBMCs, primary human monocytes, neutrophils and T-cells	n.p.	ELISA, RT-PCR, ELISPOT, NF-κBRA, FACS, TCA, CRA, RPT	HpNAP activates proinflammatory cytokine expression and T-cell responses	[38]
	AGS, MKN45	Clinical strains	EMSA, RT-PCR, WB, IFM, LGRA, IP, KA	NF-κB activation and COX-2 overexpression	[40]
	AGS, MKN45, HUVEC	Clinical strains	ELISA, DAPA, EMSA, WB, ChIP, RT-PCR, LGRA, MGTA	COX-2 induction and increased cell invasion and angiogenesis	[41]
TLR4	AGS, HEK293, MKN45, CHO	26695, LC11, 98, 99	RT-PCR, LRGA	NF-κB activation and chemokine expression	[28]
	HEK293, PBMCs, primary human monocytes and macrophages, PECs	SS1, 43504, Astra 244	ELISA, RT-PCR, cDNA-MA	Intact bacteria activate TLR-2, while LPS activates TLR-4	[29]
	AGS, MKN-7, MKN-28, MKN-45, THP-1	43504, TN583, clinical strains	LCM, IHC, RT-PCR, FACS, LGRA	NF-κB activation and chemokine expression	[42]
	MKN45, TMK1, J774A.1, THP1, PECs	TN2	ELISA, ABB, TLR-SA, RT-PCR, EMSA, RPA, IP, WB, cDNA-MA	NF-κB activation is *cag*PAI-dependent in epithelial cells, but *cag*PAI-independent in monocytes/macrophages	[43]
	AGS, MKN45, CHO, T84, THP1	LC11, LC20	ELISA, RT-PCR, WB, FACS, IFM, SEM	Upregulation of TLR expression and chemokine secretion	[44]
	Gastric mucosal cells	11637, 11638, clinical strains	RT-PCR, SB, WB, NB	Upregulation of superoxides in gastric pit cells	[45]
	HEK293 C57BL/6J mice *in vivo*	J99, B128, X47	FBA, MS, PA, TLR-SA, MCA, NF-κBRA, FM	Dephosphorylation of lipid-A reduces recognition and increases colonization	[46]
	AGS, gastric biopsies	J99, clinical strains	RT-PCR	TLR expression is not affected in gastric biopsies of infected patients	[47]
	Gastric biopsies	Clinical strains	ICH, IFM, CSLM	Shifting of subcellular localization of TLRs	[48]

Table 1: Continued.

TLR receptor	Host cells and mouse lines used	*H. pylori* strain(s) used	Applied methods	Proposed role during infection	References
TLR5	AGS, HEK293, MKN45, CHO	26695, LC11, 98, 99	RT-PCR, LRGA	NF-κB activation and chemokine expression	[28]
	HEK293	26695	ELISA, IP, WB, SPIA	MAPK activation and chemokine expression	[39]
	AGS, T84, MDCK	49503	ELISA, WB, RPT,	Flagellin evades TLR5 recognition	[49]
	CHO K1	G27, clinical strains	LRGA, WB, RPT, BIT, MA	Flagellin evades TLR5 recognition	[50]
	HEK293 BALB/c mice *in vivo*	SS1S	ELISA, CD, LRGA, RT-PCR, WB, RPT	Chimeric flagellin can activate immune responses	[51]
	HEK293, THP1	P1, P12, P310, 26695	ASPAB, ELISA, IFM, RT-PCR, TLR-SA, LRGA, WB	*cag*PAI status can change TLR activated production of cytokine/chemokine	[34]
	GES1	26695	cDNA-MA, FACS, RT-PCR, SEM	Spiral and coccoid forms can influence TLR expression	[52]
	AGS, gastric biopsies	J99, clinical strains	RT-PCR	TLR expression is not affected in gastric biopsies of infected patients	[47]
	Gastric biopsies	Clinical strains	ICH, IFM, CSLM	Shifting of subcellular localization of TLRs	[48]
	Gastric biopsies	Clinical strains	ICH, IFM, CSLM	Shifting of subcellular localization of TLRs	[53]
TLR8	PBMCs, primary human monocytes, HeLa, HEK293, HEK293T, HEK293FT, THP1	251, B128	ELISA, RT-PCR, LGRA, CSLM, BIT, FACS	Bacterial phagocytosis increases TLRs activation and cytokine secretion	[54]
TLR9	mBMDCs	SS1, J99, TX30, B128	ELISA, RT-PCR, cDNA-MA, FACS, LRGA, KD	Activates TLRs to induce production of proinflammatory cytokines	[30]
	AGS, MKN45, HUVEC	Clinical strains	ELISA, DAPA, EMSA, WB, ChIP, RT-PCR, LGRA, MGTA	COX-2 induction and increased cell invasion and angiogenesis	[41]
	AGS, MKN45	Clinical strains	EMSA, RT-PCR, WB, IFM, LGRA, IP, KA	NF-κB activation and COX-2 overexpression	[40]
	Gastric biopsies	Clinical strains	ICH, IFM, CSLM	Shifting of subcellular localization of TLRs	[48, 53]
	Primary human neutrophils	26695, G27, 8822, clinical strains	ELISA, FACS	*cag*PAI dependent production of pro- and anti-inflammatory cytokines	[55]
	Gastric tissue, C57BL/6J mice	SS1	IHC, RT-PCR, MPA, CSLM	Type-I interferon mediated anti-inflammatory response at early phase infection	[56]

AB: antibody; ABB: antibody blocking; ASPAB: activation specific phospho antibodies; BIT: bioinformatic tools; *cag*PAI: cytotoxin-associated genes pathogenicity island; CD: circular dichroism; cDNA-MA: cDNA micro/macroarray; ChIP: chromatin immunoprecipitation; COX-2: cyclooxygenase-2; CRA: chromium release assay; CSLM: confocal laser scanning microscopy; DAPA: DNA affinity protein binding assay; ELISA: enzyme-linked immunosorbent assay; ELISPOT: enzyme-linked immunospot; EMSA: electrophoretic mobility shift assay; FACS: fluorescence-activated cell sorting; FBA: fluorescent binding assay; FM: fluorescence microscopy; HPA: histopathological analysis; HSP: heat shock protein; IFM: immunofluorescence microscopy; ICC: immunocytochemistry; IHC: immunohistochemistry; IP: immunoprecipitation; KA: kinase assay; KD: knockdown of genes; LCM: laser capture microdissection; LRGA: luciferase reporter gene assay; MAPK: mitogen-activated protein kinases; MA: motility assay; mBMDCs: mouse bone marrow derived DCs; MCA: mouse colonization assay; MGTA: matrigel tube formation assay; MPA: myeloperoxidase activity assay; MS: mass spectrometry; NAP: neutrophil activating protein; NB: northern blotting; NF-κB: nuclear factor kappa B; NF-κBRA: nuclear factor kappa B reporter assay; n.p.: not provided; PA: phosphatase assay; PBMCs: peripheral blood mononuclear cells; PECs: peritoneal exudate cells; RPA: RNAse protection assay; RPT: recombinant protein techniques; RT-PCR: real-time/reverse transcriptase PCR; SB: southern blotting; SEM: scanning electron microscopy; siRNA: small interfering RNA; SPIA: signaling pathway inhibitor assay; TCA: T-cell clonal assays; TFA: transcription factor assay; TLR: Toll-like receptor; TLR-SA: TLR signaling assay; WB: western blotting.

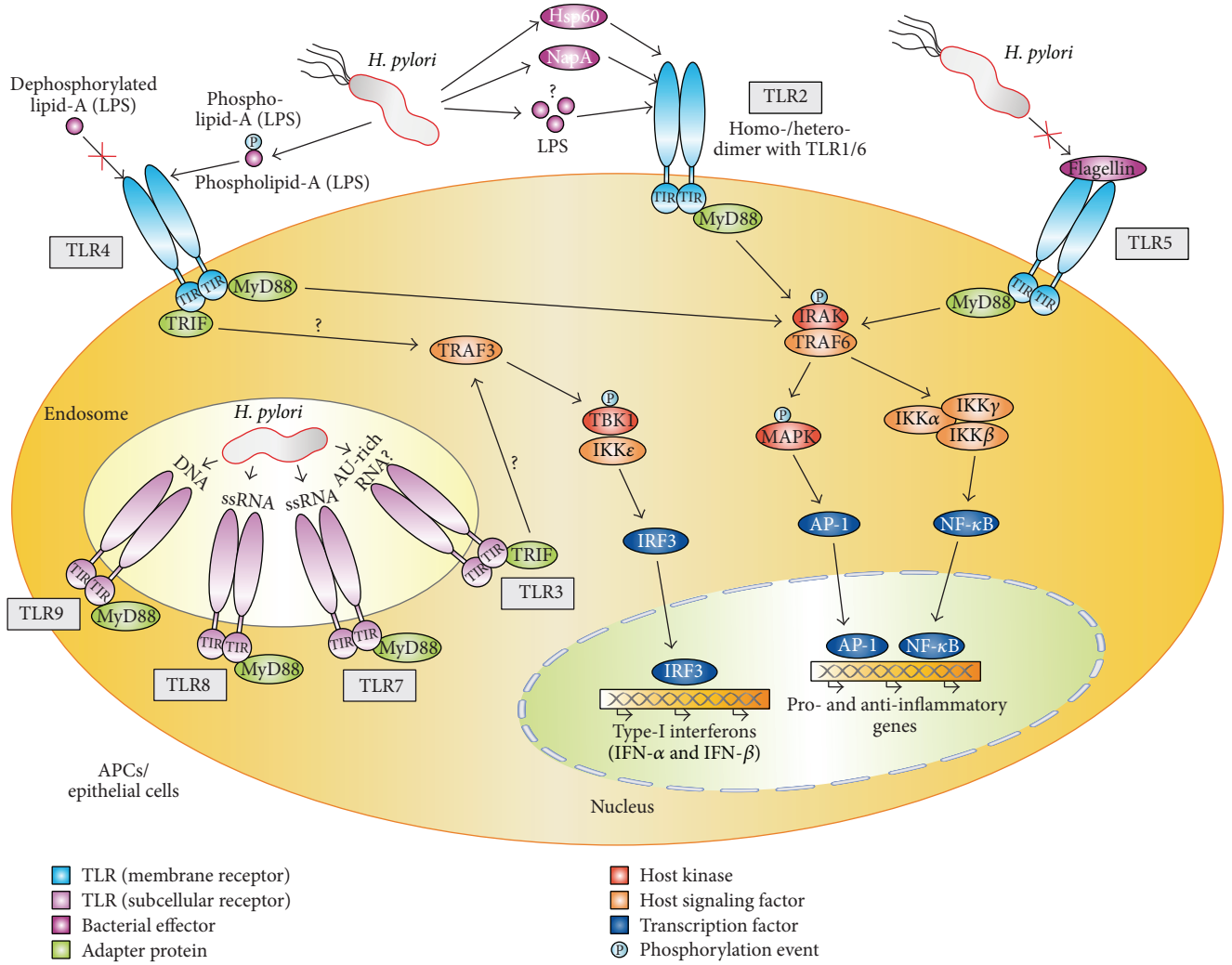

FIGURE 1: *H. pylori* mediate crosstalk with Toll-like receptors (TLRs) to manipulate signaling in innate immunity. TLRs constitute a group of cell surface and subcellular transmembrane receptors in antigen-presenting cells (APCs) and epithelial cells. *H. pylori* can interact with at least five TLR members (TLR2, TLR4, TLR5, TLR8, and TLR9) in various ways as indicated. TLRs are composed of a leucine-rich repeat-containing ectodomain, a transmembrane region and an intracellular tail with the TIR domain. *H. pylori* encodes various factors that have evolved either to target or to evade detection by the TLRs. *H. pylori* LPS, phosphorylated lipid A of LPS, HSP-60, NapA, DNA, and RNA are reported in various studies to be recognized by specific TLRs as shown. However, nonphosphorylated lipid A and flagellin evade the recognition by TLR4 and TLR5, respectively. The TIR domain in TLRs has a crucial role in adapter protein recruitment and the activation of downstream signaling cascades. TLR activity is initiated by PAMP-induced receptor dimerization and TIR engagement with the adapter proteins MyD88 or TRIF as indicated. Binding between a given TLR and MyD88 results in the recruitment of members from the IRAK kinase family. IRAK members are sequentially phosphorylated and dissociated from MyD88. This results in the activation of TRAF6, which in turn stimulates signaling through MAP kinases and IKK complex leading to the activation of transcription factors NF-κB and AP-1 and the production of pro- and anti-inflammatory mediators. The adapter protein TRIF participates in the MyD88-independent TLR4 pathway as well as in the TLR3 signaling cascade. TRAF3 is recruited for the TRIF-mediated pathway mediating the production of type-I interferons and in some cases anti-inflammatory cytokine IL-10 [23, 24]. Endosomal TLR-mediated signaling leads to the induction of type-I interferons by the engagement of the transcription factor IRF as indicated. Question marks indicate activities/pathways which are not fully clear and require further investigation. For more details, see text.

appeared to be mediated by TLR4 [29]. In addition, *cag*A-positive strains were found to be more potent in activation of TLR2 than isogenic *cag*A mutant strains, suggesting the involvement of CagA or associated genes of the *cag*PAI in signaling and cytokine secretion. Moreover, *H. pylori* was found to induce IL-8 secretion in a TLR2-dependent manner rather than TLR4. Macrophages of TLR4-deficient mice ($TLR4^{-/-}$) were also still able to produce strong cytokine and chemokine secretion (IL-6 and MCP-1), while macrophages of TLR2-deficient mice were not responsive during *H. pylori* infection [29]. In DCs derived from mice, the response to bacterial lysate also appeared to be induced via TLR2 and only to a minor extent by TLR4. On the other hand, the production of anti-inflammatory cytokine IL-10 was induced

by *H. pylori* in DCs and that is completely abrogated in TLR2$^{-/-}$ mice derived cells [30]. The production of IL-10 through activation of TLR2 could be linked to the observation that TLR2$^{-/-}$ mice clear *H. pylori* infection more efficiently than infected wild-type animals [31]. Moreover, it was also shown that TLR2/MyD88 signaling induced by *H. pylori* in mouse macrophages led to the secretion of proinflammatory cytokines such as IL-6 and IL-1β [32]. A study on human neutrophils infected with *H. pylori* showed an induced expression of TLR2 and TLR4. In addition, an increased expression of IL-8, IL-10, IL-1β, and TNF-α was also found in neutrophils, while only IL-8 and IL-10 expression was reduced by using function-blocking anti-TLR2 and/or anti-TLR4 antibodies [33]. We have shown that *H. pylori* is able to induce TLR2 expression in monocytes (THP1 cells) and HEK293 epithelial cells stably transfected with TLR2. This also resulted in significant production and secretion of the proinflammatory cytokine TNFα and chemokine IL-8 in these cells [34]. It was also reported that highly purified *H. pylori* LPS can be detected through TLR2 (but not TLR4) and the TLR2 induction was dependent on the coexpression of TLR1 or TLR6 in HEK293 transfected luciferase reporter cell lines. Among TLR2/TLR6 and TLR2/TLR1 heterodimers, TLR2/TLR1 heterodimer was preferentially recognized by *H. pylori* LPS over TLR2/TLR6 heterodimer [35]. Nevertheless, there are also studies showing opposing activation and stimulation results. For example, Viala and coworkers used HEK293 cells (not expressing TLR2) which resulted in NF-κB activation due to NOD1 recognition of peptidoglycan and thus cytokine induction irrespective of TLR expression [6]. In addition, other antigens of *H. pylori* have also been reported to trigger TLR2 activation. Addition of recombinant heat shock protein 60 (HSP60) of *H. pylori* to KATO-III gastric epithelial cells was shown to be able to trigger NF-κB activation and upregulated expression of IL-8 [36]. In accordance with this study, Zhao and colleagues have shown that HSP60 induced the secretion of IL-8 in NOMO1 monocytic cells through activation of mitogen activated protein (MAP) kinases [37]. In particular, the extracellular signal regulated kinase (ERK) and p38 MAP kinase pathways are involved and this was abrogated in the presence of anti-TLR2 function-blocking antibodies or TLR2 siRNA [37]. The neutrophil activating protein (NAP) of *H. pylori* was also reported to activate TLR2 and induced the production of Th1 inflammatory cytokines such as IL-12 and IL-23 [38]. Although some studies are highly controversial, the above data clearly affirm an important role of TLR2 in *H. pylori* recognition and induction of proinflammatory changes. However, there should be further systematic analysis of these factors and binding mechanisms to TLR2, which may help us to better understand the varied roles of this important TLR during *H. pylori* infection.

3.2. TLR4 in H. pylori Infection. TLR4 was the first identified receptor as an ortholog of drosophila toll in mammals [57]. Further studies have identified LPS of bacteria as the ligand for TLR4 and binding of this ligand was dependent on the cofactors cluster of differentiation 14 (CD14) and myeloid differentiation factor 2 (MD2) [58–60]. The classical initial immune response induced by bacterial infection is generally mediated by macrophages and derives from the activation of TLR4, which is induced by bacterial LPS [20]. However, how TLR4 is involved in recognition upon *H. pylori* infection is not really established and much debate is in the literature about this topic. TLR4 and MD2 expression was induced in gastric epithelial cells of infected patients. However, lamina propria mononuclear cells expression of TLR4 and MD2 was similar in uninfected and infected biopsies. In addition, *H. pylori* LPS was not able to induce NF-κB activation in treated AGS epithelial cells but induced NF-κB in THP1 cells. However, MD2 transfection of AGS cells alone was able to regain NF-κB activation in *H. pylori* LPS-treated cells and that was attributed to more cell surface location of TLR4 in this cell line [42]. Maeda and coworkers investigated the involvement of TLR4 and CD14 in NF-κB activation using THP-1 monocytes and MKN45 epithelial cells [43]. They showed that the expression of TLR4 and CD14 was required for activation of NF-κB in monocytes, but not in gastric epithelial cells. Interestingly, NF-κB activation in epithelial cells was dependent on a functional *cag*PAI in *H. pylori*, but not in monocytes [43]. It was also reported that *H. pylori* induced the secretion of IL-12 and IL-10 in mouse macrophages through TLR4/MyD88 signaling [32]. In addition, *H. pylori* primed DCs interaction with allogeneic CD4$^+$ T-cells, which resulted in the production of interferon gamma (IFN-γ) and IL-17A as well as the induction of transcription factor forkhead-box-protein P3 (FOXP3). This indicates that *H. pylori* induced a mixed T-cell response including Th1, Th17, and Treg. However, blocking of TLR4 signaling resulted in significant reduction of Th1 and Th17 specific cytokines as well as FOXP3 expression [61]. In contrast, other groups have shown that primary gastric epithelial cells do not express TLR4 and were also nonresponsive to LPS [62]. In another study it was demonstrated that infection of AGS and MKN45 cells with *H. pylori* resulted in an upregulation of TLR4; however, adding blocking antibodies towards TLR4 failed to inhibit LPS-induced IL-8 secretion. In line with the abovementioned studies, this study also showed that the whole *H. pylori*-induced IL-8 secretion in epithelial cells and LPS-mediated induction requires CD14 expression [44]. In one of the earlier studies using *H. pylori* LPS it was shown that impaired sensitivity to TLR4 is due to modifications of the lipid A core and this can also be responsible for reduced innate immune response to *H. pylori* [63]. Lipid A prepared from LPS of *H. pylori* strain 206-1 as well as its synthetic form resulted in low endotoxic activity when given orally to C57BL-6 mice, while the corresponding synthetic lipid A from *Escherichia coli* had strong effects as expected. Treatment with *H. pylori* LPS was also shown to decrease IL-8 and TNF-α secretion in a human gastric cancer cell line and peripheral blood mononuclear cells (PBMCs), respectively, when compared to synthetic lipid A from *E. coli* [64]. Poor recognition of *H. pylori* LPS was also suggested to depend on the degree of acylation and reduced activation of the immune system is corresponding to a low acylation level of LPS. LPS of *H. pylori* is mostly tetraacylated, while LPS from *E. coli* is hexaacylated [25, 63, 65]. However, exact role of *H. pylori*

LPS in TLR recognition is debatable because purified LPS and live bacteria have been shown to result in different pattern of recognition and signaling as reported by different groups. The bioactivity of *H. pylori* LPS to induce TLR4 activation was documented to be 1,000- to 10,000-fold less in comparison to LPS from *E. coli* [63, 66]. This has also been implicated to the variation in fatty acid distribution and phosphorylation status of the lipid A portion of *H. pylori* LPS [67]. *H. pylori* LPS was also observed to induce superoxide anions in gastric pit cells of guinea pigs, possibly through TLR4 signaling, and this was also dependent on the phosphorylation status of the lipid A disaccharide backbone [45]. In support of the above data, further studies identified two phosphatases encoded by the *H. pylori* genome, which can modify its lipid A by removal of phosphate groups from the lipid A backbone, and this modification helped to decrease the recognition of this LPS by TLR4 and also the sensitivity to cationic antimicrobial peptides [46, 68]. Interestingly, it was reported that *H. pylori* LPS can promote proliferation and progression of gastric cancer cells via a TLR4-dependent pathway as well as to attenuate cytotoxicity of PBMCs and thus promote cancer formation. The impaired cytotoxicity can be attributed to the attenuated production of Th1 cytokine IL-12, IFN-γ in mononuclear cells (MNCs), and perforin levels in natural killer (NK) cells after treatment with *H. pylori* LPS [69]. In addition, the discrepancies found in the literature regarding the activation of TLR4 by LPS might also arise from the different strains and the dosage that is used in these studies, which has already been suggested before [29]. Furthermore, the purity of LPS in different preparations is also a matter of concern because the possible presence of peptidoglycan or lipopeptides in such fractions might alter the activation pattern towards TLR2 instead of TLR4.

3.3. TLR5 in H. pylori Infection. Flagellin from many bacteria is the only ligand identified for TLR5. The binding of this ligand to TLR5 at the cell surface induces downstream signaling through a MyD88-dependent pathway. The involvement of other adaptor proteins (TRAM/TRIF) dependent downstream signaling pathway has not been reported in the case of TLR5 activation. TLR5 expression was shown to be present in most of the epithelial surfaces studied in humans and mice. This ubiquitous presence of TLR5 shows its importance in the recognition of microbial pathogens at epithelial surfaces [20]. *H. pylori* is considered to be an extracellular pathogen, mainly colonizing the human gastric mucosa and surviving there for long periods of time by defeating the efforts of host immune system. The involvement of TLR5 in the recognition and further inflammatory processes was initially proposed to be of importance on establishing a persistent infection of *H. pylori* at the mucosal surface. In one of the first studies, *H. pylori* infection of gastric epithelial cell lines has activated NF-κB through TLR5 and also induced the expression of chemokines such as IL-8, MIP3α, and GROα. They have also reported that partially purified *H. pylori* flagellin activated NF-κB, possibly through TLR5 [28]. TLR5-dependent stimulation of IL-8 secretion was activated through the increase in p38 and ERK MAP kinase activity and also showed high activating transcription factor 2 (ATF2) phosphorylation in *H. pylori* infected cells [39]. In contrast, Gewirtz and coworkers reported that infection of AGS cells with Δ*fla*A mutant of *H. pylori* did not significantly reduce the secretion of IL-8 [49]. They have also shown that purified recombinant FlaA of *H. pylori* is a weak inducer of IL-8 expression or p38 activation in gastric epithelial cells. In line with the latter observation, a classical study reported the importance of certain motifs in the flagellin for their recognition through TLR5 [50]. This study used different flagellated and nonflagellated bacteria for analyzing their capacity to activate TLR5. Nonflagellated *Staphylococcus aureus* and flagellated *Bartonella bacilliformis*, *Rhizobium meliloti*, *Campylobacter jejuni*, *H. pylori* (strain 26695 and two clinical isolates), *H. hepaticus*, *H. felis*, and *Wolinella succinogenes* were not recognized by TLR5. The strongest known ligand of TLR5, FliC of *Salmonella enterica*, stimulated TLR5-dependent NF-κB activation. Surprisingly, a chimera of FliC containing the N-terminal D0-D1 domain of *H. pylori* FlaA was completely inactive on stimulating TLR5-dependent NF-κB activation. Further experiments have located the TLR5 stimulatory and nonstimulatory effects within a specific region of the D1 domain of flagellin. Finally, replacement of amino acids 89–96 of FliC with the corresponding amino acids from *H. pylori* FlaA abolished the TLR5 agonist activity of FliC [50]. This study has clearly explained the weak recognition of *H. pylori* flagellin through TLR5 as an important immune evasion process of this long-term colonizing pathogen. However, this study did not completely rule out the possibility of other ligands from *H. pylori* or other nonstimulatory bacteria, because most of their findings were dependent on heat-killed bacteria or purified flagellin. A recent study showed that a chimeric flagellin composed of terminal regions from *E. coli* and the middle region from *H. pylori* folded correctly and was able to activate TLR5. Vaccination using this chimeric recombinant flagellin was able to provide significant protection against *H. pylori* colonization, thus making it an efficient alternative method for vaccinating against other flagellated bacteria that evade TLR5 recognition [51]. We have shown that *H. pylori* significantly induced the upregulation of TLR5 in THP1 and HEK293-TLR5 cells [34]. *H. pylori* infection of THP-1 cells induced the secretion of IL-8 and TNF-α in a *cag*PAI-dependent manner. In addition, infection of HEK293 cells expressing TLR5 with *H. pylori* induced the phosphorylation of IL-1 receptor-associated kinase 1 (IRAK-1) and inhibitor of kappa B (IκB), and this was required for the activation of NF-κB. However, induced expression of transfected TLR5 in HEK293 cells shifted *cag*PAI dependent to *cag*PAI independent proinflammatory signaling for the secretion of IL-8 and TNF-α [34]. In addition, TLR5 mRNA expression level was upregulated in the gastric epithelial cell line GES-1 during infection with spiral-shaped *H. pylori*, but not by the corresponding coccoid form [52]. In contrast, a study using gastric biopsies has ruled out the induction of TLR5 expression in *H. pylori* infection [47]. Moreover, TLR5 expression in the gastric epithelium of chronic active *H. pylori* gastritis was localized at the basolateral sides of the cells without detectable expression at the apical side, but was homogeneously distributed in the gastric epithelium

with intestinal metaplasia and dysplasia as well as in gastric carcinoma cells [48, 53]. From the above data, we can advocate an involvement of TLR5 in *H. pylori* infection for activation of proinflammatory changes, but the involved mechanism is yet unknown.

3.4. TLR8 and TLR9 in H. pylori Infection. The nucleic acid sensing TLRs are widely studied in their roles in the recognition and induction of successive immune responses against different viruses. However, the literature for microbial RNA sensing various TLRs (TLR3, TLR7, and TLR8) is limited for bacterial infections. These receptors are localized on subcellular structures and induce signaling through a MyD88-dependent pathway except for TLR3, which was reported to mediate signaling by the adaptor protein TRIF [70]. Interestingly, it has been reported that *H. pylori* phagocytosis by THP1 cells induced the expression of two functional TLR8 isoforms and TNF-α secretion. *H. pylori* phagocytosis significantly activated the sensing of synthetic TLR8 ligands in those cells for the secretion of cytokines. This emphasizes an active role of TLR8 in *H. pylori* recognition and immune responses [54]. In addition, it was shown that purified *H. pylori* RNA induced a strong IL-6 and IL-12 response in mouse DCs and this was dependent on MyD88, but not on TLR9 and TLR7 [30]. TLR9 is known to sense unmethylated CpG DNAs of bacterial, viral, or synthetic origin and signal through MyD88-dependent pathway. *H. pylori* clinical isolates induced cyclooxygenase 2 (COX2) expression through MAP kinase signaling in epithelial cells and increased the cellular invasion properties, which is partially dependent on TLR9 [40, 41]. In addition, *H. pylori* DNA was shown to induce a more pronounced invasion of a gastric cancer cell line reported through an *in vitro* invasion assay [71]. Rad and coworkers also showed a TLR9 dependent recognition of *H. pylori* DNA and induction of proinflammatory cytokines in mouse DCs [30]. TLR9 expression was predominantly localized at the gastric surface epithelium; however, chronic active gastritis in *H. pylori* infection changed the expression exclusively to the basolateral side and the incoming neutrophils also showed high TLR9 expression [48]. Similarly, infection of isolated neutrophils has also shown an elevated TLR9 expression in a *cag*PAI-independent manner [55]. It has also been demonstrated that TLR9 expression increased in epithelial and immune cells infiltrating to the lamina propria and submucosa of *H. pylori* infected mice. TLR9$^{-/-}$ mice showed the same level of *H. pylori* colonization; however, the myeloperoxidase (MPO) activity and mRNA expression of TNF-α and IFN-γ were increased in the gastric tissue during the initial phase of infection. In addition, type-I IFN such as IFN-α and IFN-β mRNA expression was significantly reduced in the TLR9$^{-/-}$ mice during infection. Moreover, recombinant IFN-α administration significantly reduced MPO activity and mRNA expression of TNF-α and IFN-γ in these infected TLR9$^{-/-}$ mice. These data indicate the anti-inflammatory role of TLR9 during the early phase of *H. pylori* induced gastritis and that could be possibly through the production of type-I IFN [56]. Apart from the latter study, it has also been reported that genomic *H. pylori* DNA contained more immune-regulatory sequences (IRS) and was found to be a weak inducer of type-I IFN or IL-12 secretion from mouse DCs and also suppressed the induction of these cytokines by *E. coli* DNA. In concurrence with the anti-inflammatory response reported in the above studies, *H. pylori* DNA administration before the induction of dextran sodium sulfate (DSS) mediated colitis significantly ameliorated the severity of colitis. This also supports the hypothesis of inverse relationship between *H. pylori* and inflammatory bowel diseases [72]. *H. pylori* genome specific IRS 5′-TTTAGGG-3′ with other IRSs might constitute the inhibitory effect of *H. pylori* genomic DNA through TLR9 signaling pathway [73]. Taken together, the above studies indicate that *H. pylori* nucleic acids are recognized by TLRs and inducing both pro- and anti-inflammatory responses during infection. These contrasting observations must be studied in detail to understand differential factors influencing shifting of responses and to see if this is helping the host or bacteria in the hostile interaction.

4. Role of TLR Gene Polymorphisms in *H. pylori* Infection

It has been noted that the sequence of *TLR* genes can slightly vary between patients. Hundreds of small nucleotide polymorphisms (SNPs) have been identified in various *TLR* genes, but the functional consequences of the majority of SNPs remain unknown. Many associations have been reported between TLR polymorphisms and infectious diseases or cancers. In the case of *H. pylori* infection, TLR polymorphisms have been specifically implicated to enhance the susceptibility for infection (TLR1) and also the risk of developing *H. pylori*-induced gastric cancer (TLR4).

4.1. Susceptibility to H. pylori Infection. Several small case control studies have addressed the role of genetic polymorphisms in the risk of *H. pylori* infection, but only one Genome Wide Association Study (GWAS) has so far been published [74]. Mayerle and colleagues reported two independent GWAS studies from two independent population-based cohorts from north-eastern Germany (Study of Health in Pomerania) and Netherlands (Rotterdam Study) [74]. Fecal *H. pylori* antigen testing was used to determine the presence of infection in these individuals. GWAS meta-analysis identified 2 genome-wide significant loci in terms of their association to *H. pylori* seropositivity, namely, the *TLR* locus on chromosome 4p14 and the *FCGR2A* locus on chromosome 1q23.3. The lead SNP on the *TLR* locus with the lowest *P* value was rs10004195 (OR = 0.70, 95% CI = 0.65–0.76), closely followed by rs4833095 (OR = 0.70, 95% CI = 0.65–0.76). Three different TLRs are located along the 4p14 region: TLR1, TLR6, and TLR10. In an additional study conducted on 1,763 participants from both cohorts, analysis of whole blood RNA gene expression profiling showed that among the three *TLR* genes, only TLR1 was differentially expressed in relation to the rs10004195 genotype (in the presence of the rs10004195-A allele [beta = −0.23, 95% CI = −0.34 to −0.11]). Furthermore, analysis of TLR1, TLR6, and

TLR10 mRNA amounts also showed that there was a specific and genotype-independent transcriptional upregulation of TLR1 in the presence of *H. pylori*. These results imply that the increase in TLR1 mRNA expression as a result of the rs10004195 SNP is strongly associated with an increased risk of *H. pylori* seropositivity.

The mechanism for the relationship between increased TLR1 expression and a higher *H. pylori* seroprevalence remains unexplained. However, TLR1 has been shown to interact with TLR2 to form a heterodimer [75], which is responsible for the initiation of cellular downstream signaling in response to the recognition of triacylated lipopeptides from the Gram-negative bacterial cell wall [76]. This is particularly relevant to *H. pylori* infection as triacylated lipopeptides can be found in the structure of *H. pylori* lipid A, allowing it to be recognized by the TLR1-TLR2 complex. It has been suggested that the resulting activation of the immune cascade could reduce the anti-inflammatory response of the host against *H. pylori*, thus allowing persistent infection [76]. Another explanation proposed recently is that the SNP at the *TLR1* gene causes less effective anti-inflammatory signaling initiated by the TLR1-TLR2 complex in response to the presence of *H. pylori*, thus increasing the risk of persistent infection [77].

4.2. Risk of TLR SNPs in H. pylori-Induced Gastric Cancer. Hold and coworkers reported that the *TLR4*+896A>G polymorphism was associated with risk of gastric cancer and its precursors [78]. *TLR4*+896G carriers had an 11-fold (95% confidence interval [CI], 2.5–48) increased odds ratio (OR) for hypochlorhydria and also had significantly more severe gastric atrophy and inflammation. Seventeen percent of gastric carcinoma patients in the initial study and 15% of the noncardia gastric carcinoma patients in the replication study had 1 or 2 TLR4 variant alleles *versus* 8% of both control populations (combined OR = 2.3; 95% CI = 1.6–3.4) [78]. In a case-control study and meta-analysis, Castaño-Rodríguez and coworkers reported that the TLR signaling pathway was implicated in gastric carcinogenesis, with *TLR4* Asp299Gly and *TLR2* −196 to −174 del showing associations with gastric cancer in an ethnic-specific manner [79]. Although not fully understood, these observations highlight the importance of TLR2 and TLR4 in gastric disease development.

5. Conclusions and Outlook

H. pylori is one of the most successful bacterial pathogens infecting about half of the human world population and is responsible for a considerable global health burden, including peptic ulcer disease and gastric cancer. Studies of host-bacterial interactions using their fundamental virulence-associated factors have provided us with remarkable insights into *H. pylori* biology. Here we have reviewed the interference of a multitude of bacterial factors with five TLRs (TLR2, TLR4, TLR5, TLR8, and TLR9). The current data suggest a model in which *H. pylori* can interact with or evade these TLRs (Figure 1). In addition, we have reviewed our current knowledge on the bacterial factors including HSP-60, NapA, LPS, DNA, or RNA and how they may target TLRs and downstream signaling cascades. It can be therefore assumed that there is a highly dynamic system of extensive crosstalk between TLRs and their bacterial ligands to make up a scenario of complex host cellular processes leading to persistent colonization and chronic pathogenicity. However, there are some discrepancies with regard to corresponding *H. pylori* ligands or new molecular patterns to induce different signaling pathways for the production of mediators of the host immune system as discussed here. In the future, it will therefore be important to solve some of the conflictive reports discussed above. For example, the bacterial factors activating TLR2 and TLR4. In addition, it will be fundamental to unravel if new *H. pylori* factors can target TLRs for inducing downstream signaling. Finally, SNPs were found in various TLRs and they are known to crucially influence the clinical outcome of *H. pylori* infections. These factors therefore appear to play a key role in the pathophysiology of gastric disease development and their importance should be investigated in animal models which mimic human gastric neoplasia. It can be expected that more genetic polymorphisms both in the host and in *H. pylori* will be uncovered with advancing technologies, so that there is every prospect of defining full genetic risk profiles in near future. This will also aid in improving the current testing and treatment strategies of *H. pylori* infections. Thus, it appears that *H. pylori*-TLR receptor interactions will continue to be a fascinating and rewarding research topic in future studies.

Abbreviations Used

AP-1:	Activator protein 1
APC:	Antigen presenting cell
IFN:	Interferon
IKKs:	IκB kinases
IRF3:	Interferon regulatory factor 3
Lipid A:	A component of LPS
LPS:	Lipopolysaccharide
MAPK:	Mitogen-activated protein kinase
MyD88:	Myeloid differentiation primary response gene 88
NF-κB:	Nuclear factor kappa B
PAMP:	Pathogen-associated molecular pattern
TBK1:	Tank-binding kinase 1
TIR:	Toll/interleukin-1 receptor domain
TRAF3/6:	TNF receptor associated factor 3/6
TRIF:	TIR-domain-containing adapter-inducing interferon-β.

Conflict of Interests

The authors declare no potential conflict of interests.

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Research Article

Helicobacter pylori Outer Membrane Protein 18 (Hp1125) Is Involved in Persistent Colonization by Evading Interferon-γ Signaling

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Outer membrane proteins (OMPs) can induce an immune response. Omp18 (HP1125) of *H. pylori* is a powerful antigen that can induce significant interferon-γ (IFN-γ) levels. Previous studies have suggested that IFN-γ plays an important role in *H. pylori* clearance. However, *H. pylori* has multiple mechanisms to avoid host immune surveillance for persistent colonization. We generated an *omp18* mutant (*H. pylori* 26695 and *H. pylori* SS1) strain to examine whether Omp18 interacts with IFN-γ and is involved in *H. pylori* colonization. qRT-PCR revealed that IFN-γ induced Omp18 expression. qRT-PCR and western blot analysis revealed reduced expressions of virulence factors CagA and NapA in *H. pylori* 26695 with IFN-γ treatment, but they were induced in the Δ*omp18* strain. In C57BL/6 mice infected with *H. pylori* SS1 and the Δ*omp18* strain, the Δ*omp18* strain conferred defective colonization and activated a stronger inflammatory response. Signal transducer phosphorylation and transcription 1 (STAT1) activator was downregulated by the wild-type strain but not the Δ*omp18* strain in IFN-γ-treated macrophages. Furthermore, Δ*omp18* strain survival rates were poor in macrophages compared to the wild-type strain. We concluded that *H. pylori* Omp18 has an important function influencing IFN-γ-mediated immune response to participate in persistent colonization.

1. Introduction

Helicobacter pylori is the principal pathogenic factor in gastritis, peptic ulcer, and even gastric cancer and mucosa-associated lymphoid tumors [1, 2]. Almost half of the world's population has had an *H. pylori* infection, especially in China [3]. Without treatment, *H. pylori* colonizes in the stomach for the host's entire life [4]. Therefore, *H. pylori* has near-perfect niche adaptation and can avoid human immune responses [5, 6].

Most outer membrane proteins (OMPs) of bacteria are surface-exposed and therefore may be important in interfacing bacteria with the mammalian host and its defenses [7]. For example, *Pseudomonas aeruginosa* OprF can recognize IFN-γ and mount an effective countermeasure to immune activation by the host [8]. *Francisella novicida* FopC plays a role in inhibiting the IFN-γ-mediated host immune defense [9]. *H. pylori* contains an OMP family of approximately 33 genes [10]. Omp18 (HP1125), located on bacteria's outer membrane surfaces, is expressed by all known *H. pylori* strains and can react specifically with sera from all *H. pylori*-infected patients. Omp18 is habitually recognized by the immune system [11] and primes a T helper 1 cell (Th1) response from proliferating splenocytes by inducing IFN-γ production [12].

Table 1: Primers used in this study.

Primers used to construct Δ*omp18* strain	
omp18-1: 5′-CCATCGATAACAAACGCTCTTTGGCTTC-3′	*omp18*-2: 5′-CGGAATTCGGCAATACCGATGAATTTGG-3′
omp18-3: 5′-CGGGATCCATGAAGAGATCTTCTGTATTTAG-3′	*omp18*-4: 5′-AAAACTGCAGTTACTTCATTAATTTGACATCC-3′
Primers for RT-PCR	
*napA*F: 5′-TGAAGAGTTTGCGGACAT-3′	*napA*R: 5′-R AGAGTGGAAGCTCGTTTT-3′
*cagA*F: 5′-AGCAAAAAGCGACCTTGAA-3′	*cagA*R: 5′-AGCCAATTGCTCCTTTGAGA-3′
*omp18*F: 5′-TGCTTTTGGAAGGCAATACC-3′	*omp18*R: 5′-CATTTGGGTTTGGTTTCACC-3′
*16SrRNA*F: 5′-GCTCTTTACGCCCAGTGATTC-3′	*16SrRNA*R: 5′-GCGTGGAGGATGAAGGTTTT-3′

F: forward primer; R: reversed primer.
Underlined letters indicate nucleotides added at the 5′ end to create a restriction site.
Restriction sites for *Cla*I (*omp18*-1), *Eco*RI (*omp18*-2), *Bam*HI (*omp18*-3), and *Pst*I (*omp18*-4) are underlined letters.

H. pylori infection is dominated by the Th1-type immune response [13, 14]. IFN-γ is a characteristic Th1 response cytokine [15], and IFN-γ activity, mediated by a $CD4^+$ T-cell response to *H. pylori* infection, is essential for clearance [16, 17]. IFN-γ can induce nitric oxide (NO) production in macrophages by activating the transcription factor signal transducer and activator of transcription 1 (STAT1) [18], and NO is a key component of the innate immune system and an effective antimicrobial agent [19]. However, *H. pylori* can disrupt STAT1-mediated IFN-γ-induced signal transduction in epithelial cells [20]. Moreover, when *H. pylori* is exposed to IFN-γ; the main virulence factor cytotoxin-associated gene A (CagA) is downregulated [21], which is beneficial for persistent colonization [22]. Thus, *H. pylori* may actively respond to altered IFN-γ levels for persistent colonization.

Considering Omp18's importance to *H. pylori*, we constructed an *omp18* mutant strain to study this protein's contribution to *H. pylori*'s persistent colonization. Omp18 helped to inhibit IFN-γ-mediated *H. pylori* virulence factors and host immune response, thereby promoting colonization.

2. Materials and Methods

2.1. Bacteria and Culture Conditions. *H. pylori* 26695 and the SS1 strain were kindly provided by Dr. Zhang Jianzhong (Chinese Disease Control and Prevention Center). The bacteria were revived from frozen stocks and grown on Skirrow agar with 5% (v/v) sheep's blood under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C. The liquid culture media for *H. pylori* consisted of Brucella broth containing 10% fetal bovine serum for incubation in a microaerobic environment at 37°C on a shaker set at 120 rpm. For Δ*omp18* isogenic mutants, kanamycin (10 mg/mL, Sigma-Aldrich, St. Louis, MO) was supplemented in solid and liquid medium. We supplemented 10 mL aliquots of liquid overnight-cultured *H. pylori* 26695 and Δ*omp18* isogenic mutants with IFN-γ concentrations (Sigma-Aldrich) to examine the effects on *omp18, cagA, and napA*.

2.2. Construction of the omp18 Mutant Strain. The *omp18* mutant strains for *H. pylori* 26695 and SS1 were constructed as described [23]. Plasmids pILL570 and pUC18K2 were kindly provided by Dr. Agnes Labigne (Département de Microbiologie, Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris). The mutant strains were constructed as follows: fragment 1 containing the 5′ region of the *omp18* gene flanked by *Cla*I and *Eco*RI restriction sites was amplified by PCR with the first pair of primers (*omp18*-1 and *omp18*-2). Fragment 2, containing the 3′ region of *omp18* flanked by *Bam*HI and *Pst*I restriction sites, was amplified by PCR with the second pair of primers (*omp18*-3 and *omp18*-4). *H. pylori* 26695 and SS1 genomic DNA were used as the template, and the primers are in Table 1. Following PCR amplification, fragment 1 was digested by *Cla*I and *Eco*RI, and fragment 2 was digested by *Bam*HI and *Pst*I. We obtained the nonpolar kanamycin cassette from pUC18K2 by *Eco*RI and *Bam*HI digestion. Then, we ligated the resulting 3 fragments with the *Cla*I- and *Pst*I-digested vector pILL570 by T4 ligase (Fermentas), generating the plasmid pILL570-*omp18*, in which about a 200-bp *omp18* deletion was replaced by the kanamycin cassette. Finally, *H. pylori* 26695 and SS1 were electrotransformed with the plasmid pILL570-*omp18*, and we selected kanamycin-resistant (Kanr) recombinants. The *omp18* mutation in the Kanr recombinant was verified by PCR with the primers for *omp18*-1 and *omp18*.

2.3. Care and Infection of Experimental Mice, Gastric Tissue Preparation, and Assessment of Colonization and Histopathology. The use of animals in this experiment was approved by the Ethics Committee of Shandong University School of Medicine (number 001 in 2011 for Animal Ethics Approval), and all efforts were made to minimize the mice's suffering. We obtained 80 6-week-old, female, specific-pathogen-free (SPF) C57BL/6 mice from Zhejiang University that were bred at the Shandong University pathogen-free facility. We allowed the mice to adapt to their new environment for 2 weeks before experimentation. We housed them in individual ventilated cages and, at 8 weeks old, divided them into 2 groups (n = 40/group) for inoculation by oral gavage twice over 3 days with 100 μL *H. pylori* SS1 (~10^8 colony-forming units [cfu] mL^{-1}) or 100 μL *H. pylori* SS1 Omp18 isogenic mutant (~10^8 cfu mL^{-1}).

Five mice from each group were euthanized by CO_2 asphyxiation at 2, 4, 6, and 8 weeks after inoculation. We retrieved and cleaned their stomachs and removed the

forestomach. We opened the remaining piece containing the corpus and antrum along the lesser curvature and spread it out in the form of a trapeze. We then dissected the tissue longitudinally (i.e., from the forestomach/corpus junction down to the antrum/duodenum junction) into 3 equal, parallel pieces with nearly identical antral and corpus tissue proportions. To quantitatively assess *H. pylori* colonization, we transferred one section from each stomach to a tube containing Brucella broth and homogenized them. We placed serial dilutions on horse blood plates to determine bacterial loads. We homogenized one section from each stomach for ELISA, fixed the last section in 10% neutralized buffered formalin, and then embedded them in paraffin. Paraffin blocks were sectioned and stained with haematoxylin and eosin for histopathological evaluation or with Steiner's modified silver stain to grade bacterial load. Polymorphonuclear and mononuclear cells in the antrum and body were graded as described [24]: 0, none; 1, some infiltrates; 2, mild infiltrates (few aggregates in submucosa and mucosa); 3, moderate infiltrates (several aggregates in submucosa and mucosa); 4, marked infiltrates (many large aggregates in submucosa and mucosa); 5, nearly the entire mucosa contained a dense infiltrate; and 6, the entire mucosa contained a dense infiltrate.

2.4. Cell Culture and Infection Conditions. We maintained murine macrophage RAW264.7 cells (from BOSTER, Wuhan, China) in DMEM (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS). The human gastric epithelial cell line AGS, obtained from the cell repository for Academia Sinica (Shanghai), was grown in F12 (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS). All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37°C without antibiotics. Before infection, *H. pylori* 26695 bacteria was washed and resuspended in a total volume of 0.05 mL antibiotic-free tissue culture medium. The cell lines were infected with *H. pylori* 26695 at a multiplicity of infection (MOI) of 1 : 100 in antibiotic-free medium and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After infection, we rinsed the cell monolayer and added medium alone or medium containing IFN-γ (50 pg/mL) to the remaining adherent cells. We confirmed bacteria viability after the experiments concluded by visualizing their motility under light microscopy.

2.5. ELISA. AGS cells were seeded at 1×10^5/well in 24-well plates and incubated in 95% air and 5% CO_2 humidified air for 24 h at 37°C. After infection with *H. pylori* strain with or without IFN-γ (50 pg/mL) for 15 h, the supernatant was harvested and stored at −80°C after aspiration. We detected interleukin-8 (IL-8) secreted by AGS cells after stimulation for 15 h using a human IL-8 ELISA kit (NeoBioscience, China). The homogenized stomach was centrifuged at 20,000 g for 10 min at 4°C to precipitate the insoluble cellular debris, and the supernatant was stored at −80°C. IFN-γ, macrophage inflammatory protein 2 (MIP-2), and IL-12p70 protein levels in supernatants were assayed in duplicate with mouse ELISA kits specific for IFN-γ (eBioscience, San Diego, CA), MIP-2 (Cusabio Biotech, China), and IL-12p70 (NeoBioscience, China), respectively.

2.6. Quantitative Real-Time PCR. To determine the mRNA expressions of *Omp18*, *NapA*, and *CagA* with or without IFN-γ treatment, we inoculated *H. pylori* 26695 and 26695 Δ*omp18* isogenic mutants into liquid bacterial cultures with preliminary OD_{600} 0.05 and harvested them at different times. We extracted the total bacterial RNA using TRIzol (Invitrogen). Then, we obtained cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas). Quantitative RT-PCR amplification involved the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) with one cycle at 95°C for 10 s and 40 cycles at 95°C for 5 s and 60°C for 31 s. Each reaction mixture contained 10 μL SYBR Premic Ex TaqTM (Takara, Otsu, Shiga, Japan) and 0.4 μL ROX Reference Dye (Takara) added to each 20 μL PCR reaction mixture. We performed a melting curve analysis for each PCR reaction to ensure the amplified product's purity. The data were normalized to 16sRNA (*H. pylori*) expression in each sample, with 3 biological replicates performed. We calculated the relative gene expression using the $2^{-(\Delta\Delta Ct)}$ method. PCR amplification involved the primers listed in Table 1.

2.7. Western Blot Analysis. To detect the *NapA* and *CagA* protein expressions with or without IFN-γ treatment, we collected the liquid bacterial culture of *H. pylori* 26695 and 26695 Δ*omp18* isogenic mutants at 8 h. Bacterial cell lysates were prepared as described [25]. To determine phosphotyrosine STAT1 protein expression, we seeded macrophages at 2×10^6/well in flat-bottomed 6-well microplates for 24 h and then infected them with *H. pylori 26695* with or without IFN-γ (50 pg/mL) for 6 h. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in RIPA buffer (Beyotime Biotechnology, China) with 1% PMSF. We spun down the lysates and collected the supernatant. We used the Bradford method to determine protein concentration. Approximately 25–30 μg protein for each sample was loaded and separated by SDS-PAGE. They were then probed with specific antibodies against NapA (obtained from our lab) [25], STAT1 (Cell Signaling Technology, #5375), and CagA (Abcam, ab90490), followed by anti-mouse or rabbit horseradish peroxidase-conjugated IgG. They were then developed with the enhanced chemiluminescence method. β-Actin was a loading control, and each experiment was repeated 3 times.

2.8. Griess Assay of Nitrite Concentration. Macrophages were seeded at 2×10^6/well in six-well plates and incubated in 95% air and 5% CO_2 humidified air for 24 h at 37°C. After infection with *H. pylori* 26695 with or without IFN-γ (50 pg/mL) for 3 h, we collected the supernatant and stored it at −80°C. We estimated the nitrite content in infected macrophages and gastric tissue supernatant by a colorimetric assay based on the Griess reaction [26]. Briefly, 50 μL supernatant was mixed with 50 μL Griess reagent I and II (Beyotime, China). We measured the absorbance at 540 nm (Bio-Rad) and

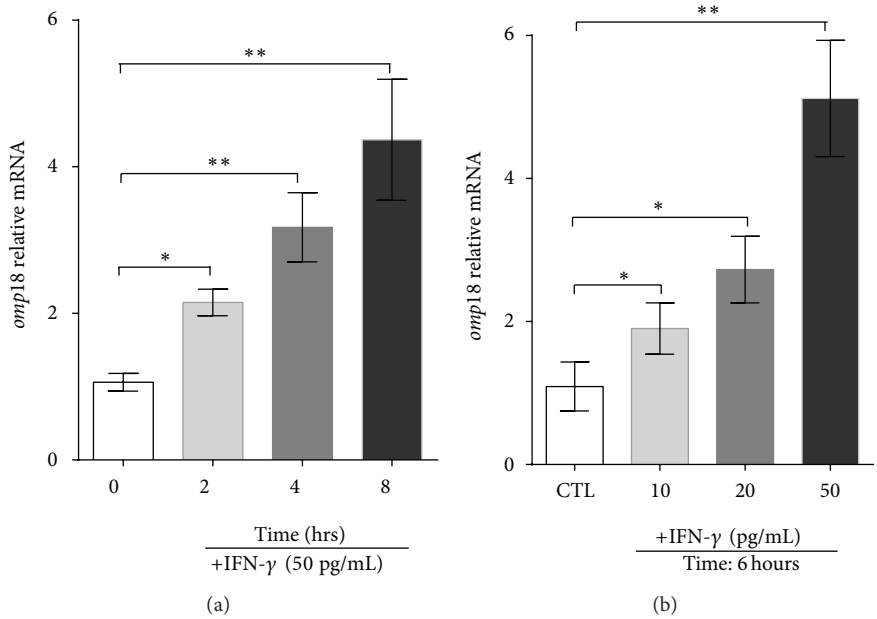

Figure 1: Interferon γ (IFN-γ) induces high *omp18* expression in *Helicobacter pylori 26695*. Quantitative RT-PCR analysis of *Omp18* mRNA expression with (a) exposure to different concentrations of IFN-γ and (b) exposure to IFN-γ (50 pg/mL) at different times. Quantitative RT-PCR data were standardized to that of 16s rRNA. Data are means ± SEM from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

determined nitrite concentration by extrapolation using a $NaNO_2$ standard curve (1–100 mM).

2.9. Survival of Wild-Type and Δomp18 H. pylori Exposed to Sodium Nitroprusside (SNP). This experiment was performed as we previously described [27], with some modifications. We added overnight-cultured *H. pylori* ($OD_{600} \approx 0.8$) to SNP (8 mM) and added *H. pylori* suspensions at 0, 2, 4, 6, and 12 h to SA plates with 5% (v/v) sheep's blood and then incubated them under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C for 3 to 4 days before viability assessment. To assess viability at each time, we determined the number of colony-forming units by plating serial dilutions of cultures in duplicate on Skirrow agar plates with 5% (v/v) sheep's blood. Each assay was replicated at least 3 times.

2.10. Confocal Microscopy. To determine shape and survival ability of *H. pylori* exposed to SNP, we stained *H. pylori* ($OD_{600} \approx 0.8$) using LIVE/DEAD BacLight Bacterial Viability kits (Molecular Probes, Invitrogen, USA) and then performed confocal microscopy as described [25]. SYTO-9 is a green fluorescent membrane-permeant dye that labels all bacteria by staining nucleic acid, whereas PI is a red-fluorescent membrane-impermeant dye that labels only bacteria with damaged membranes.

2.11. Intracellular Bacterial Survival Assay. Survival of wild-type and Δ*omp18 H. pylori* in macrophages was demonstrated as previously described [28], with some modifications. Cells were seeded at 5×10^5/well in 24-well plates and incubated in 37°C, 95% air, and 5% CO_2 humidified air conditions for 24 h. Macrophages were infected at 100 MOI with chilled wild-type or Δ*omp18 H. pylori* and then incubated in 37°C, 95% air, and 5% CO_2 humidified air conditions. After 1 h, infected monolayers were washed once with PBS and then incubated in 500 μL DMEM containing 10% (v/v) FBS and 100 μg gentamicin/mL for 1 hr to kill extracellular bacteria but not macrophages. Infected cells were then lysed at different times (2, 6, and 24 h). To lyse macrophage monolayers and release *H. pylori*, we added 500 μL sterile water to each well. Finally, we determined the number of viable bacteria in macrophage lysates by plating serial dilutions on solid plates.

2.12. Statistical Analysis. Data are presented as means ± SEM. Statistical significance was determined by unpaired Student's *t* test, and the *P* values were corrected by the Sidak-Bonferroni method for multiple comparisons. $P < 0.05$ was considered statistically significant. Results were analyzed using a Graphpad Prism (Graphpad Software Inc., La Jolla, CA, USA).

3. Results

3.1. IFN-γ Induced Higher Expression of H. pylori 26695 Omp18. Because IFN-γ is a predominant component of the anti-*H. pylori* protective immune response [16, 17] and because Omp18 induces IFN-γ production [12], we wondered whether IFN-γ could affect Omp18 expression, and our results displayed that IFN-γ induced higher expression of *H. pylori* 26695 Omp18 by dose (Figure 1(a)) and time (Figure 1(b)).

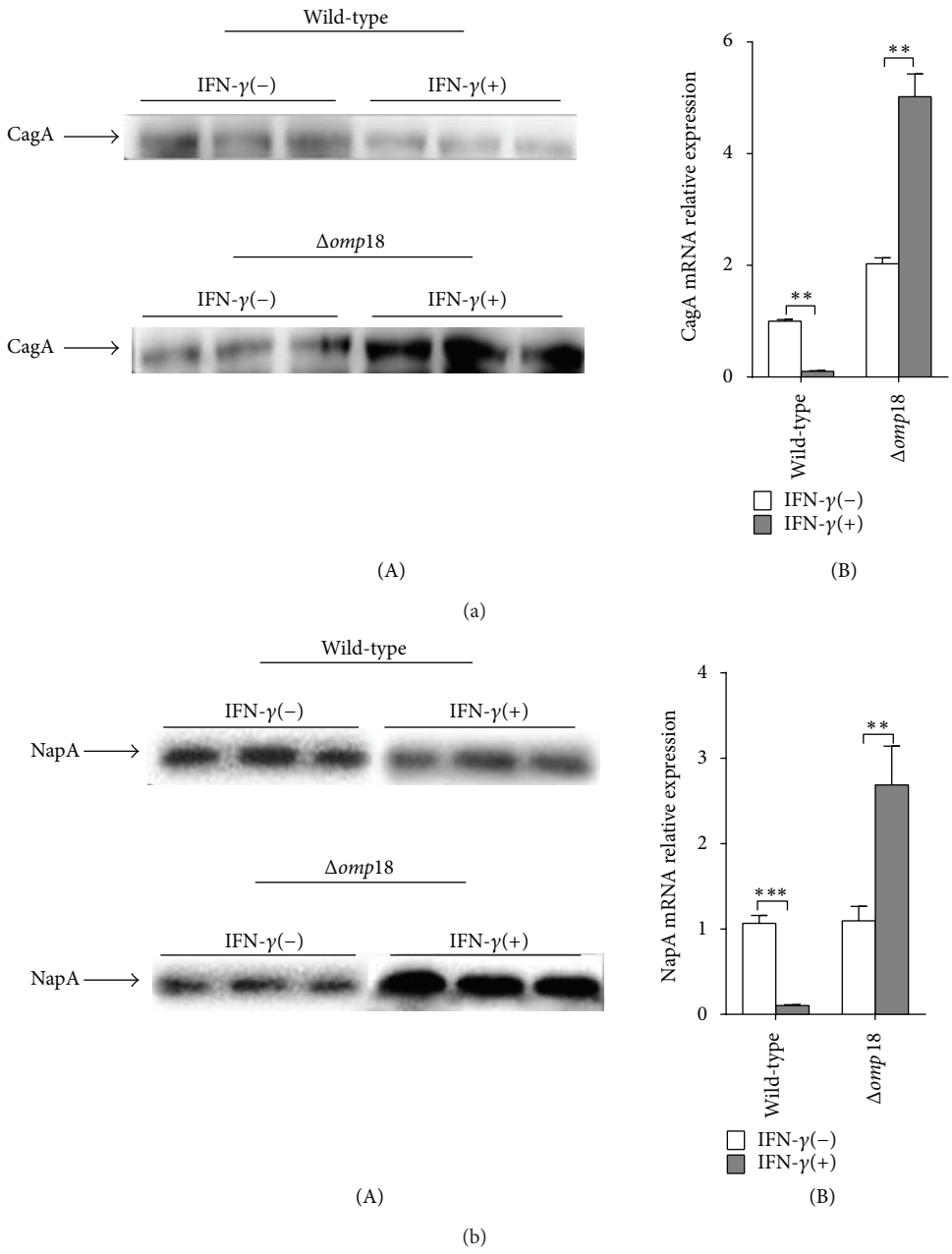

FIGURE 2: IFN-γ reduces wild-type *H. pylori* virulence factor expressions. Wild-type and Δ*omp18 H. pylori* were cultured to $OD_{600} \approx 0.8$ and then exposed or not to IFN-γ (50 pg/mL) for 6 h. Western blot and qRT-PCR analysis of protein (A) and mRNA (B) levels of CagA (a) and NapA (b). Quantitative RT-PCR data were standardized to that of 16s rRNA. Data are means ± SEM from 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. IFN-γ Reduces the Expressions of H. pylori 26695 Virulence Factors CagA and NapA. CagA and NapA are important virulence factors involved in the *H. pylori* pathogenic process [1, 2]. Therefore, we sought to determine their expression when *H. pylori* was exposed to IFN-γ. CagA and NapA were downregulated in wild-type *H. pylori* 26695 exposed to IFN-γ but could not be reduced in the Δ*omp18* strain with IFN-γ treatment (Figure 2).

3.3. Δomp18 H. pylori Shows Defective Colonization in Mice's Gastric Systems. Previous studies reported that several OMPs participate in *H. pylori* colonization [29]. To evaluate the difference in colonization efficiency between *H. pylori* SS1 and the Δ*omp18 H. pylori* SS1 strain, we inoculated mice with these two kinds of bacteria and euthanized them from 2 to 8 weeks after inoculation. Compared to *H. pylori* SS1, the Δ*omp18* strain showed gradually decreased colonization from

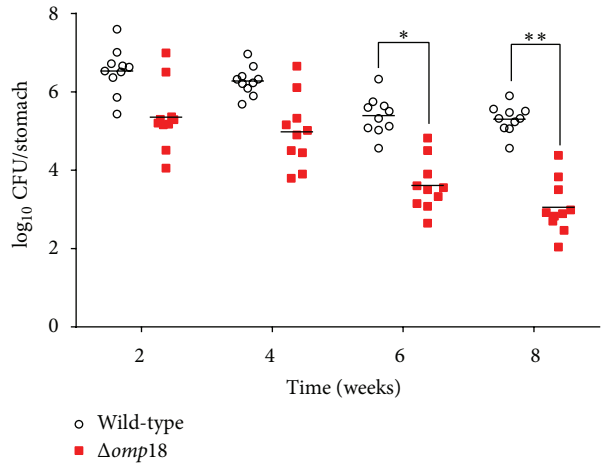

FIGURE 3: Δ*omp18 H. pylori* shows defective colonization in mice gastric systems. C57BL/6 mice (n = 10/group, 6–8 weeks old) were infected with *H. pylori* SS1 and Δ*omp18 H. pylori* SS1 (10^9/mL). $^*P < 0.05$, $^{**}P < 0.01$. CFU: colony formation unit.

weeks 2 to 8 and especially weeks 6 and 8 in C57BL/6 mice (Figure 3).

3.4. Omp18 Isogenic Mutant Strain's Effect on Infection Severity in Mice. Because the inflammation score is likely affected by *H. pylori* density, we measured the inflammation score in relation to *H. pylori* density (score/$\log_{10}$ cfu). Histologic changes in the gastric mucosa infected by *H. pylori* SS1 were very mild or undetected from 2 to 6 weeks after inoculation. However, gastric mucosa infected with the Omp18 isogenic mutant strain showed histologic changes at 8 weeks of inoculation, and the inflammation score was higher than that for wild-type-infected mice (Figures 4(a) and 4(b)). Δ*omp18*-infected mice showed increased neutrophil infiltration and severe gastric tissue damage, with the greatest damage at week 8 ($P = 0.0186$).

3.5. Δomp18 H. pylori Infection Induces More Cytokine and Chemokine Production. At 8 weeks after inoculation, compared with wild-type infection, Δ*omp18* infection increased MIP-2, IFN-γ, and IL-12p70 production in gastric tissues (Figures 5(a), 5(c), and 5(d)). More IL-8 expression was induced in human gastric cancer AGS cells infected with Δ*omp18 H. pylori* SSI with or without IFN-γ incubation (Figure 5(b)).

3.6. Δomp18 H. pylori Infection Induces More NO Production. Phosphorylated STAT1 is associated with NO production from macrophages and could be induced by IFN-γ [18]. With IFN-γ treatment, STAT1 phosphorylation in macrophages was inhibited with wild-type but not Δ*omp18 H. pylori* (Figure 6(a)). Also, Δ*omp18* infection increased NO production from macrophages with or without IFN-γ (Figure 6(b)). At 8 weeks after inoculation, compared with wild-type infection, Δ*omp18 H. pylori* infection induced more NO secretion (Figure 6(c)).

3.7. Omp18 Is Involved in H. pylori 26695 Survival with Oxidative Stress and Antiphagocytosis. H. pylori colonization is inevitably attacked by reactive oxygen species and eliminated by macrophages. With SNP as a simulation for NO, Δ*omp18* bacteria survival rates decreased sharply compared to wild-type *H. pylori* (Figure 7(a)), and most bacteria transformed from normal helical bacillary morphology to a coccoid morphology (Figure 7(c)). The Δ*omp18* strain also showed weakened survival rates in macrophages (Figure 7(b)).

4. Discussion

H. pylori can colonize the human stomach in childhood, and, without treatment, it can persist throughout life [30]. *H. pylori* can successfully cope with innate immune responses and continuous attack by the adaptive immune response [6] and has evolved complex mechanisms to escape immune reactions. For example, *H. pylori* lipopolysaccharide is not well recognized by Toll-like receptor 4 (TLR4) [31], and flagellin is a poor TLR5 stimulator [32]. Also, vacuolating cytotoxin A interferes with antigen processing in B cells [33]. In this report, we focused on *H. pylori* Omp18's role in avoiding IFN-γ signaling to achieve persistent colonization in an experimental mouse model. First, the expression of Omp18 was induced by IFN-γ *in vitro*, and the expression of virulence factors CagA and NapA was reduced in *H. pylori* 26695 with IFN-γ treatment but induced in the Δ*omp18* strain. Second, in C57BL/6 mice infected with *H. pylori* SS1 and the Δ*omp18* strain, the Δ*omp18* strain conferred defective colonization and activated a stronger inflammation response. Third, STAT1 phosphorylation is downregulated by the wild-type but not Δ*omp18* strain in IFN-γ-treated macrophages. Furthermore, Δ*omp18* strain survival rates are poor in macrophages compared to the wild-type strain. Our data showed that Omp18 in *H. pylori* may actively

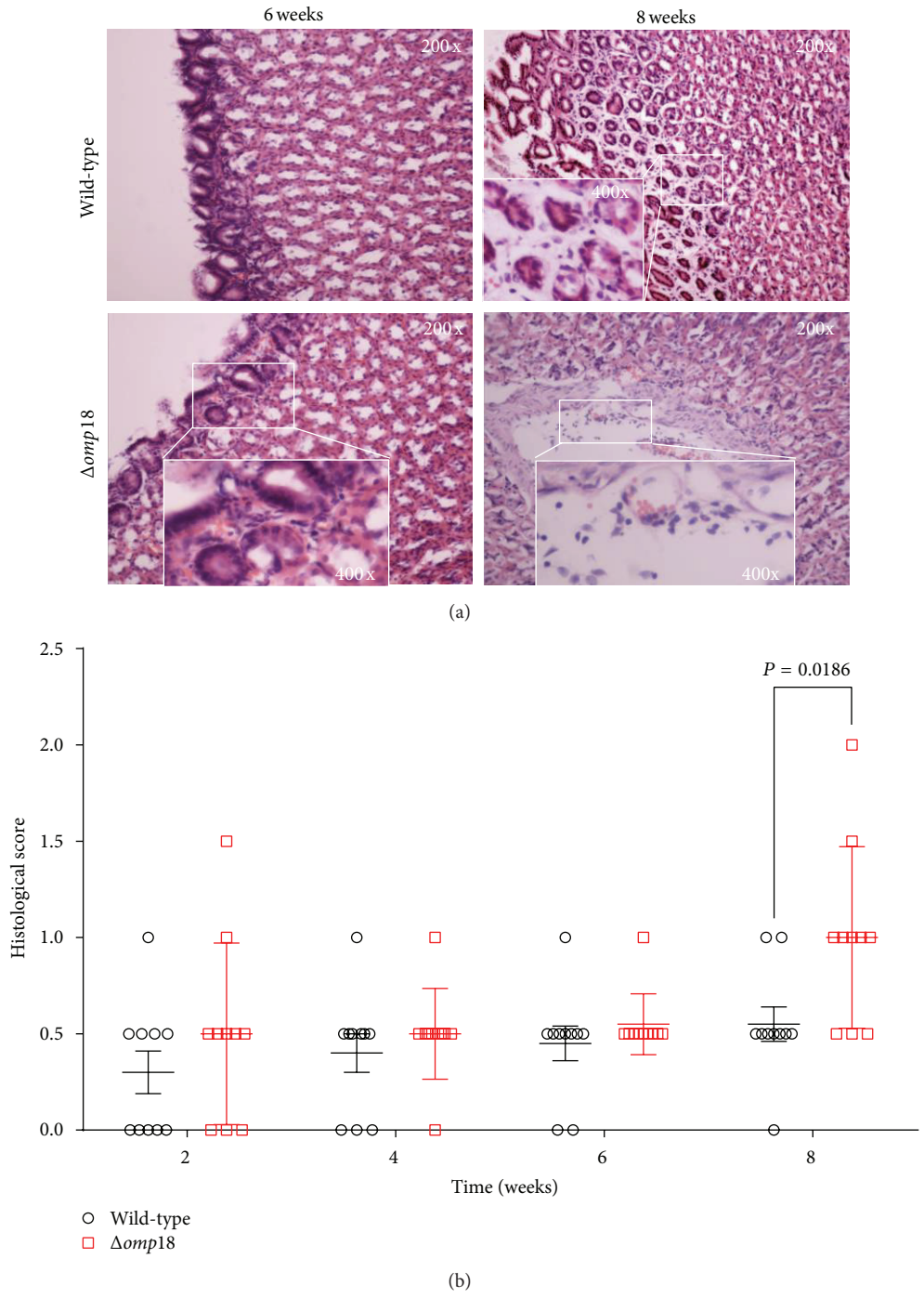

Figure 4: Δ*omp18 H. pylori* SS1 infection induces inflammation response in C57BL/6 mice. (a) Histology of antrum from C57BL/6 mice infected with *H. pylori* SS1 or Δ*omp18 H. pylori* SS1 at weeks 6 and 8. All sections were stained with hematoxylin and eosin. Magnification: 200x, 400x. Areas of lymphocytic inflammation were marked by rectangles. (b) Histopathology scores for gastric tissues from C57BL/6 mice (n = 10/group, 6–8 weeks old).

sense altered IFN-γ levels and respond to avoid the IFN-γ-mediated immune defense(s) involved in *H. pylori* colonization.

Although most *H. pylori* cells are found in the mucus layer covering the gastric epithelium [34], some bacteria are even found in deeper tissues or intracellular locations [35, 36]. IFN-γ has an important function for clearing *H. pylori* [16]. Outer membrane protein reportedly evades IFN-γ-mediated host immune defenses for several intracellular bacteria [9, 37]. Several OMPs are also known to be involved

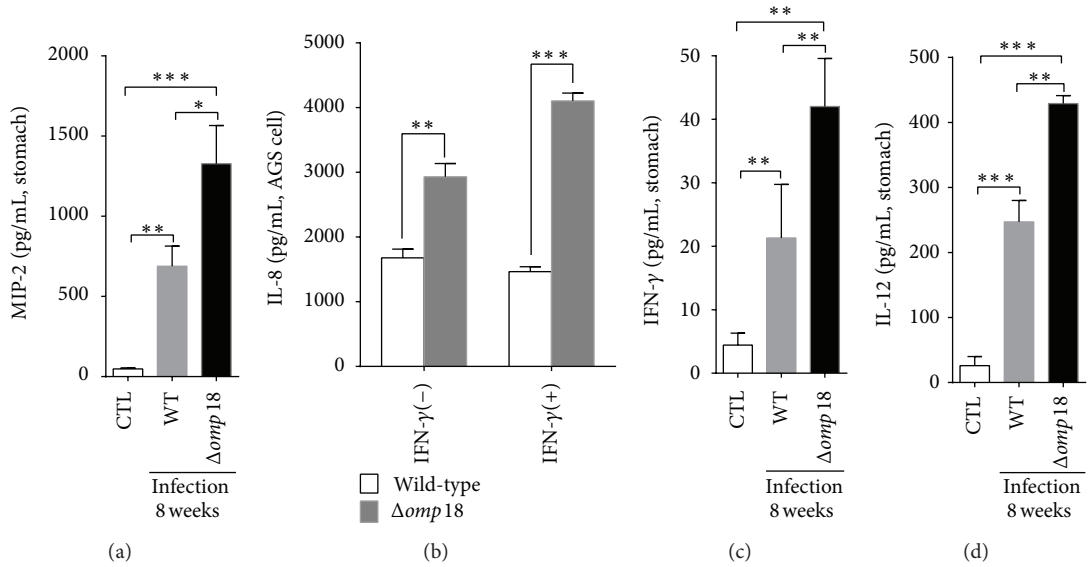

Figure 5: Δ*omp18 H. pylori* infection increased cytokine and chemokine production. (a), (c), and (d) Analysis of MIP-2, IFN-γ, and IL-12 protein expressions in gastric tissues from C57BL/6 mice infected by *H. pylori* SS1 or Δ*omp18 H. pylori* SS1 at week 8. (b) Analysis of IL-8 secretion from AGS cells infected by *H. pylori* 26695 or Δ*omp18 H. pylori 26695* with or without IFN-γ (50 pg/mL). Data are means ± SEM from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

in *H. pylori* adhesion, such as BabA, AlpA (HopC), AlpB (HopB), and HopZ [29, 38–40]. Omp18 is a peptidoglycan-associated lipoprotein (PAL) precursor, and the previous study saw it as a major antigenic molecule [11]. In this study, the increased Omp18 expression induced by IFN-γ suggested that Omp18 may be involved in *H. pylori*'s adaptation to the host immune response. Correspondingly, the next set of experiments confirmed the conclusion. Δ*omp18 H. pylori* conferred defective colonization in C57BL/6 mice's gastric systems with relatively severe inflammatory responses and increased production of the proinflammatory cytokine MIP-2 in mouse gastric tissues, as well as IL-8 in AGS cells. IL-8 (CXCL-8) and its functional murine counterpart MIP-2 induce neutrophil attraction, activation, and transendothelial migration. IL-8 is strongly induced in human gastric cancer cells, such as the commonly used AGS cell line on coculture with *H. pylori*, and this signal is generally assumed to initiate the acute (neutrophil-dominated) inflammation in the early stages of *H. pylori* infection [41]. More IFN-γ was also induced in C57BL/6 gastric tissues infected by the Δ*omp18* strain, combined with increased IL-12p70 expression, which is involved in the differentiation of naive T cells into Th1 cells [42]. A stronger Th1 immune response may have been induced in Δ*omp18*-rather than wild-type-infected C57BL/6 mice. The relatively strong inflammation and immune responses induced by Δ*omp18* infection in C57BL/6 mice's gastric systems may contribute to its defective colonization and attribute to the high expressions of virulence factors CagA and NapA in the Δ*omp18* strain induced by IFN-γ.

CagA and NapA, two important *H. pylori* virulence factors, can activate a strong innate and adaptive immune response in the host. CagA induces IL-8 expression in gastric epithelial cells [43], and NapA promotes Th1 immune responses [44]. To avoid being cleared by the host immune system, *H. pylori* must downregulate virulence genes' expressions. For example, after persistent colonization, some of the *H. pylori* population may delete their Cag genes [22]. In previous studies, we demonstrated that *H. pylori* CagA is suppressed by IFN-γ treatment [21]. In this study, NapA expression was also suppressed by IFN-γ. Conversely, these two virulence factors were upregulated in Δ*omp18 H. pylori* exposed to IFN-γ, so Δ*omp18 H. pylori* infection may induce a more serious immune response in the host than wild-type *H. pylori*. This represents a key factor leading to their weak colonization in C57BL/6 mice. Because IFN-γ can induce high *H. pylori* Omp18 expression and because virulence factors CagA and NapA were downregulated by IFN-γ in wild-type rather than Δ*omp18 H. pylori*, our results indicate that the expressions of virulence factors CagA and NapA of *H. pylori* exposed to IFN-γ may be modulated by Omp18. Similiarly, IFN-γ reportedly binds to an outer membrane protein in *Pseudomonas aeruginosa*, OprF, resulting in the expression of a quorum-sensing dependentvirulence determinant, the PA-I lectin [8], and sigma E activity is regulated by OMP expressions in *E. coli* [45].

NO is a key component of the innate immune system and an effective antimicrobial agent [19]. However, *H. pylori* has evolved countermeasures against it. For example, *H. pylori* can disrupt STAT1-mediated IFN-γ-induced signal transduction in epithelial cells [20], and macrophage Arg2 induced by *H. pylori* inhibits inducible NO synthase translation, NO production, and bacteria killing *in vitro* [46]. We found that wild-type but not Δ*omp18 H. pylori*

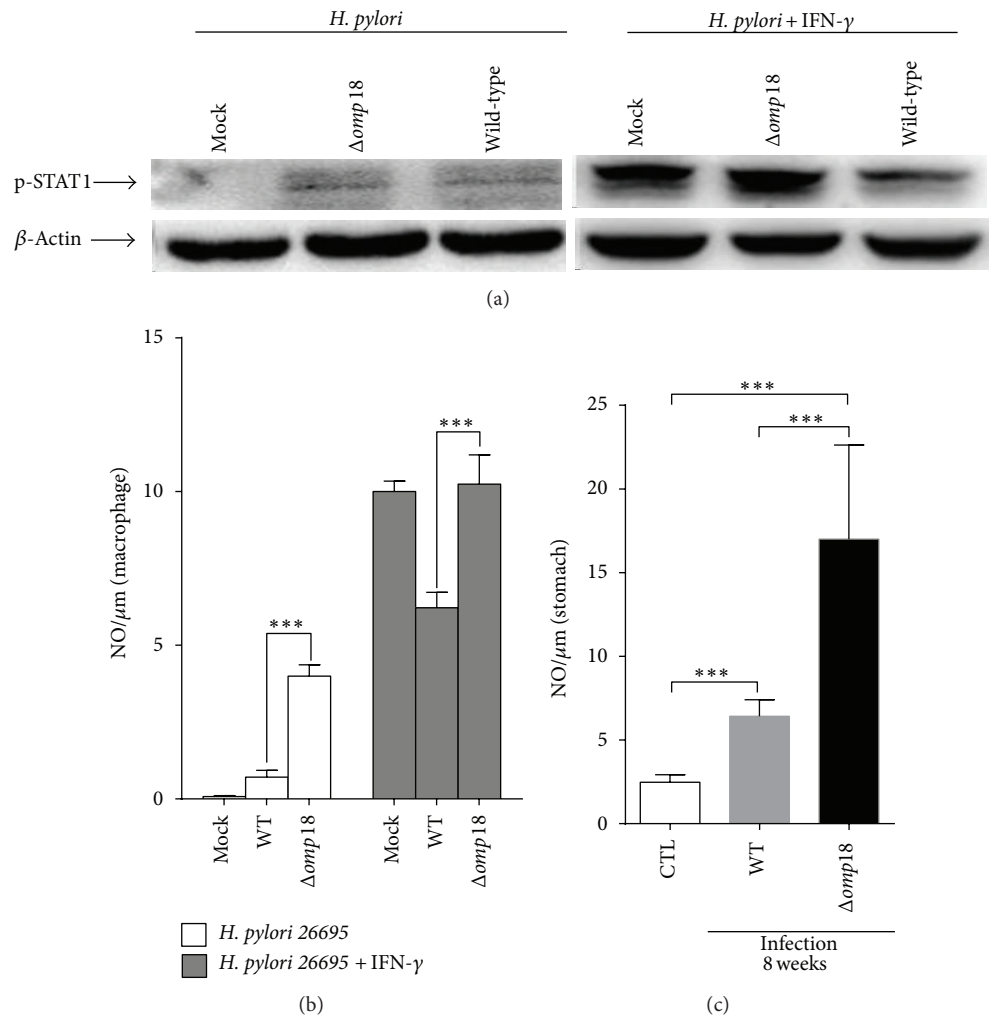

Figure 6: Δ*omp18 H. pylori* induces more nitric oxide (NO) production in macrophages and mice stomachs. The murine macrophage RAW264.7 was used in this experiment and was infected by *H. pylori* 26695 or Δ*omp18 H. pylori 26695* with or without IFN-γ (50 pg/mL). (a) Western blot analysis of phosphorylated signal transducer and regulator of transcription 1 (STAT1) protein expression in murine macrophage RAW264.7 cells. Actin was a loading control. (b) Analysis of NO secretion from murine macrophage RAW264.7 cells by ELISA. (c) Analysis of NO secretion in gastric tissues from C57BL/6 mice infected by *H. pylori* SS1 and Δ*omp18 H. pylori* SS1 at week 8 by ELISA. n = 10/group, 6–8 weeks old. $^{***}P < 0.01$. Data are means ± SEM from 3 independent experiments.

could disrupt STAT1-mediated IFN-γ-induced signal transduction in macrophages. Moreover, *omp18* mutant-infected macrophages produced greater NO activity in the supernatant, and more NO was produced from the gastric systems of C57BL/6 mice infected with the *omp18* mutant strain, which was attributed to the invalid STAT1 disruption and associated with stronger immune response activated by Δ*omp18* infection. Furthermore, Δ*omp18 H. pylori* survival rates were weakened compared to the wild-type with NO exposure. Most Δ*omp18* strains transformed their normal helical bacillary features to coccoid features. Omp18 has an important function in helping *H. pylori* avoid NO oxidative stress. Additionally, Δ*omp18 H. pylori* showed reduced survival rates in macrophages, another reason why Δ*omp18* could not effectively colonize C57BL/6 mice's gastric tissues.

5. Conclusions

Omp18 is involved in *H. pylori* persistent colonization. *H. pylori* may actively sense altered IFN-γ levels by Omp18 and respond by optimizing the virulence phenotype to avoid inducing a strong immune response, thereby guaranteeing persistent colonization. Omp18 is involved in *H. pylori* surviving NO oxidative stress and antiphagocytosis. Our studies provide new insight into *H. pylori*'s immune evasion.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

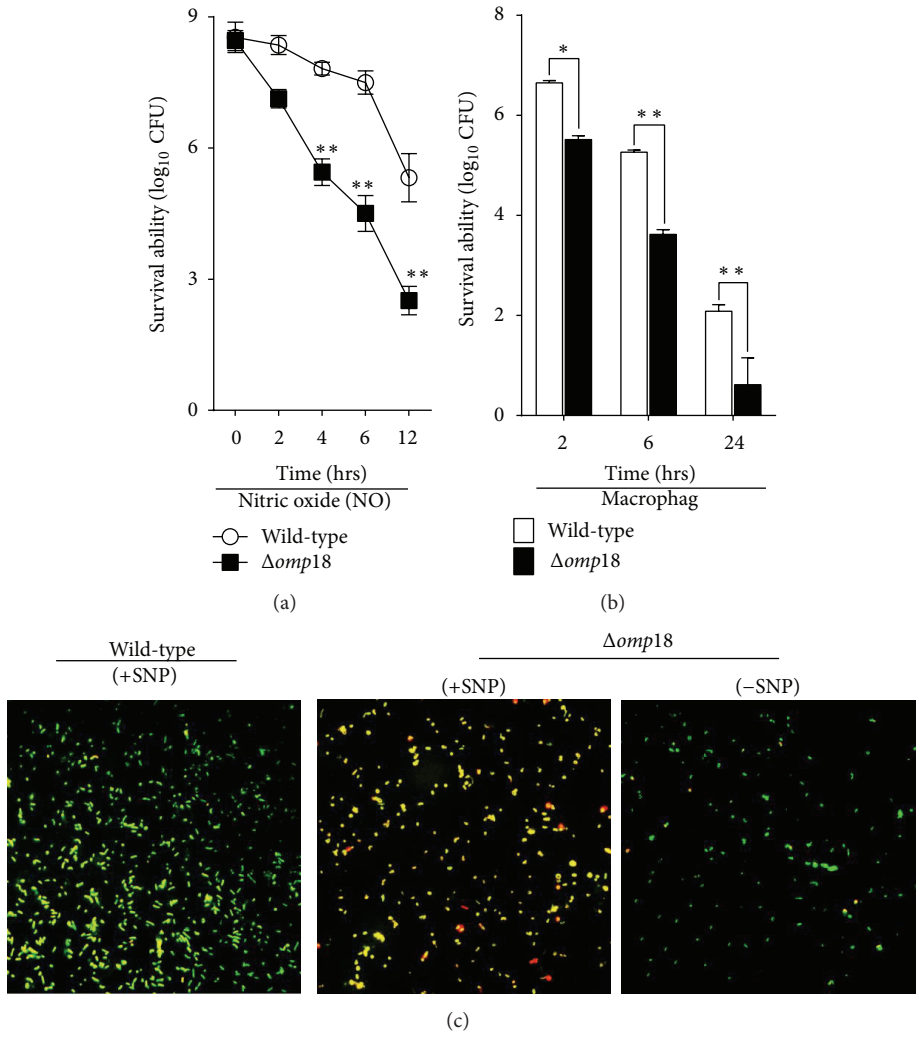

FIGURE 7: Omp18 is involved in *H. pylori* survival with NO oxidative stress and in macrophages. (a) Survival of wild-type and Δ*omp18 H. pylori* with exposure to NO oxidative stress (simulation with sodium nitroprusside, SNP). CFU: colony formation unit. (b) Survival of wild-type and Δ*omp18 H. pylori* in macrophages. Data are means ± SEM from 3 independent experiments. (c) SNP treatment for 6 h induces coccoid transformation and death of Δ*omp18* bacteria, and Δ*omp18 H. pylori* without SNP treatment was a negative control. Confocal microscopy of cells stained with membrane-permeant SYTO-9 (green) and membrane-impermeant PI (red). Data are representative of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Authors' Contribution

Yuqun Shan and Xingxiao Lu contributed equally to this work.

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Letter to the Editor

Comment on "*Helicobacter pylori* Outer Membrane Protein 18 (Hp1125) Is Involved in Persistent Colonization by Evading Interferon-γ Signaling"

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We read with interest the paper by Shan et al. [1] in a recent issue. It is an interesting paper concluding that *Helicobacter pylori* (*H. pylori*) omp18 is indirectly affecting long term bacterial colonization by successfully influencing IFN-γ-mediated immune response. Nevertheless, we found that some statements could not support the final conclusion. *H. pylori* infects the gastric mucosal layer of half of the human population worldwide and causes various digestive disorders such as chronic gastritis, gastric ulcer, duodenal ulcer, and gastric cancer [2]. To date, it has been established that such complex mechanism of bacterial interaction with human host can shape the successful and persistent colonization of *H. pylori* [3, 4]. Undoubtedly, understanding the mechanisms of immune evasion could provide new options for better management of infection. To our knowledge, the host immune response to the infection is ineffective; accordingly, the bacterium persists and remains for decades. In brief, Shan et al. [1] reported the *oipA* as a critical factor affecting bacterial colonization. However, we know that, in chronic process of colonization adopted by *H. pylori*, the connection of a unique factor to the drive of the final pattern of this phenomenon could be too speculative. Despite the interesting report of Shan et al. [1], we may hypothesize more factors involved in *H. pylori* colonization. Surprisingly, *H. pylori* colonization is not comparable with that of other pathogens [5]. Indeed, different mechanisms are contributing to this mysterious and long term biologic function. Conclusively, more studies are necessary to draw a direct and final conclusion on "the mystery" of *H. pylori* colonization.

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Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Research Article

Nordihydroguaiaretic Acid Disrupts the Antioxidant Ability of *Helicobacter pylori* through the Repression of SodB Activity *In Vitro*

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Iron-cofactored superoxide dismutase (SodB) of *Helicobacter pylori* plays an indispensable role in the bacterium's colonization of the stomach. Previously, we demonstrated that FecA1, a Fe^{3+}-dicitrate transporter homolog, contributes to SodB activation by supplying ferrous iron (Fe^{2+}) to SodB, and *fecA1*-deletion mutant strains have reduced gastric mucosal-colonization ability in Mongolian gerbils, suggesting that FecA1 is a possible target for the development of a novel eradication therapy. This study aimed to identify novel FecA1-binding compounds *in silico* and then examined the effect of a predicted FecA1-binding compound on *H. pylori* SodB activity *in vitro*. Specifically, we demonstrated that nordihydroguaiaretic acid (NDGA) is a predicted FecA1-binding compound. NDGA reduced intracellular Fe^{2+} levels in *H. pylori* and reduced SodB activity. Additionally, NDGA increased H_2O_2 sensitivity of *H. pylori* and increased the metronidazole (Mtz) sensitivity. The present study demonstrated that NDGA repressed SodB activity associated with the gastric mucosal-colonization via inhibition of intracellular Fe^{2+} uptake by FecA1, suggesting that NDGA might be effective for the development of a novel eradication therapy.

1. Introduction

Helicobacter pylori infection is one of the most common infectious diseases worldwide. Chronic infection of *H. pylori* plays a causative role in gastritis, peptic ulcer, and gastric carcinomas [1]. Excessive reactive oxygen species (ROS) are generated by neutrophils and macrophages in *H. pylori* colonized human stomachs [2]. Although the generation of ROS is an important host immune response against persistent pathogens, *H. pylori* counteracts oxidative stress using a variety of enzymes to establish a chronic infection [3, 4]. Therefore, persistent and excessive ROS induces oxidative stress injuries to the gastric mucosa.

The *H. pylori* genome encodes iron-cofactored superoxide dismutase (SodB) [5, 6]. Mutants with a deletion of *sodB* show a reduced gastric mucosal-colonization ability in mice, suggesting that SodB plays an indispensable role in the colonization of host gastric mucosa owing to its defensive role against excessive ROS [7]. Ferrous ion (Fe^{2+}) is required for the activation of SodB. We recently demonstrated that the deletion mutant of the *fecA1* gene, a Fe^{3+}-dicitrate transporter homolog, shows reduced SodB activity [8]. Our previous

findings suggested that the Fe^{2+}-supply system associated with SodB activation proceeds as follows: Fe^{3+}-dicitrate transport is enhanced by FecA1, and then intracellular ferric ion (Fe^{3+}) is reduced to Fe^{2+} by Fe^{3+}-reductase (ribBA), providing Fe^{2+} to SodB [8, 9]. In addition, *fecA1*-deletion mutant strains show reduced gastric mucosal-colonization ability in Mongolian gerbils [8]. These results indicated that *fecA1*-deletion mutant strains were eradicated by excessive ROS in host gastric mucosa via disruption of SodB activity. In fact, FecA1 associated with SodB activation is an important determinant of the establishment of chronic infections.

Recently, we demonstrated that SodB overexpression is caused by amino acid mutations of ferric uptake regulator (Fur), which is associated with the development of metronidazole (Mtz) resistance [10]. The deletion of *fecA1* in Mtz-resistant strains (KS0048 and KS0145) results in increased Mtz sensitivity, suggesting that FecA1 is also associated with the development of Mtz resistance [8].

Our previous results indicated that FecA1 is a possible target for the development of a selective *H. pylori* eradication therapy mediated by excessive ROS accumulation in the human stomach and for avoiding the development of Mtz resistance associated with SodB overexpression. In the present study, we identified a compound that demonstrated an inhibitory effect on SodB activity via the inhibition of FecA1.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions. *H. pylori* strains ATCC700392, KS0048, and KS0145 were used in this study. The KS strains were clinically isolated and maintained at −80°C in Brucella broth (Becton–Dickinson, Franklin Lakes, NJ, USA) containing 25% (vol/vol) glycerol. The KS0048 and KS0145 strains were used as the Mtz-resistant strains with SodB overexpression owing to Fur amino acid mutations. ATCC700392 pHel3::*sodB* strain was used as the SodB-overexpressing strain of *H. pylori* ATCC700392, and ATCC700392 pHel3 *ctrl* strain was used as the control strain of ATCC700392 pHel3::*sodB* strain [10]. It was confirmed that the SodB activity in ATCC700392 pHel3::*sodB* was higher as compared with that of ATCC700392 pHel3 *ctrl* strain [10]. ATCC700392Δ*fecA1*, KS0048Δ*fecA1*, and KS0145Δ*fecA1* strains were used as *fecA1*-deletion mutant strains of *H. pylori* ATCC700392, KS0048, and KS0145, respectively [8]. It was confirmed that the SodB activities in *fecA1*-deletion mutant strains were significantly decreased as compared with wild-type strains [8]. These strains were maintained at −80°C in Brucella broth (Becton–Dickinson, Franklin Lakes, NJ, USA) containing 25% (vol/vol) glycerol. The bacteria were cultured on Brucella agar containing 7% sheep blood and 7% fetal bovine serum for 2 days at 37°C under microaerobic conditions maintained with AnaeroPack MicroAero (Mitsubishi Gas, Tokyo, Japan).

2.2. In Silico Screening of Chemical Compounds with Binding Affinity to FecA1. The FecA1 amino acid sequence of *H. pylori* (UniProt ID: O25395) was inserted into the COPICAT (Comprehensive Predictor of Interactions between Chemical Compounds and Target Proteins) program, which is an *in silico* screening system to predict the comprehensive interactions between target proteins and chemical compounds [11]. A chemical compound that is likely to interact with FecA1 was predicted.

2.3. Measurement of Intracellular Fe^{2+} Levels. The bacteria normalized to an OD_{600} of 0.5 were incubated with or without nordihydroguaiaretic acid (NDGA) for 3 hr. The bacteria were washed three times with HBSS and then 10 μM RhoNox-1 (from 1 mM stock solution in DMSO) was added [12]. After incubation for 1 hr at 37°C, the bacteria were washed with HBSS and then normalized to an OD_{600} of 0.1. Fluorescence intensity was measured using 560 nm excitation and 595 nm emission.

2.4. Measurement of SOD Activity. The bacteria normalized to an OD_{600} of 0.5 were incubated with or without NDGA for 3 hr. The bacteria were centrifuged, were washed three times with PBS, and then were sonicated (1.5 min at 25% power). The bacterial lysates were centrifuged, and the SOD activity was measured using the SOD Assay Kit (Dojindo, Kumamoto, Japan) following the manufacturer's guidelines [8]. Protein concentrations of the bacterial lysates were measured by BCA assays (Thermo Scientific, Rockford, IL, USA).

2.5. Measurement of the MICs of Mtz. The bacteria normalized to an OD_{600} of 0.5 were incubated with or without 50 μM NDGA for 3 hr. The bacteria (at an OD_{600} of 0.1) were inoculated on an agar plate containing Mtz in serial twofold dilutions (0.5–128 μg/mL). All plates were incubated at 37°C under microaerobic conditions, and the minimum inhibitory concentration (MIC) values were determined [8].

2.6. Disk Assays for H_2O_2 Susceptibility. The bacteria normalized to an OD_{600} of 0.5 were incubated with or without 50 μM NDGA for 3 hr. The bacteria were centrifuged and washed three times with PBS. The bacteria, normalized to an OD_{600} of 0.1, were plated for confluent growth on Nissui Helicobacter agar (Nissui, Tokyo, Japan). Sterile 5 mm disks saturated with 10 μL of 5 M hydrogen peroxide (H_2O_2) were placed on the plates. After 3 days, the zone of inhibition around the disks was measured [8].

2.7. Statistical Analysis. All values were expressed as means ± SD. The statistical significance of differences between three or more groups was evaluated using the Tukey test. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Screening of Chemical Compounds with Binding Affinity to FecA1 of H. pylori. To search for chemical compounds with binding affinity to FecA1 of *H. pylori*, we used the COPICAT web-based software system [11]. Using the FecA1

protein (UniProt ID: O25395) as the COPICAT input, nordihydroguaiaretic acid (NDGA), sevoflurane, and enflurane were a predicted FecA1-binding compound. Considering the clinical application and toxicity of these compounds, sevoflurane and enflurane were excluded. We focused on the effect of NDGA on the intracellular Fe^{2+} levels and SodB activity in *H. pylori* (Figure 1).

3.2. Effect of NDGA on Intracellular Fe^{2+} Levels and SodB Activity in H. pylori. We examined the effect of NDGA on intracellular Fe^{2+} levels using a turn-on fluorescent probe, RhoNox-1, for the selective detection of Fe^{2+} [12]. The intracellular Fe^{2+} levels were significantly lower in NDGA-treated strains than in control strains, and this effect was dose-dependent (Figure 2(a)). Previously, we demonstrated that *fecA1*-deletion mutant strains show lower SodB activity than the wild-type strain [8]. Therefore, we investigated the effect of NDGA on SodB activity. The SodB activity was significantly decreased after exposure to NDGA in a dose-dependent manner (Figure 2(b)). Treatment with 50 μM NDGA decreased the SodB activity to the same level observed in the *fecA1*-deletion mutant strains (Δ*fecA1*) (Figure 2(b)). To evaluate the effect of NDGA on the bacterial viability, we estimated growth rates after NDGA exposure by measuring optical density (OD_{600} nm). Although the bacterial growth was suppressed after exposure to 100 μM NDGA, it was hardly influenced by exposure to 10 and 50 μM NDGA (Figure 2(c)). Similarly, deletion of the *fecA1* gene had little influence on the bacterial growth (Figure 2(c)). These results suggested that 50 μM NDGA was a potent selective inhibitor of SodB activity in *H. pylori* via the repression of intracellular Fe^{2+} levels, without bacteriostatic activity.

Recently, we demonstrated that SodB activities of Mtz-resistant strains (KS0048 and KS0145) increase due to Fur amino acid mutations [10]. Therefore, we investigated whether NDGA repressed the enhanced SodB activities of KS0048 and KS0145. The intracellular Fe^{2+} levels of the SodB-overexpressing strain (ATCC700392 pHel3::*sodB*) as well as that of the control strain (ATCC700392 pHel3 *ctrl*) decreased significantly for exposure to 50 μM NDGA (Figure 3(a)). Similarly, the intracellular Fe^{2+} levels of KS0048 and KS0145 decreased significantly to levels comparable to the *fecA1*-deletion mutant strains (KS0048Δ*fecA1* and KS0145Δ*fecA1*, resp.) (Figure 3(a)). The SodB activities of these strains decreased significantly to the same levels as those of *fecA1*-deletion mutant strains (KS0048Δ*fecA1* and KS0145Δ*fecA1*, resp.) (Figure 3(b)). These results suggested that NDGA also repressed the enhanced SodB activities of KS0048 and KS0145.

3.3. Effect of NDGA on Mtz Susceptibility of H. pylori. Previously, we demonstrated that *fecA1*-deletion mutants of KS0048 and KS0145 have decreased MICs of Mtz [8]. Therefore, we examined whether Mtz resistance of KS0048 and KS0145 was reversed by NDGA treatment. The MICs of Mtz for KS0048 and KS0145 decreased from 32 to 8 and from 128 to 16 μg/mL, respectively (Table 1). In particular, the Mtz resistance of ATCC700392 pHel3::*sodB* was completely reversed by treatment with 50 μM NDGA (MIC < 8 μg/mL) (Table 1).

Table 1: Effect of NDGA on MICs (μg/mL) of Mtz-resistant strains.

Strain numbers	Without NDGA	With 50 μM NDGA
ATCC700392 pHel3::*sodB*	32	4
KS0048	32	8
KS0145	128	16

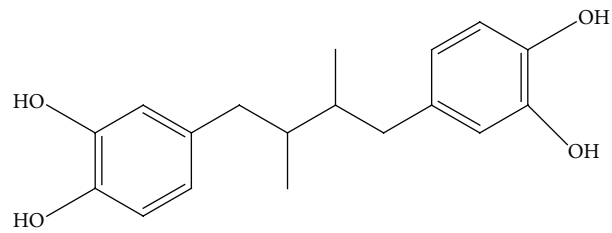

Figure 1: Chemical structure of nordihydroguaiaretic acid (NDGA). Other name of NDGA is Masoprocol. Chemical structural formula of NDGA was described using the ChemBioDraw13.0.

3.4. Effect of NDGA on H. pylori H_2O_2 Sensitivity. To investigate whether NDGA repressed the antioxidant ability of *H. pylori*, we examined the H_2O_2 sensitivity of *H. pylori* by disk assays. The H_2O_2 sensitivity of *H. pylori* was significantly increased by NDGA treatment in a dose-dependent manner (Figures 4(a) and 4(b)). On the other hand, enhanced H_2O_2 sensitivity of *fecA1*-deletion mutant strains (ATCC700392Δ*fecA1*) was not affected by NDGA treatment (Figure 4(b)). Similarly, the H_2O_2 sensitivity of SodB-overexpressing strains (ATCC700392 pHel3::*sodB*, KS0048, and KS0145) was significantly increased after exposure to NDGA in a dose-dependent manner (Figure 5). Enhanced H_2O_2 sensitivity by deletion of *fecA1* genes (KS0048Δ*fecA1* and KS0145Δ*fecA1*) was not affected by NDGA treatment (Figure 5). From these results, it was thought that NDGA disrupted the SodB-dependent antioxidant ability of *H. pylori* via FecA1 inhibition.

4. Discussion

Previously, we demonstrated that *fecA1*-deletion mutant strains show reduced host colonization owing to the inactivation of SodB, suggesting that *H. pylori* was eradicated by excessive ROS mediated by host immune responses [8]. In this *in vitro* study, we showed that NDGA treatment inhibited the SodB activity and increased the H_2O_2 sensitivity to the same levels observed in *fecA1*-deletion mutant strains. Therefore, it was expected that NDGA might reduce the host-colonization ability of *H. pylori*.

Although the growth of *H. pylori* was suppressed under 100 μM NDGA exposure, it was minimally influenced under 50 μM NDGA, suggesting that high concentrations of NDGA showed bacteriostatic activity (Figure 2(c)). Since the lower intracellular Fe^{2+} levels under 100 μM NDGA exposure were

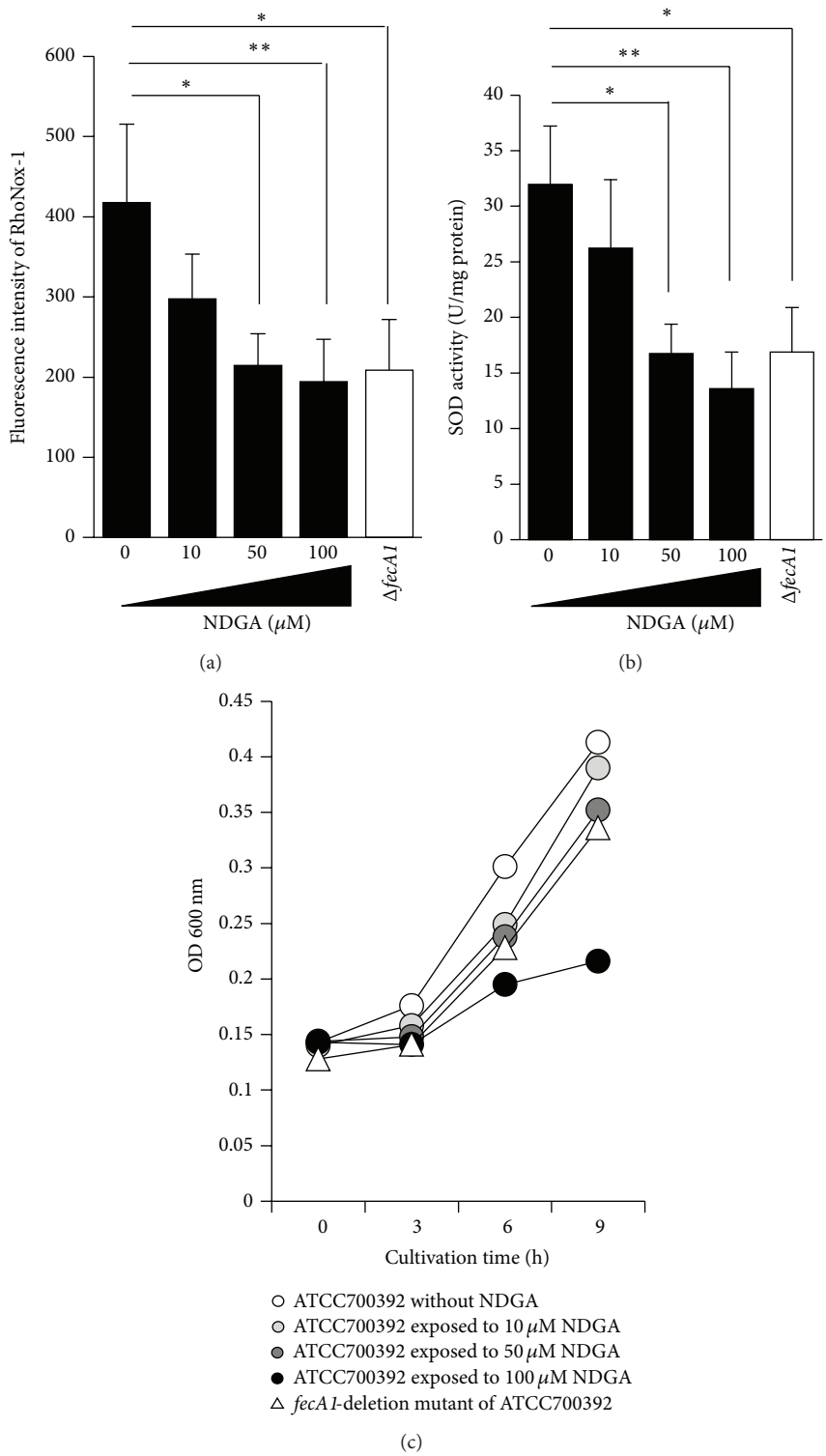

Figure 2: Effect of NDGA on intracellular Fe^{2+} levels, SOD activity, and bacterial growth. (a) Intracellular Fe^{2+} levels in ATCC700392 exposed to NDGA and *fecA1*-deletion mutant of ATCC700392 (Δ*fecA1*) were measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test). (b) The SodB activities in ATCC700392 exposed to NDGA and *fecA1*-deletion mutant of ATCC700392 (Δ*fecA1*) were measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test). (c) The growth rates of ATCC700392 without NDGA (white circle), ATCC700392 exposed to 10 μM NDGA (gray circle), ATCC700392 exposed to 50 μM NDGA (deep-gray circle), ATCC700392 exposed to 100 μM NDGA (black circle), and *fecA1*-deletion mutant of ATCC700392 (white triangle) were measured by measuring optical density (OD 600 nm).

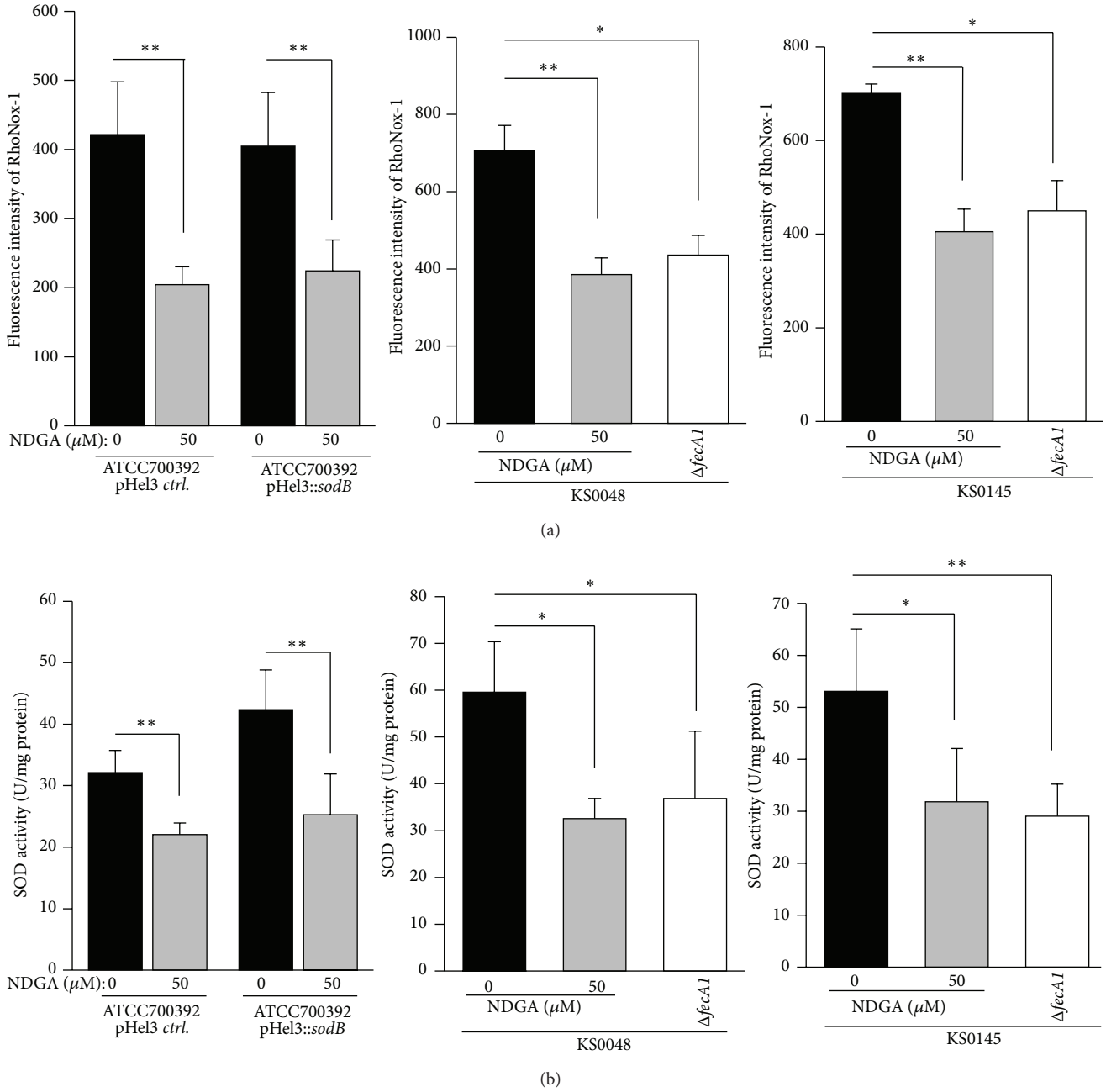

FIGURE 3: The intracellular Fe^{2+} levels and the SOD activities of Mtz-resistant strains under exposure to 50 μM NDGA. (a) The intracellular Fe^{2+} levels of control strain (ATCC700392 pHel3 *ctrl.*), SodB-overexpressing strain (ATCC700392 pHel3::*sodB*), KS0048, *fecA1*-deletion mutant of KS0048 (KS0048Δ*fecA1*), KS0145, and *fecA1*-deletion mutant of KS0145 (KS0145Δ*fecA1*) were measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test). (b) The SOD activities of control strain (ATCC700392 pHel3 *ctrl.*), SodB-overexpressing strain (ATCC700392 pHel3::*sodB*), KS0048, *fecA1*-deletion mutant of KS0048 (KS0048Δ*fecA1*), KS0145, and *fecA1*-deletion mutant of KS0145 (KS0145Δ*fecA1*) were measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test).

the same as those observed for 50 μM NDGA exposure, the bacteriostatic activity and the repression of intracellular Fe^{2+} levels are caused by different mechanisms.

The present study showed that NDGA repressed the SodB activity without bactericidal activity by itself (Figure 2). As a result, NDGA enhanced the bactericidal activity by ROS exposure (Figure 4). These results suggested that NDGA is effective for selective eradication therapy of pathogenic bacteria of inducing inflammatory response without affecting intestinal microbiota that does not induce an inflammatory response. Therefore, *H. pylori* eradication therapy with NDGA is expected to contribute to the development of

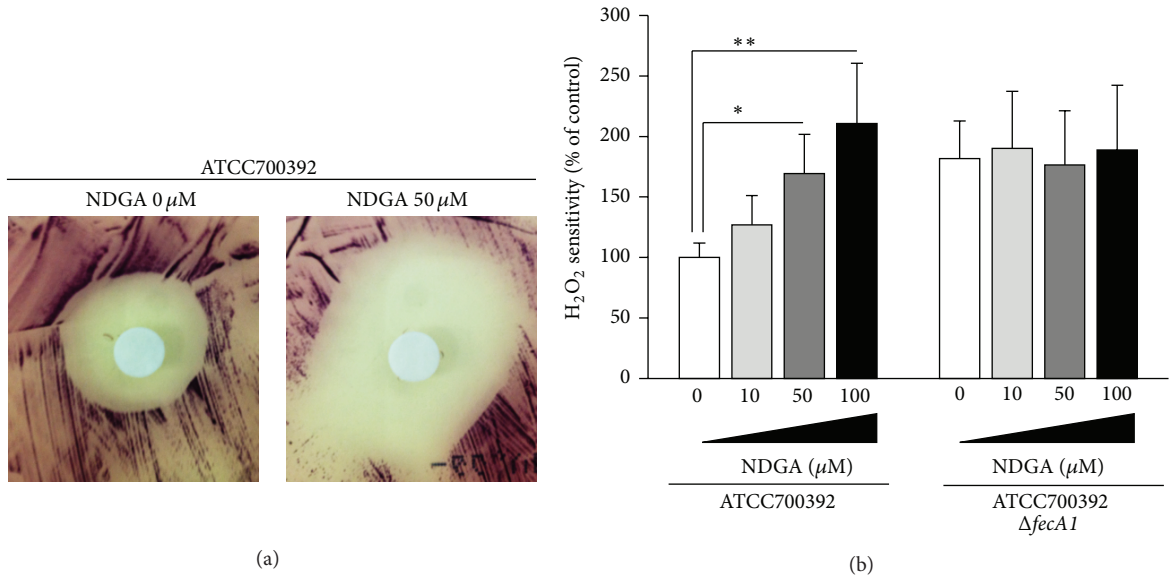

Figure 4: Effect of NDGA on H_2O_2 sensitivity. (a) Representative disk assays for H_2O_2 sensitivity of ATCC700392 exposed to 50 μM NDGA. (b) The H_2O_2sensitivity of ATCC700392 and *fecA1*-deletion mutant of ATCC700392 (ATCC700392Δ*fecA1*) under exposure to NDGA was measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test).

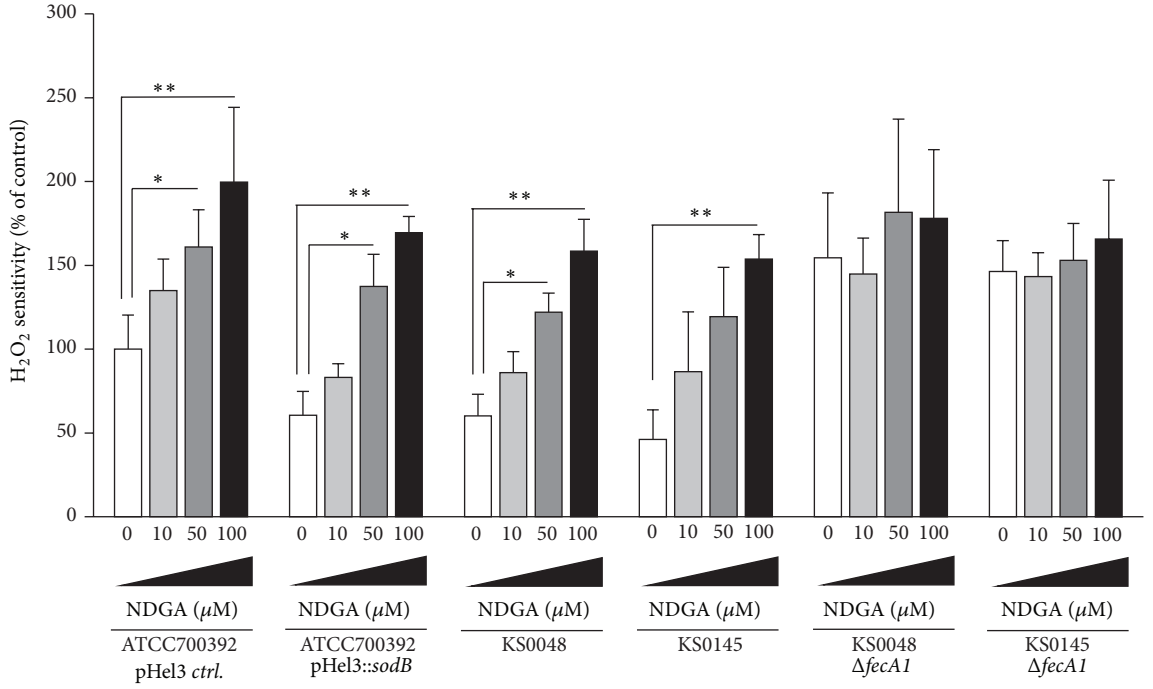

Figure 5: The H_2O_2 sensitivity of Mtz-resistant strains under exposure to NDGA. The H_2O_2sensitivities of control strain (ATCC700392 pHel3 *ctrl.*), SodB-overexpressing strain (ATCC700392 pHel3::*sodB*), KS0048, KS0145, *fecA1*-deletion mutant of KS0048 (KS0048Δ*fecA1*), and *fecA1*-deletion mutant of KS0145 (KS0145Δ*fecA1*) were measured by the method described under Materials and Methods. Results are means ± SD of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Tukey test).

selective eradication therapy of *H. pylori* by excessive ROS in host gastric mucosa without affecting intestinal microbiota. *H. pylori* eradication therapy often induces side effects such as diarrhea and soft stools. It was reported that 10–30% of ulcer patients receiving eradication therapy experienced diarrhea and soft stools [13, 14]. The reason for these side effects was assumed to be a disturbance in the composition of intestinal microbiota by antibiotics. Therefore, *H. pylori* eradication therapy with NDGA is expected to be effective in reducing the side effects such as diarrhea and soft stools.

Recently, the prevalence of Mtz resistance has increased in Asia and Europe [15–17]. It is suggested that the widespread use of Mtz may contribute to the increasing prevalence of Mtz resistance [16, 18]. Mtz is a prodrug, and its bactericidal activity is dependent on the generation of superoxide radicals mediated by the reduction of its nitro group to nitro anion radicals [19, 20]. Therefore, enhanced SodB activity is associated with the development of Mtz resistance [10]. In the present study, it was shown that NDGA repressed the SodB activity and then increased Mtz sensitivity in Mtz-resistant strains with SodB overexpression (Figure 3(b) and Table 1). From these results, it is expected that Mtz and NDGA combination therapy is effective for eradicating Mtz-resistant strains and preventing the development of Mtz resistance.

NDGA is the main metabolite of the creosote bush, *Larrea tridentata*, which is commonly known as chaparral or greasewood in the United States and as gobernadora or hediondilla in Mexico [21]. The creosote bush has been widely used in Mexican traditional herbal medicine [22]. It is known that NDGA is a potent scavenger of ROS such as peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\bullet}OH$), superoxide anion ($O_2^{-\bullet}$), H_2O_2, and hypochlorous acid (HOCl) [23]. Because the H_2O_2 sensitivity of *H. pylori* exposed to NDGA was significantly increased, it was thought that the SodB-inactivation caused by NDGA is independent of its ROS-scavenging activity. The present study is the first demonstration that NDGA suppresses the SodB activity of *H. pylori* via repression of intracellular Fe^{2+} by FecA1 *in vitro*. Recent studies have indicated the inhibitory effect of NDGA against *N*-methyl-*N*-nitrosourea-initiated and *H. pylori*-promoted gastric carcinogenesis in Mongolian gerbils [24]. This inhibitory effect of NDGA might be associated with antioxidant activity and inhibitory effects on the progression of gastritis [24]. Therefore, it is expected that NDGA might be effective for both the eradication of *H. pylori* and the prevention of gastric carcinogenesis.

From our *in vitro* findings, it is expected that NDGA would bind to FecA1 *in vivo*. To investigate whether NDGA binds to FecA1 *in vivo*, further experiments in which fluorescence intensity and fluorescence localization are examined by immunohistochemical analysis using *H. pylori* infection Mongolian gerbil treated with fluorescently labeled NDGA are needed.

The reported acute NDGA LD_{50} ranges between 800 and 500 mg/kg body weight orally in rodents [22]. Although administration of 3% NDGA results in renal toxicity in rats, 0.5% and 1% NDGA did not exhibit toxicity [22]. Further studies are needed to determine the effect and toxicity of 50 μM NDGA *in vivo*. In addition, it is important to analyze the binding mode of NDGA with FecA1 *in vitro*, which would contribute to the chemical modification of NDGA that mediates the toxicity.

In conclusion, NDGA repressed the SodB activity associated with the gastric mucosal-colonization ability via repression of intracellular Fe^{2+} by FecA1 and increased the H_2O_2 sensitivity and the Mtz sensitivity of *H. pylori in vitro*.

Conflict of Interests

During the last 2 years, the author Hidekazu Suzuki received scholarship funds for the research from Astellas Pharm Inc., Astra-Zeneca K.K., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd., and received service honoraria from Astellas Pharm Inc., Astra-Zeneca K.K., Eisai Co., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd.

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Research Article

Prevalence of *Helicobacter pylori vacA*, *cagA*, and *iceA* Genotypes in Cuban Patients with Upper Gastrointestinal Diseases

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Virulence factors of *Helicobacter pylori* can predict the development of different gastroduodenal diseases. There are scarce reports in Cuba about *H. pylori* isolates genotyping. The aim of the present investigation was to identify allelic variation of the virulence genes *vacA*, *cagA*, and *iceA* in sixty-eight patients diagnosed as *H. pylori* positive by culture. In seven out of 68 patients, strains from both gastric regions were obtained and considered independent. DNA was extracted from all the *H. pylori* strains and evaluated by PCR-genotyping. The *vacA* s1 allele, *cagA* gene, and *iceA2* allele were the most prevalent (72.0%, 56.0%, and 57.3%, respectively). Alleles from m-region showed a similar frequency as s1a and s1b subtypes. The presence of multiple *H. pylori* genotypes in a single biopsy and two gastric region specimens were found. Significant statistical association was observed between *iceA2* allele and patients with non-peptic ulcer dyspepsia (NUD) ($P = 0.037$) as well as virulence genotypes (s1, *s1m2*) and patients over 40 years old ($P < 0.05$). In conclusion, the results demonstrated a high prevalence of *H. pylori* virulent genotypes in Cuban patients over 40 years old while *iceA2* alleles demonstrated a good specificity in patients with NUD.

1. Introduction

Helicobacter pylori is associated with the development of chronic gastritis, peptic ulcer disease (PUD), and gastric cancer (GC). Hence, since 1994, the World Health Organization has classified it as class I carcinogen [1]. Interestingly, despite the high prevalence of *H. pylori* infection in some countries, the frequency of severe diseases is much lower than other populations. In addition to host factors and diet, the varying outcomes of *H. pylori* infection could be related to the virulence of *H. pylori* strains differences [2].

Different virulence factors that play a role in the pathogenesis of the disease such as cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), and *iceA* gene have been described [2, 3]. The *cagA* gene, which encoded the CagA protein, is reported to be found in more than half of the *H. pylori* isolates. It is known that *cagA* is a marker for the cag pathogenicity island and is associated with increased IL-8 production, nuclear factor-kB activation, mucosal inflammation, and development of PUD and GC [3].

The protein VacA is responsible for the gastric epithelial erosion observed in infected hosts. The *vacA* gene encoding the vacuolating toxin comprises three variable parts, the s-region (encoding the signal peptide) and two alleles, s1 and s2. Within the s1 allele, several subtypes (s1a, s1b, and s1c) can be distinguished. For the m-region (middle), two alleles, m1 and m2, have been recognized [4]. The VacA activity level is defined by *vacA* s- and m-regions combination; *s1m1* produces high amount of toxin and is considered the most virulent; however, *s2m2* produces an inactive toxin [4, 5]. In Western countries, infection with *vacA s1m1* strain is more common in patients with PUD than those with chronic gastritis [5]. Recently, a third polymorphic determinant of vacuolating activity (located in the middle of s- and

Table 1: Primers used in PCR for amplification of *cagA*, *vacA,* and *iceA* sequences and s1 region sequencing.

DNA amplified region	Primer	Primer sequence (5′-3′)	PCR product (bp)	PCR program	Reference
cagA	CAG-L	TGCTAAATTAGACAACTTGAGCGA	289	30 cycles (1 min at 95°C, 1 min at 50°C, and 1 min at 72°C)	[13]
	CAG-R	AATAATCAACAAACATCACGCCAT			
vacAs1a	SS1-F	GTCAGCATCACACCGCAAC	190	35 cycles (1 min at 95°C, 1 min at 56°C, and 1 min at 72°C)	[5]
vacAs1b	SS3-F	AGCGCCATACCGCAAGAG	187		
vacAs1c	SS1C-F	CTAGCTTTAGTGGGGATA	213		[14]
vacAs2	SS2-F	GCTAACACGCCAAATGATCC	199		[5]
	VA1-R*	CTGCTTGAATGCGCCAAAC			
vacA m1/m2	VAG-F	CAATCTGTCCAATCAAGCGAG	567/645±		[5]
	VAG-R	GCGTCAAAATAATTCCAAGG			
VacAs1	SIG-F	ATGGAAATACAACAAACACACCG	338		[8]
	SIG-R	CAACCTCCATCAATCTTACTGGA			
	VA1-F	ATGGAAATACAACAAACACAC	259		[5]
iceA1	iceA1-F	GTGTTTTTAACCAAAGTATC	246	30 cycles (1 min at 95°C, 1 min at 50°C, and 1 min at 72°C)	[8]
	iceA1-R	CTATAGCCATTATCTTTGCA			
iceA2	iceA2-F	GTTGGGTATATCACAATTTAT	229/334¥		
	iceA2-R	TTTCCCTATTTTCTAGTAGGT			

*Used as reverse primer with SS1-F, SS3-F, SS1C-F, SS2-F, and VA1-F. ±The size of the product is variable depending on the present subtype 567 bp for m1 and 645 pb for m2. ¥The primers yield a fragment of 229 or 334 bp depending on the presence of a repetitive sequence of 105 nucleotides codifying for 35 amino acids in some *iceA2* alleles.

m-regions) has been described as an intermediate (i) region [6].

The *iceA* gene has two alleles: *iceA1* and *iceA2*. The *iceA1* allele, encoding a CATG-specific restriction endonuclease, is regulated by the contact of *H. pylori* with the human gastric cells [7]. In Western countries the presence of *iceA1* allele is strongly associated with PUD [7, 8].

In Cuba, there are scarce investigations regarding the pattern of virulence genes in *H. pylori* strains [9, 10], but none have examined the s1 allele subtypes of *vacA* gene, the *iceA* gene nor the *H. pylori* strains genotypes isolated from younger or older patients. The aim of this study was to investigate the prevalence of *cagA*, *vacA,* and *iceA* genotypes of *H. pylori* isolates recovered from Cuban patients with dyspepsia.

2. Materials and Methods

2.1. Patients. Gastric biopsies from 150 patients referred to gastroscopy at two Cuban hospitals in Havana from 2009 to 2010 were collected. Patients with a history of gastric surgery, active gastrointestinal bleeding or who had received antibiotics, proton pump inhibitors, or bismuth compounds in the last four weeks were excluded. Sixty-eight patients (35 male and 33 female) with a mean age of 39.2 (years range = 9 to 68) reported positive for *H. pylori* infection by culture were included. The protocol was approved by the Ethical Review Committee of the Tropical Medicine Institute "Pedro Kourí" (IPK) and all patients provided an informed consent.

2.2. Culture and Genomic DNA Isolation. Antrum and corpus biopsy specimens from each patient were kept in sterile saline solution (0.9%) at 4°C. The endoscopic biopsy specimens were smeared on the surface of Columbia chocolate agar plates enriched with Dent supplement (Oxoid, England) and 1% of fetal calf serum (Gibco, USA) and incubated under microaerophilic conditions (Campy Packs, Oxoid) for up to 3 days. *H. pylori* isolates were identified by typical Gram-staining morphology and positive biochemical urease, oxidase, and catalase [11]. Biopsies of 68 patients yielded 75 *H. pylori* isolates; those obtained from two gastric biopsy sites of seven patients were considered independent. Primary cultures of *H. pylori* were conserved at −80°C in brain heart infusion with 20% glycerol and later on were subcultured as it was described above.

All colonies from the subculture were used for chromosomal DNA extraction by Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. DNA content and purity were determined by measuring the absorbance at 260–280 nm (Spectrophotometer MRC, Spain) and by amplification of *ureA* gene [12]. Samples were stored at −20°C before polymerase chain reaction (PCR) amplification was performed.

2.3. PCR. Primers used in this study are shown in Table 1. Amplification of *ureA*, *cagA*, *vacA,* and *iceA* genes by PCR was made in a volume of 50 μL containing 1X PCR buffer (pH = 7), 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.5U Taq polymerase (Sigma, USA), 25 pmol of each primer, 2 μL of chromosomal DNA, and sterile distilled water (Sigma). PCR amplifications were performed in an automated thermal cycler (Techne, Belgium). All runs included a negative DNA control consisting of PCR grade water and two positive DNA controls from *H. pylori* reference strains, ATCC43504 (genotype *vacAs1am1/cagA+/iceA1*) and 26695

Table 2: Association of *H. pylori* subtypes/genotypes with endoscopic findings and age of patients.

Genotypes	Clinical status			Age groups		
	NUD $n = 44$ (%)	PUD $n = 24$ (%)	P value	Group 1 $n = 35$ (%)	Group 2 $n = 33$ (%)	P value
vacA						
s1	31 (70.5)	19 (79.2)	0.249	23 (65.7)	27 (81.8)	0.008*
s2	11 (25.0)	3 (12.5)		12 (34.3)	2 (6.1)	
s1-s2±	2 (4.5)	2 (8.3)		0 (0.0)	4 (12.1)	
m1	20 (45.5)	15 (62.5)	0.179	17 (48.6)	18 (54.5)	0.622
m2	24 (54.5)	9 (37.5)		18 (51.4)	15 (45.5)	
s1m1	20 (45.5)	14 (58.4)	0.095	17 (48.6)	17 (51.5)	0.589
s1m2	11 (25.0)	5 (20.8)	0.728	6 (17.1)	10 (30.4)	0.003*
s2m1	0 (0.0)	1 (4.2)	—	0 (0.0)	1 (3.0)	—
s2m2	11 (25.0)	2 (8.3)	0.395	12 (34.3)	1 (3.0)	0.016*
s1-s2m2±	2 (4.5)	2 (8.3)		0 (0.0)	4 (12.1)	
cagA						
cagA+	22 (50.0)	16 (66.7)	0.186	18 (51.4)	20 (60.6)	0.446
cagA−	22 (50.0)	8 (33.3)		17 (48.6)	13 (39.4)	
iceA						
iceA1	14 (31.8)	11 (45.8)	0.037*	12 (34.3)	13 (39.4)	0.455
iceA2	28 (63.6)	12 (50.0)		23 (65.7)	17 (51.5)	
iceA1-iceA2±	2 (4.6)	1 (4.2)		0 (0.0)	3 (9.1)	
Clinical status						
NUD	—	—		28 (80)	16 (48.5)	0.007*
PUD	—	—		7 (20)	17 (51.5)	

NUD: non-peptic ulcer dyspepsia. PUD: peptic ulcer disease. Group 1: under 40 years old. Group 2: those within 40 years and older. *Statistically significant ($P < 0.05$). ±Strains with multiple genotypes and seven strains recovered from gastric corpus were excluded from analysis. (—) This analysis is impossible to do.

(genotype *vacAs1bm1/cagA+/iceA1*). The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide (Promega) and visualized under UV light. Standards of 100 bp DNA Step Ladder (Promega) were used as molecular size marker.

2.4. Sequencing of s-Region. To analyze nucleotide sequence similarity of *vacA* genotypes among nontypable *H. pylori* strains for s1 region, entire s-region of *vacA* gene was amplified using both the forward and reverse primers (Table 1). Amplified products purified by High Pure PCR Product Purification Kit (Roche, Switzerland) were directly sequenced (Beckman Coulter, Belgium) using DTCS Quick Start Master Mix (GenomeLab, USA). Sequence comparison was carried out using the BLASTn program and the GenBank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Statistical Analysis. Data were analysed using Chi-square test. Any P value < 0.05 was considered to be statistically significant. Association between clinical findings and genotypes was tested independently in two groups, defined according to age group (patients aged less than 40 years were grouped in group 1 and those within 40 years and older in group 2).

3. Results

3.1. Distribution of vacA, cagA, and iceA Genotypes. The *vacA* s-region was amplified in all 75 *H. pylori* strains studied: 54 (72.0%) were identified as s1 and 17 (22.7%) as s2 and the remainder 4 strains (5.3%) harboured both alleles (Table 2). The s1 variants were detected in 94.4% (51/54 strains) and three strains could not be typed by the primers used in this study. In 49 of 51 strains only two single s1 subtypes were identified (25 as s1a and 24 as s1b) and two strains were classified as s1a-s1b subtype. Two of the three selected strains for sequencing the s1 region were identified as s1a-s1b variant with more than 91% of homology (IPK56C, IPK191C) and as s1a variant with 89% of homology (IPK201A) (GenBank accession numbers: KP462879, KP462880, and KP462878). All strains were typed by *vacA* m-region, resulting in 50.7% (38 strains) with m2 allele and 49.3% (37 strains) with m1 allele. Four possible single combinations of the s/m alleles were detected and the most frequent was *s1m1* (36 strains, 48.0%) followed by *s1m2* (18 strains, 24.0%), *s2m2* (16 strains, 21.4%), and *s2m1* (1 strain, 1.3%). Four strains (5.3%) corresponded with *s1-s2m2* combination. According to s1 subtypes (54 strains) the most frequent combination was *s1bm1* (19 strains, 35.2%), *s1am1* (15 strains, 27.8%), *s1am2* (12 strains, 22.2%), *s1a-s1bm1* (2 strains, 3.7%), and *s1bm2* (1 strain, 1.9%).

The *cagA* gene was detected in 42 (56.0%) strains. Overall 68 (90.7%) isolates had a single *iceA* allele; *iceA2* was detected in 43 (57.3%) and *iceA1* in 25 (33.3%) strains. Seven strains were identified with both *iceA* alleles (7 strains, 9.3%).

3.2. H. pylori Genotypes and Clinical Association. Regarding endoscopy aspects of the mucosa, patients were distributed

Table 3: *H. pylori* strains recovered from gastric antrum and corpus of seven patients.

Patients	Stomach region	Endoscopic diagnostic	*vacA* gene subtypes		*cagA* gene presence*	*iceA* gene subtypes
73	A	NUD	s1b	*m1*	−	*iceA2*
	C		s1a, *s2*	*m2*	+	*iceA1, iceA2*
71	A	NUD	*s2*	*m2*	+	*iceA2*
	C		*s2*	*m2*	−	*iceA2*
72	A	NUD	s1b	*m2*	−	*iceA2*
	C		*s2*	*m2*	+	*iceA1, iceA2*
62	A	NUD	s1a	*m1*	+	*iceA1, iceA2*
	C		s1a, s1b	*m1*	+	*iceA1, iceA2*
69	A	NUD	*s2*	*m2*	−	*iceA1*
	C		*s2*	*m2*	−	*iceA1, iceA2*
78	A	NUD	*s2*	*m2*	+	*iceA2*
	C		s1a, *s2*	*m2*	−	*iceA2*
81	A	NUD	s1a	*m1*	+	*iceA2*
	C		s1a	*m1*	+	*iceA2*

A: gastric antrum. C: gastric corpus. NUD: non-peptic ulcer dyspepsia. *Negative (−), *cagA* gene absent; positive (+), *cagA* gene present.

into non-peptic ulcer dyspepsia (NUD) in 64.7% (44/68), PUD in 35.3% (24/68: 10 gastric and 14 duodenal ulcers). Despite the fact that s1 allele of *vacA* gene and *cagA*+ *H. pylori* strains were more frequently identified in patients with NUD (31 patients, 70.5%) and with PUD (16 patients, 66.7%), respectively, no statistical association was observed. However, the presence of *iceA2* allele in 63.6% (28 strains) of isolated strains from patients having NUD was associated statistically ($P = 0.037$) (Table 2).

3.3. Association between Age of Patients and H. pylori Genotypes. The 50.7% (38 strains) of *H. pylori* strains were isolated from patients under 40 years old (group 1) while the 49% (37 strains) were recovered from patients over 40 years old (group 2). Genotypes s1 and *s1m2* were more frequently found in patients belonging to group 2 while the genotypes s2 and *s2m2* were more often detected in group 1, both with a significant statistical association ($P < 0.05$). *H. pylori* strains isolated from patients over 40 years old were more frequent in those with PUD; between these two variables a statistical association was observed ($P = 0.007$) (Table 2).

3.4. H. pylori Strains Recovered from Different Gastric Regions. Fourteen *H. pylori* strains were isolated from two stomach regions, antrum and corpus of seven patients. In only one of these patients, a strain with identical genotype (*s1am1/cagA+/iceA2*) was found. However, in the majority of them, at least a variation in one of the investigated genes was observed. The variation percentages of the following genes, *cagA*+, *vacA* (s-region), and *iceA1,* were 57.1% (8/14 strains), 71.4% (10/14 strains), and 42.9% (6/14 strains), respectively (Table 3).

4. Discussion

In the current investigation the predominant types in Cuban *H. pylori* strains were the s1 (50/68 strains, 73.5%), *iceA2* (40/68 strains, 53.3%), and *cagA* gene (38/68 strains, 55.9%). Our results are in agreement with other developed studies in Cuba, Europe, and East Africa where a higher prevalence (70% or more) of the s1 subtype had been reported [9, 15, 16]. In contrast, an elevated frequency of strains belonging to s2 subtype was described in Jordan [17]. These data demonstrated the high genetic variability of strains in different countries. A low percentage of the strains (5%) harbouring both alleles s1 and s2 has been reported previously [18].

The current research is the first study developed in Cuba to analyze both the s1 variants of *vacA* gene and the gene *iceA* in *H. pylori* strains. Concerning previous research, the dominant *vacA* gene subtype in North and South America, Central Europe, and Australia was s1a [19] but in Portugal and Brazil was s1b [20, 21]. The same frequency of s1a and s1b subtypes, as it was observed in our study, is similar to reports from industrialised countries as France, Italy, USA, and Canada [22].

The existence of nontypeable *H. pylori* strains has been described previously, using similar primers [23]. In our investigation, two of the three sequenced strains were identified as s1a-s1b subtypes. Similar results have been informed by other authors, defining the consensus sequence as a recombinant of two originals with different subtypes each [24]. For m-region of the *H. pylori* strains, m1 and m2 subtypes were approximately equally prevalent as in Europe and Latin America [25].

The percentage of *vacA* genotype s1b/m1 in this study is similar to research developed in Mexico, Brazil, and Costa Rica [26]. However, in Japan and China, the most prevalent genotypes are s1c/m1 and s1c/m2, respectively [27].

In European, Venezuelan, and North American populations, only 60% of *H. pylori* isolates harboured *cagA* gene [16, 28], as what occurred in the current study. However, in Japan and Korea, the proportion of *cagA* + strains is usually over 90% [29]. In our investigation, the prevalence of *cagA* + strains is similar to the report of Torres [9] but is lower in comparison with another Cuban study (70%) [10].

The predominance of *iceA2* subtype is in agreement with reports from Colombia and USA [30]. Both subtypes of the *iceA* gene have been identified in Brazil, Malaysia, and Korea [31, 32].

Patients with *H. pylori* strains recovered from gastric antrum and corpus were infected with different *cagA*, *vacA*, and *iceA* genotypes. Also, multiple *H. pylori* genotypes were observed in a single biopsy specimen. Previous detailed molecular analysis has shown that each of the *H. pylori* strains contains only one *cagA* allele and each one of the s- and m-region subtypes of *vacA* gene. Therefore, it is an exact indicator for the presence of multiple strains of this organism if different genotypes are found [33]. The coexistence of more than one strain in the same individual may reflect the capacity of *H. pylori* to evolve genetic variations during the long-term colonization from childhood [3].

The absence of association between virulence genes explored and PUD could be influenced by the small number of patients studied with this pathology. Similar results have been described previously [3, 9, 10]. Moreover, the predominance of high virulence Cuban *H. pylori* strains in the group of patients with benign gastric diseases has unusual results in Western population. As it was reported in previous Cuban studies [9, 10], despite the presence of highly virulent *H. pylori*, the incidence of gastric cancer is lower in dyspeptic patients (gastric cancer death rate in Cuba: 7.5/100 000, http://files.sld.cu/dne/files/2014/05/anuario-2013-esp-e.pdf). Although this behavior has been observed in several previous studies [3, 7], it is probable that these findings suggest the action of environmental and host factors in Cuban patients. Further research studies to examine the role of host immunological factors might help to explain the different outcomes of *H. pylori*-induced disease in Cuban individuals.

We found an association between *H. pylori* strains harbouring the *iceA2* allele in patients with NUD. This behaviour has also been described in Europe, Saudi Arabia, and Turkey [34]. Several studies suggest an association of the *iceA1* variant and PUD and between *iceA2* variants with gastritis [34, 35]. However, this association varies among populations; in Brazil, for instance, *iceA1* allele is associated with gastritis [31]. A recent meta-analysis confirms the relationship between the *iceA* allelic types and clinical outcomes [7].

The current investigation also showed statistical association of more virulent variants of *H. pylori* (s1 and *s1m2*) strains in the group of older patients. In Portugal and Tunisia, virulent strains have been detected in adult patients more frequently than children [36]. It has been described that *H. pylori* strains experiment recombination with others more virulent and better adapted strains to host changes, resulting in genotypes variable distribution between age groups [37, 38].

In summary, our results show a high prevalence of main virulence factors in Cuban isolates similar to that observed in other Western populations. In addition, we found strains with multiple genotypes, as it has been observed in countries with a high prevalence of *H. pylori* infection. Notably, a significant association was found among *iceA2* allele and NUD as well as strains with more virulent types and older patients. The *iceA* gene may be considered a useful marker in patients with gastroduodenal diseases. The relationship between *H. pylori* virulence factors and clinical outcomes in Cuban population is still unclear; therefore, further studies are required to determine the role of environmental and immunological factors.

Ethical Approval

The project was approved by the Ethical Review Committee of the IPK, Cuba.

Conflict of Interests

The authors declare that they have no conflict of interests.

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