Distinct Genetic Variation of *Helicobacter pylori* *cagA*, *vacA*, *oipA*, and *sabA* Genes in Thai and Korean Dyspeptic Patients


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Introduction

Infection with *Helicobacter pylori* is the most commonly diagnosed bacterial infection worldwide [1]. This bacterium plays an important role in the development of gastrointestinal diseases and is indicated as a class I carcinogen that is involved with the development of gastric carcinoma and mucosal-associated lymphoid tissue lymphoma (MALT) [2]. Gastric cancer is a leading cause of cancer-related deaths worldwide, especially in East Asian countries. However, it has been postulated that more than 50% of the world’s population is infected with *H. pylori*, but less than 2% develop gastric cancer [1]. The incidence of *H. pylori* infection and gastric cancer varies geographically and ethnically, even among countries in East and Southeast Asia. An epidemiological
study showed that the prevalence of *H. pylori* infection was similar in Thailand and Korea [3], although the morbidity and mortality rates of gastric cancer were significantly greater in Korea [4–7]. Due to Thailand is classified in Asian Enigma with high prevalence of *H. pylori* infection but does not translate into high gastric cancer incidence [8]. It is possible that the differences in geography and host ethnicities of these two countries might affect the genetic variation of *H. pylori* strains.

The clinical outcome of *H. pylori* infection varies according to the involvement of various bacterial, host, and environmental factors. Besides the host genetic and environmental components, the divergence of the virulence factors of *H. pylori* in different area should be emphasized. Several studies of the variation in *H. pylori* virulence-associated genes have suggested that the cytotoxin-associated gene A (*cagA*) is an important marker of the virulent strains of *H. pylori* [9, 10]. The CagA protein is delivered into the host cell via a type IV secretory system and has been associated with the induction and production of interleukin 8 (IL-8) by gastric epithelial cells [11]. The genotypes of the *cagA* gene have been classified into East Asian and Western types according to the amino acid sequences surrounding the EPIYA (Glu-Pro-Ile-Tyr-Ala) motif. The East Asian type of CagA strongly binds to Src homology 2 domain-containing protein tyrosine phosphatase and induces IL-8 secretion more than the Western type of CagA, indicating that the East Asian type of *cagA* is more virulent than are strains of the Western type [12, 13]. Vacuolating cytotoxin A (*vacA*), which is produced by all *H. pylori* strains, plays a predominant role in vacuolating activities and host cell injury [14]. Studies of the variations of *vacA* due to the sequence heterogeneity at the signal (s; s1/s2) and middle (m; m1/m2) regions have shown that the combination of *vacA* s1/m1 type exhibits higher levels of cytotoxic activity than do the *vacA* s1/m2 or s2/m2 types [15].

The outer membrane proteins (OMPs) of bacterial pathogens play important roles in bacterial colonization, antibiotic resistance, interactions with host receptors, and immunological responses [16]. The *H. pylori* outer inflammatory protein A (*oipA*) gene encodes the OMP and induces IL-8 production by gastric epithelial cells [17]. The putative virulence factor, sialic acid-binding adhesion (*sabA*), is believed to play a critical role in *H. pylori* colonization, which leads to persistence of infection. Besides adherence properties, the SabA protein binds specifically to the sialyldimeric-Lewis^x^ glycosphingolipid on the neutrophil membrane, suggesting that it might play an important role in pro-inflammatory and immune responses.

Although, several *H. pylori* virulence genes have been previously revealed, but data of other genes were lacking in Thailand and Korea, which need to be investigated, including *oipA* and *sabA* genes. Importantly, there was no simultaneous comparison of *H. pylori* virulence genes in high risk gastric cancer and Asian Enigma regions, which may explain to the low gastric cancer incidence in Thailand. We aimed to elucidate the prevalence of the variations of the *H. pylori* virulence genes involving inflammation and cell damage detected between Thai and Korean dyspeptic patients, including *cagA* (Western and East Asian), *vacA* (s- and m-), *oipA*, and *sabA*.

Materials and Methods

Patients and specimen collection

To date, a total of 45 *H. pylori* Korean strains (30 associated with gastritis, 10 with peptic ulcer diseases, and 5 with gastric cancer) have deposited to the *H. pylori* Korean Type Culture Collection (http://hpktcc.knrrc.or.kr/) of the Gyeongsang National University School of Medicine. A total of 50 Thai dyspeptic patients, including 35 with gastritis, 10 with peptic ulcer diseases, and 5 with gastric cancer, underwent gastro-endoscopy at the Unit of Endoscopy Medicine of Supprasittiprasong Hospital (Ubon Ratchathani, Thailand). Gastric biopsy specimens were screened for *H. pylori* infection using a rapid urease test (RUT) kit (Pronto Dry®, Herford, Germany) according to manufacturer’s instructions. The RUT-positive biopsy specimens were subsequently collected for DNA extraction and *H. pylori* genotyping. The study protocol was approved by the Human Ethics Committee of Mahidol University (COA No. MU-CIRB 2016/053.1804), and all patients provided informed consent.

DNA extraction and *H. pylori* genotyping

*H. pylori* Korean strains were derived from frozen stock cultures of *H. pylori* Korean Type Culture Collection (http://hpktcc.knrrc.or.kr/) and subsequently cultured in Brucella agar supplemented with 10% bovine...
serum and incubated at 37°C for 48 h under 5% CO₂ atmosphere. Genomic DNA from \textit{H. pylori} Korean strains and the RUT-positive biopsy specimens collected from Thai patients was extracted using DNAzol reagent (Invitrogen Corporation, Paisley, Scotland, UK) in accordance with the manufacturer’s instructions. The DNA pellet was solubilized in TE buffer and stored at -20°C until amplification by polymerase chain reaction (PCR).

The PCR was performed in a reaction volume of 25 µl of a ready-to-use OnePCR™ Plus (GeneDireX, Taiwan) containing 0.5 µM of each primer, 100 ng of genomic DNA from gastric biopsy specimens, or 10 ng of \textit{H. pylori} genomic DNA. The primer sequences used in this study were designed as reported elsewhere [18–23] with slight modifications and are listed in Table 1. The 16SrRNA gene was amplified to confirm the presence of \textit{H. pylori} in gastric biopsy specimens. PCR amplifications were performed in an automated thermal cycler (BioRad T100™, USA). The PCR amplicons were detected by 1.5% agarose gel electrophoresis and visualized under ultraviolet light. The amplified DNA of the oipA and sabA genes was purified and sequenced. The nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov), and the functional status of these two genes was determined as either “on” or “off.”

The cagA genotypes (Western and East Asian type of cagA) were determined by real-time PCR using the LightCycler® 96 Real-Time PCR System (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). PCR was performed in a 20 µl reaction mixture containing 1× concentration of a ready-to-use SYBR Green master mix (FastStart DNA Green Master; Roche Diagnostics Deutschland GmbH), a 0.5 µM concentration of each primer, and 100 ng of DNA template. The PCR cycling conditions comprised an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. Fluorescence was measured at each extension step. Melting curve analysis was performed to assess the specificity of the amplicons.

**Data analysis**

Data were analyzed using the chi-square ($\chi^2$) test. A probability ($P$) value of <0.05 was considered statistically significant.

**Results**

**Prevalence of \textit{H. pylori} cagA and vacA genotypes in Thai and Korean dyspeptic patients**

To determine the presence of \textit{H. pylori} infection in RUT-positive samples of Thai dyspepsia, we performed

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (5’-3’)</th>
<th>PCR size (bp)</th>
<th>Conditions</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrRNA</td>
<td>F: GCGACCTGCTGGAACATTAC</td>
<td>139</td>
<td>35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R: CGTTAGCTGCAATTACTGGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA</td>
<td>F: TTGACCAAACAACCAAAAACCGAAG</td>
<td>183</td>
<td>35 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R: CTTCCCTTAAATGGCAGATTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA (Western)</td>
<td>F: AGGCATGATAAAAGTTGATGAT</td>
<td>91</td>
<td>40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>R: AAGGTCGCGCGAGATCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA (East Asian)</td>
<td>F: AAAAGGATGGGCGCGTTCA</td>
<td>91</td>
<td>40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>R: CCTGCGTTGATTTGGCCTCATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vacA s1/s2</td>
<td>F: ATGGAAAATAACAAAACACAC</td>
<td>259/286</td>
<td>35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R: CTGCGTTAGGCGCCAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vacA m1/m2</td>
<td>F: CAATCTGTCAATCAACGCGAG</td>
<td>567/642</td>
<td>35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R: GCCGCAAAATAATCCCAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oipA</td>
<td>F: TTTTGTGATGATGCGATTTTTTCT</td>
<td>401</td>
<td>35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>R: GTGCATCTTATGCGTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sabA</td>
<td>F: TTTTGTGATGATGCGATTTTTCT</td>
<td>650</td>
<td>35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C</td>
<td>19</td>
</tr>
</tbody>
</table>
PCR using primers specific for the H. pylori 16S rRNA gene (Fig. 1). As shown in Table 2 and Fig. 1, the prevalence of the cagA gene was significantly greater in Korean than in Thai dyspeptic patients (45/45, 100% vs. 35/50, 70%, respectively). However, there was no significant association between the cagA gene and the dyspeptic clinical outcomes of the Thai and Korean populations. The cagA genotypes were also examined by real-time PCR analysis. The Western type cagA gene was significantly more common in Thai dyspeptic patients (28/35, 80%), whereas the East Asian type cagA gene was significantly more common in Korean dyspeptic patients (39/45, 86.67%).

Only vacA s1 type was found in both Thai and Korean dyspeptic patients (Fig. 1). As revealed by statistical analyses, the prevalence of vacA s1/m1 genotypes of Korean dyspeptic patients was significantly higher than that of Thai dyspeptic patients (40/45, 88.89% vs. 21/50, 42%, respectively). However, there was no significant association between the vacA s- and m- genotypes and dyspeptic clinical outcomes in the Thai and Korean populations.

Prevalence of the H. pylori oipA gene in Thai and Korean dyspeptic patients

As shown in Table 2 and Fig. 1, the oipA gene was detected significantly more often in Korean than in Thai dyspeptic patients (43/45, 95.56% vs. 16/50, 32%, respectively; p < 0.05). Nevertheless, there was no significant relationship between oipA and dyspeptic clinical outcomes in either population. The functional status of the oipA gene was determined by DNA sequence analysis. The oipA “on”-status was similar between Thai and Korean dyspeptic patients (15/16, 93.75% vs. 41/43, 95.35%, respectively; p > 0.05). In this study, the number of cytosine-thymine dinucleotide repeats (CT repeats) at the signal-peptide coding region was observed. The number of oipA “on”-status patterns with 4-CT repeats was most commonly found in both Thai and Korean dyspeptic patients. The oipA “off”-status with 8-CT repeats was found in Thai dyspeptic patients, whereas 3-CT repeats were detected in Korean dyspeptic patients (Table 3).

Table 2. Prevalence of the H. pylori cagA, vacA, oipA, and sabA genes in Thai and Korean dyspeptic patients.

<table>
<thead>
<tr>
<th>H. pylori genes</th>
<th>Thai dyspeptic patients</th>
<th>Korean dyspeptic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GT (n = 35)</td>
<td>PUD (n = 10)</td>
</tr>
<tr>
<td>cagA+</td>
<td>22 (62.86%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>cagA Western</td>
<td>18 (81.82%)</td>
<td>7 (77.78%)</td>
</tr>
<tr>
<td>cagA East Asian</td>
<td>4 (18.18%)</td>
<td>2 (22.22%)</td>
</tr>
<tr>
<td>vacA s1 m1</td>
<td>15 (42.86%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>vacA s1 m2</td>
<td>20 (57.14%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>oipA+</td>
<td>13 (37.14%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>oipA on-status</td>
<td>12 (92.31%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>sabA+</td>
<td>14 (40%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>sabA on-status</td>
<td>8 (57.14%)</td>
<td>2 (66.67%)</td>
</tr>
</tbody>
</table>

Abbreviations: GT, gastritis; PUD, peptic ulcer diseases; GC, gastric cancer.
*Significant difference between the Thai and Korean populations (p < 0.05).
Prevalence of the *H. pylori* *sabA* gene in Thai and Korean dyspeptic patients

The 650-bp PCR product indicating the presence of the *sabA* gene are shown in Table 2 and Fig. 1. Statistical analysis showed that the *sabA* gene was more dominant in Korean than in Thai dyspeptic patients (41/45, 91.11% vs. 17/50, 34%, respectively; *p* < 0.05). Comparisons of three clinical outcomes showed no significant relationship between the presence of the *sabA* gene and dyspeptic clinical outcomes in the Thai and Korean populations.

In addition, *sabA* gene sequence analysis showed a similar frequency of *sabA* “on”-status in the Thai and Korean dyspeptic patients (10/17, 58.82% vs. 29/41, 70.73%, respectively; *p* > 0.05). Further analysis of the number of CT repeats at the signal-peptide coding region in Thai dyspeptic patients showed that only 3 and 12-CT repeats were found in the *sabA* gene with an “on” and “off” statuses, respectively (Table 3). In contrast, the number of CT repeats varied in the *sabA* gene with “on” and “off” statuses in Korean dyspeptic patients.

Discussion

Thailand is a Southeast Asian country that is at the cultural crossroads between South and East Asia and has two major ethnicities: Thai and Chinese. Conversely, Korea is an East Asian country between Japan and China. It is noteworthy that the rate of *H. pylori* infection in these two countries seems to be equal, although there are significant differences in the severity of dyspeptic symptoms, especially in peptic ulcer diseases and gastric cancer. In Korea, a high-risk area of gastric cancer, *H. pylori* infection is highly associated with peptic ulcer diseases and gastric cancer, but much lower in Thailand [24, 25]. It is now clear that Asian Enigma, which included Thailand, refers to regions where *H. pylori* infection is high but gastric cancer is relatively low [7]. Enigmas have been explained by several factors, such as host genetics, bacterial genetics behavioral and environmental factors [8]. Among factors, infection with different genotypes of *H. pylori* strains, especially the virulence genes involving inflammation and cell damage, might be involved in differences in disease severity between these two countries.

We examined the putative virulence of the *cagA* gene, which is part of the *cag* pathogenicity island (*cag*PAI) and involved in the inflammatory process by induction of IL-8 production via the bacterial type IV secretory system [26]. Infection with *H. pylori* *cagA*-positive strains increases the risk of gastric cancer more than infection with *H. pylori* *cagA*-negative strains [27]. There are two major genotypes of the *cagA* gene: East Asian and Western types. Additionally, the East Asian type of *cagA* was found to be more virulent than the Western type of *cagA* [28]. It has been reported that the East Asian *cagA*-positive strain is associated with a greater mortality rate of gastric cancer in Asia [29]. Korean dyspeptic patients had a higher prevalence of *cagA*-positive strains and East Asian type *cagA*, as compared with Thai dyspeptic patients who more often carry the Western type of *cagA*. Our data support the findings of several previous studies, showing that the East Asian type *cagA* was more common in East Asian countries [30], whereas the Western type of *cagA* is widely distributed in south and southeastern areas of Asia and Thai-
land [4]. Thus, our finding suggest that the East Asian type of cagA, which is associated with a greater risk of gastric carcinogenesis, was more common in Korean than in Thai populations.

The vacA gene, which is present in all H. pylori strains that induce vacuolization and cell damage, contains two variable parts, the vacA-s and vacA-m regions. Of the two variable regions, the vacA s1 type possesses higher toxin activity and causes more severe damage to gastric epithelial cells than do the other alleles [31]. The m-region plays an important role in binding to the receptor on the host cell membrane [32]. Amino acid sequence analysis showed that VacA m2 strain, but not VacA m1, has a 23-amino acid insert, which forms an additional β-helix [33]. It has been suggested that H. pylori m1 strains are associated with a higher risk of gastric ulceration and cancer than are the m2 strains owing to differences in amino acid sequences, which may affect receptor binding affinities of VacA m1 and m2 [34]. The results of the present study showed that strains carrying the vacA s1/m1 types were significantly more common in Korean than in Thai populations, which is in accordance with a previous report by Yamaoka et al. [35]. We concluded that the difference in vacA m-region genotypes might affect the receptor binding affinity and toxin production of the host cells, which might have led to the differences in H. pylori pathogenesis between these two populations.

The H. pylori OMP plays a pivotal role in bacterial attachment during initial colonization in gastric epithelial cells. The expression of H. pylori OMPs is regulated by several factors, such as gene conversion, gene duplication, and phase variation. The H. pylori oipA gene codes for a surface protein involved in the in vitro production of IL-8 by gastric cells via the PI3/Akt pathway, although the host receptor of OipA remains unknown [36]. Previous studies reported that the presence/absence of the oipA gene and its functional status varied among countries [37]. Overall, the association of the oipA gene with clinical outcomes remains unclear. Expression of the H. pylori OipA protein is controlled by phase variation within a CT dinucleotide repeat motif at the 5′ end of the signal-peptide coding region [17]. Several studies have shown that OipA is significantly related with more severe gastric diseases, including high level of mucosal IL-8 production in gastric tissues, cytotoxic effects in the AGS human gastric cancer cell line, H. pylori density in epithelial cells, and promotion of neutrophil infiltration [38, 39]. Thus, OipA may indirectly contribute to the tight adhesion between H. pylori and epithelial cells, leading to strong cellular signaling of the type IV secretory system and CagA [40]. Our study showed that oipA-positive samples were significantly predominant in the Korean dyspeptic patients. It is possible that the high prevalence of the oipA gene might partly account for the increased risk of developing severe gastroduodenal diseases in the Korean population, as compared with the Thai population.

SabA is the most well-characterized adhesion protein of H. pylori and has been associated with binding of the bacterium to the sialyl-dimeric-Lewis’ glycosphingolipid on the membrane of gastric epithelial cells [41]. The interaction between SabA and its ligand enhances attachment to the host epithelial cell, thereby facilitating successful colonization [42]. Besides these attachment properties, SabA has been identified as a hemagglutinin, which specifically binds to sialylated carbohydrate structures on neutrophils and stimulates neutrophils to produce reactive oxygen species, which leads to gastric epithelial cell damage by oxidative stress [43]. The sabA gene appears to be the one of the most divergent genes in the H. pylori genome [44]. It was found that the sabA expression was regulated by the CT dinucleotide repeats present at the 5′ end of the signal-peptide coding region caused by phase variation through a slipped strand mispairing, similar to that observed with the oipA gene. Our findings demonstrated that the sabA “on”-status was similar in Korean and Thai dyspeptic patients, although there were differences in sabA CT repeat patterns among the two populations. Hence, we hypothesized that the variation of the sabA gene detected in two populations was due to differences in bacterial adaptation among host ethnicities and environments. However, owing to the greater prevalence of sabA-positive samples in Korean dyspeptic patients, as compared with the Thai population, the differences in the severity of gastric cell damage in Thai and Korean dyspeptic patients might be involved by SabA-induced immune responses.

This is the first study to simultaneously compare H. pylori virulence genes between areas of a high risk of gastric cancer and Asian Enigma. The genetic variation

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of *H. pylori* virulence genes in Korean dyspeptic patients, as compared with Thai dyspeptic patients, showed a high frequency of cagA, East Asian cagA type, vacA s1/m1, oipA-positive, and sabA-positive genes in Korean. However, within Thai or Korean dyspeptic patient groups, the significant association of these virulence-associated genes among three clinical manifestations was not found. Taken together, we hypothesized that the *H. pylori* virulence-associated genes might be distinctly diverse and antigenically different in patients from different geographic areas and the Korean population was at a greater risk of exposure and colonization with the virulent *H. pylori* strains than the Thai population was, which might explain the *H. pylori* pathogenesis between a high gastric cancer epidemiology and Asian Enigma regions. However, other host genetic, environmental, and behavioral factors may be involved in the *H. pylori* pathogenesis of these two countries and should be further elucidated.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


