Synergistic Effects of *Helicobacter pylori* Outer Inflammatory Protein A (*oipA*) and *cag* Pathogenicity Island (*cag PAI*) on Interleukin-1β and Interleukin-8 Gene Expression Levels in Gastric Tissues of Thai Gastroduodenal Patients

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*Helicobacter pylori* outer inflammatory protein A (*OipA*) has been found to associate with inflammation that is similar to *cag* pathogenicity island (*cagPAI*). However, the roles of the presence of *oipA* gene involving inflammatory responses *in vivo* need to be clarified. We investigated the association of *oipA* and *cagPAI* on the expression of pro-inflammatory cytokine genes (*IL-1β* and *IL-8*) in gastric tissues of Thai gastroduodenal patients. We detected the *oipA* and *cagPAI* genes in 35.36% and 68.89%, respectively. The *oipA* “on” status was mostly found (93.75%) in *oipA*-positive samples. We observed higher levels of *IL-1β* and *IL-8* gene expression in *oipA*-positive tissues (with “on” status), similar to those with *cagPAI*-positive tissues. Interestingly, samples positive for both *oipA* and *cagPAI* genes showed significantly higher levels of *IL-1β* and *IL-8* gene expression, when compared with tissues single-positive for either *oipA* or *cagPAI*, or double-negative for these two genes. We conclude that *H. pylori* induces *IL-1β* and *IL-8* gene expression via *oipA*-dependent mechanisms. Furthermore, synergy in the presence of both *oipA* and *cagPAI* genes associated with increased *IL-1β* and *IL-8* gene expression levels in gastric tissues, which suggested that *oipA* plays a critical role in the *H. pylori* pathogenesis.

**Keywords**: *H. pylori*, *oipA*, *cag PAI*, IL-1β, IL-8, inflammation.

*Helicobacter pylori* (*H. pylori*) is a Gram-negative microaerophilic bacterium and a causative agent in various gastroduodenal diseases in humans. The association between *H. pylori* infection and gastric cancer led to its classification as a class I carcinogen 1. Infection with this bacterium has been found to stimulate of various pro-inflammatory cytokines from gastric cells such as interleukin-1 (*IL-1*), *IL-6* and *IL-8*, that is play important roles in gastric inflammation 2. The most important *H. pylori* virulence factor is the *cag* pathogenicity island (*cagPAI*) which encodes a type 4 secretory system (T4SS) and immunodominant antigen of CagA. Strains expressing the *cagPAI* have been associated with a more severe inflammatory response than that induced by *cagPAI*-negative strains. *H. pylori* has been reported to depend upon both T4SS- and CagA-dependent mechanisms to induce IL-8 production in gastric cells, resulting in increased...
inflammation. It has also been shown that studies of gastric biopsies from patients infected with *H. pylori* cag-positive strains induce significantly more IL-1α and IL-1β than do cag-negative strains. Therefore, several studies have suggested that the *H. pylori* cagPAI is a putative marker for virulent strains of *H. pylori* that are associated with severe gastroduodenal diseases, especially gastric adenocarcinoma. Interestingly, however, some IL-1β and IL-8 responses have also been found to be independent of the cagPAI mechanisms. Therefore, we speculated that several *H. pylori* virulence factors may be involved together in promoting inflammation and disease severity.

Bacterial adherence to gastric epithelial cells is a critical step in *H. pylori* pathogenesis. *H. pylori* contains a large family of outer membrane proteins (OMPs), comprising 32 members involved in adhesion, immunostimulatory functions and micropore formation. The outer inflammatory protein A (OipA), or HopH, encodes one of the outer membrane proteins, with the *oipA* gene located approximately 100 kb from the cag PAI in the *H. pylori* genome. Aside from promoting bacterial adherence to gastric epithelial cells, OipA (status “on”) increases inflammatory responses by inducing IL-8 production and regulating its secretion through the PI3/Akt pathway in these cells. Using purified OipA protein was shown to cause cell cytotoxicity and apoptotic cell death by modulation of Bax/Bcl-2 levels in the AGS human gastric cell line, indicating that *H. pylori* OipA may play a pathogenic role by damaging host cells. However, previous studies on the role of OipA-induced inflammation have been reported and revealed that mutagenesis of the *H. pylori* oipA gene did not alter IL-8 production in gastric cells in vitro nor in animal model studies. Currently, the effect of *H. pylori* OipA on inflammatory responses of IL-8 is still controversial, in particular its role in vivo. Additionally, the association of OipA with the expression of IL-1β has not yet been examined. We hypothesized that *H. pylori* OipA may associate with pro-inflammatory responses by regulating the expression of IL-1β and IL-8 in the human gastric cells in vivo. To investigate this question, the purpose of the current study was to determine the association between the *oipA* and cagPAI gene status of *H. pylori* infections with IL-1β and IL-8 gene expression levels in the gastric tissue samples from Thai subjects with gastroduodenal disease (gastritis and peptic ulcer disease).

**MATERIALS AND METHODS**

**Patients and Sample Collection**

Gastric tissues were obtained from subjects undergoing gastroduodenal examination at the unit of endoscopy medicine, Suprasitiprasong Hospital, Ubon Ratchathani, Thailand. *H. pylori* infection status of the subjects was determined by the Rapid Urease Test kit (RUT). A total of 45 gastric tissues were collected for this study from subjects with confirmed gastritis (GT=35) and peptic ulcer disease (PUD=10). Ethic committee approval was received for this study from The Institutional Review Board of Mahidol University (COA.NO. 2016/157.0912). Written informed consent was obtained from patients who participated in this study.

**Genomic DNA Extraction and PCR**

Genomic DNA was extracted from RUT positive gastric tissues using DNA purification kits (RBC Real Genome, RBCBiascience), according to the manufacturer’s protocol. Isolated DNA was solubilized by Tris-EDTA (TE) buffer and kept at -20°C until polymerase chain reaction (PCR) was performed.

PCR was used to detect *H. pylori* using primers specific for *16SrRNA*, *oipA*, and *cagPAI*. All primer sequences were selected from published data with slight modifications, as shown in Table 1. The PCR were prepared in a volume of 25 µl using a ready-to-use PCR master mix (OnePCR Ultra®, Bio-Helix Co., LTD. Taiwan), containing 0.5 µM of each primer. PCR amplifications were performed in an automated thermal cycler (BioRad T100™, USA) with the following cycle conditions: *16SrRNA*: 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C; *cagPAI*: 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C; *oipA*: 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C. PCR products were analyzed on 1.5% agarose gels and visualized under a UV illuminator. *H. pylori* oipA amplicons were subsequently sequenced to determine their functional status by observing the stop codons of a signal-peptide coding region.
RNA Extraction and mRNA Quantification

Total RNA was extracted from RUT positive tissues using Ribozol™ (Amresco, VWR Company, USA), according to the manufacturer’s protocol. RNA pellets were solubilized with RNAse-free distilled water. The RNA yield and quality were measured by spectrophotometer. RNA (1 µg) was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen), as described by the manufacturer. IL-1β and IL-8 gene expression were quantified by the SYBR Green assay on a ThermalCycler96® Real-time PCR (Roche Molecular Systems, Inc.) with an initial denaturation at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 30 sec, and normalized to 18SrRNA. The primer sequences for IL-1β, IL-8 and 18SrRNA genes are listed in Table 1. Gene expression levels were quantified using the ∆∆CT-method.

Data Analysis

All data were performed using GraphPad Prism software (version 5.0) and significance evaluated with the Student’s t-test. A value of p < 0.05 was considered statistically significant.

RESULTS

As shown in table 2, 45 RUT positive samples were confirmed to harbor *H. pylori* infection by PCR amplification. A 401-bp PCR product, indicating the presence of the *oipA* gene, was obtained in 16 (35.56%) of these subjects. No significant differences between the gastritis and PUD were detected. Subsequently, the *oipA* functional status (“on” / “off”) was determined by PCR-based sequencing. We found that in 15 of 16 (93.75%) subjects, the *oipA* status was “on”.

The *cagPAI*-positive samples was found 68.89% (31/45) of gastric tissues of Thai gastroduodenal subjects (Table 2). The vital components of *cagPAI*, *cagA* and *cagE*, were also found in the *cagPAI* positive samples (data not shown). There were no significant differences between the gastritis and PUD. The frequency

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| 16SrRNA F: GCACGCTGTGAGAACATTAC  
  R: CGTACGTCATTACCTGGAGA  |
| oipA F: GTTTTTGATGATCATTGAGGATT  
  R: GTGCACCTCTCTATGGCTTT  |
| cagPAI F: ACATTGTGCTAATAAAAGCCTG  
  R: TCTCCATGTCATGTCCATTATGCT  |
| IL-1β F: GCACGATGACCTGTACGAT  
  R: GTCATCTCTATGGCTTT  |
| IL-8 F: ACTGAGAGTGTAGGATGGAC  
  R: AACCCTCTGCACCCAGTTTTC  |
| 18SrRNA F: CCGGCGGACCACCCATTCGAAC  
  R: GAATCGAACCCTGATTCGCCGC  |

<table>
<thead>
<tr>
<th>Genes</th>
<th>Patient groups</th>
<th>Total (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastritis(n=35)</td>
<td>PUD(n=10)</td>
</tr>
<tr>
<td>oipA+</td>
<td>13 (37.14%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>oipA’ status on</td>
<td>12 (92.31%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>cagPAI+</td>
<td>22 (62.86%)</td>
<td>9 (90%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene combinations</th>
<th>Patient groups</th>
<th>Total (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastritis(n=35)</td>
<td>PUD(n=10)</td>
</tr>
<tr>
<td>cagPAI+ / oipA+</td>
<td>10 (28.57%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>cagPAI+ / oipA’+</td>
<td>12 (34.29%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>cagPAI+ / oipA’+</td>
<td>3 (8.57%)</td>
<td>1 (10%)</td>
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<tr>
<td>cagPAI+ / oipA’+</td>
<td>10 (28.57%)</td>
<td>2 (20%)</td>
</tr>
</tbody>
</table>
distributions of the combined genotypes of *H. pylori* cagPAI and oipA in the 45 samples of Thai subjects are presented in table 3. We clustered a total 4 different cagPAI / oipA genotype combinations, out of which the most prevalent genotypes was cagPAI / oipA; but the genotype of cagPAI/oipA was not found in PUD.

In order to study the association of *H. pylori* oipA and cagPAI genes with inflammatory responses, we determined *IL-1β* and *IL-8* gene

![Figure 1](image1.png)

**Fig. 1.** *IL-1β* gene expression levels in the presence/absence of oipA and cagPAI genes in gastric tissues of subjects with gastritis (A) and PUD (B).

*significant differences when compared with double-negative cagPAI / oipA gene status (*p*<0.05).

*significant differences when compared with single-positive oipA, cagPAI gene status, or double-negative for these two genes (*p*<0.05).

![Figure 2](image2.png)

**Fig. 2.** *IL-8* gene expression levels in the presence/absence of oipA and cagPAI genes in gastric tissues of subjects with gastritis (A) and PUD (B).

*significant differences when compared with double-negative cagPAI / oipA gene status (*p*<0.05).

*significant differences when compared with single-positive oipA, cagPAI gene status, or double-negative for these two genes (*p*<0.05)
expression levels in the gastric tissues of Thai subjects with gastroduodenal disease. As shown in fig. 1 and 2, IL-1β and IL-8 gene expression levels in samples containing single-positive gene status of cagPAI (cagPAI/oipA) were significantly higher than in those containing double-negative genes (cagPAI/oipA) (p<0.05). Similarly, samples containing single-positive oipA gene status (cagPAI/oipA) were found to have significantly increased IL-1β and IL-8 gene expression levels when compared with those with a double-negative gene status (cagPAI/oipA) (p<0.05), however, the levels were slightly lower than for the cagPAI+ samples (cagPAI/oipA). Our study, only one sample carrying oipA-functional “off” status was combined with the cagPAI-negative samples that showed the decreasing of IL-1β and IL-8 gene expression. Interestingly, we found that samples with double-positive gene status for oipA and cagPAI (cag PAI/oipA), had higher IL-1β and IL-8 levels compared with single-positive and double-negative samples. These results were consistent for subjects with gastritis and PUD.

DISCUSSION

H. pylori has been shown to stimulate the production of various pro-inflammatory cytokines from gastric epithelial cells 23. IL-1β is a pro-inflammatory cytokine that has been involved in pain, inflammation and autoimmune condition 24. The biological effects of IL-1β is involved to induce expression of many cytokine genes by either initiating their transcription or stabilizing their mRNA, including tumor necrosis factor α (TNF-α), IL-2, IL-6, IL-12, interferon α, β, and γ, granulocyte-colony stimulating factor and macrophage-colony stimulating factor 25. The in vitro study revealed that H. pylori cagA and cagE mutant strains showed a marked decrease in the induction of IL-1β production of human peripheral blood mononuclear cells. It was suggested that the components of cagPAI more frequently associated with the production of IL-1β 26, especially H. pylori requires the expression of cagE for the full induction of IL-1, IL-12, and TNF-α in the immature dendritic cells 27. However, the molecular mechanisms of H. pylori lipopolysaccharide (LPS) and vacuolating cytotoxin A (VacA) induced IL-1β have been determined 9,28. Thus, based on the previous data, we assumed that other components of H. pylori might be associated with the production of IL-1β.

IL-8 is also a pro-inflammatory cytokine and is well known to be a chemoattractant for neutrophils and an activator of macrophages that plays important role in pro-inflammatory responses. This chemokine is also associated with cell proliferation, migration and survival of endothelial cells, thereby potentiating the epithelial-mesenchymal transition and survival of cancer cells, required for cancer progression 29. IL-8 has been found to play a dominant role in gastric epithelial cells exposed to H. pylori infection and in pathological processes leading to gastroduodenal disease 30. It was shown that H. pylori can also stimulate gastric epithelial cells to produce IL-8 through a CagA-dependent mechanism. In addition to the CagA protein, bacterial cell wall components, such as peptidoglycan and heptose-1,7-bisphosphate, are also translocated via the T4SS into host epithelial cells and recognized by the nucleotide-binding oligomerization domain receptor (NOD) and the TRAF-interacting protein with forkhead-associated domain (TIFA), respectively, to induces NF-κB activation and up-regulation of pro-inflammatory immune responses 31,32. Although previous studies reported that H. pylori cagPAI mutant strains induced reduced levels of IL-8 secretion in vitro, other H. pylori components may also be involved 33. This suggests that although the cagPAI may promote clinical disease, other factors important for H. pylori pathogenicity remain to be investigated 34.

Approximately 4% of the H. pylori genome is composed of genes encoding OMPs. Among them, OipA has emerged as another virulence factor involved in promoting PUD and gastric cancer 12. Previous in vitro study showed that IL-8 production was significantly reduced in epithelial cells stimulated with H. pylori oipA mutant strains on a cag-positive background, indicating the important role of OipA in IL-8 production12. Conversely, OipA-positive strains on a cag-negative background, did not induce higher IL-8 amounts than OipA-negative strains. These results suggest that the OipA protein alone is not able to stimulate IL-8 secretion in AGS cells, but that OipA expression seems to support

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Cag-induced cytokine secretion 35. However, the effect of OipA on IL-8 secretion in vitro might not be sufficient to explain the exact role of OipA under physiological conditions in vivo. Additionally, the association of OipA on the expression of IL-1β has not been extensively studied. Thus, we evaluated the association of the oipA gene, in the presence or absence of the cagPAI, on IL-1β and IL-8 gene expression levels in gastric tissues of subjects with gastroduodenal disease. We found that IL-1β and IL-8 gene expression levels in gastric tissues was dependent on the cagPAI gene (cagPAI/oipA). We also found that tissue samples positive for the oipA gene (cagPAI/oipA) also exhibited higher levels of IL-1β and IL-8 gene expression, when compared with those double-negative for the oipA and cagPAI genes. Our data indicated that increasing levels of IL-1β and IL-8 gene expression might be associated with the oipA gene. Additionally, the highest levels of IL-1β and IL-8 gene expression were found in the tissue samples that were positive for both oipA and cagPAI, when compared to single-positive gene and double-negative genes of oipA and/or cagPAI. It is possible that the association of oipA and cagPAI might be associated with heightened levels of IL-1β and IL-8 gene expression in gastric tissues. The results of the present study are consistent with the findings of previous in vitro study which showed that H. pylori oipA mutant bacteria displayed reduced levels of cell adherence and induced less IL-8 secretion in the AGS cell line. Furthermore, these mutant bacteria were unable to translocate CagA into gastric epithelial cells 36. Taken together, these data suggest that H. pylori OipA might involve in bacterial adherence leading to IL-1β and IL-8 production through direct signaling and that, moreover, this OMP might cooperate with the cagPAI to induce inflammation.

CONCLUSIONS

Our study investigate that H. pylori oipA gene associates with increased IL-1β expression in gastric tissues of subjects with gastroduodenal disease, as well as IL-8 expression. Our findings conclude that IL-1β and IL-8 gene expression levels were dependent on the H. pylori oipA gene. In addition, synergistic effects of oipA combined with cagPAI have been found to associate with increased levels of IL-1β and IL-8 gene expression. These data suggest that OipA might be an important H. pylori virulence factor associated with inflammation. However, the molecular mechanisms of oipA-induced IL-1β expression should be further determined.

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REFERENCES


normal, and neoplastic gastroduodenal mucosa. 


36. Horridge DN, Begley AA, Kim J, Aravindan N, Fan K, Forsyth MH. Outer inflammatory protein a (OipA) of Helicobacter pylori is regulated by host cell contact and mediates CagA translocation and interleukin-8 response only in the presence of a functional cag pathogenicity island type IV secretion system. Pathog Dis 2017;75(8).