Increased Risk of Severe Gastric Symptoms by Virulence Factors vacAs1c, alpA, babA2, and hopZ in Helicobacter pylori Infection

Dong-Hae Lee1,2, Jong-Hun Ha1,2, Jeong-Ih Shin1,2, Kyu-Min Kim1,2, Jeong-gyu Choi1,2, Seorin Park1,2, Jin-Sik Park1, Ji-Hyeun Seo3,4, Ji-Shook Park3,4, Min-Kyoung Shin1,2,4,5, Seung-Chul Baik1,4, Woo-Kon Lee1,4, Myung-Je Cho1,5, Hyung-Lyun Kang1,4,5, and Myunghwan Jung1,2,4,5*

1Department of Microbiology and 2Pediatrics, College of Medicine, Gyeongsang National University, Jinju 52727, Republic of Korea
2BK21 Center for Human Resource Development in the Bio-Health Industry, Department of Convergence Medical Science, Gyeongsang National University, Jinju 52727, Republic of Korea
3Institute of Health Science, Gyeongsang National University, Jinju 52727, Republic of Korea
4Research Institute of Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea

Two virulence factors of Helicobacter pylori, cagA and vacA, have been known to play a role in the development of severe gastric symptoms. However, they are not always associated with peptic ulcer or gastric cancer. To predict the disease outcome more accurately, it is necessary to understand the risk of severe symptoms linked to other virulence factors. Several other virulence factors of H. pylori have also been reported to be associated with disease outcomes, although there are many controversial descriptions. H. pylori isolates from Koreans may be useful in evaluating the relevance of other virulence factors to clinical symptoms of gastric diseases because the majority of Koreans are infected by toxigenic strains of H. pylori bearing cagA and vacA. In this study, a total of 116 H. pylori strains from Korean patients with chronic gastritis, peptic ulcers, and gastric cancers were genotyped. The presence of virulence factors vacAs1c, alpA, babA2, hopZ, and the extremely strong vacuolating toxin was found to contribute significantly to the development of severe gastric symptoms. The genotype combination vacAs1c/alpA/babA2 was the most predictable determinant for the development of severe symptoms, and the presence of babA2 was found to be the most critical factor. This study provides important information on the virulence factors that contribute to the development of severe gastric symptoms and will assist in predicting clinical disease outcomes due to H. pylori infection.

Keywords: Helicobacter pylori, vacAs1c, alpA, babA2, hopZ, gastric diseases

Introduction

Helicobacter pylori is a gram-negative, spiral-shaped capnophilic bacterium [1] which is closely associated with the epithelial surface or the surface mucus of gastric mucosa [2]. H. pylori is the causative agent of chronic gastritis and peptic ulcer, and prolonged carriage is known to be a significant risk factor for the development of gastric disorders including gastric cancer in Korea [9]. The incidence of gastric cancer differs geographically [10], and East Asian countries have the highest incidence rates of this disease worldwide [11]. This regional discrepancy can be partly explained by different prevalence rates of H. pylori infection [11], which in East Asian countries are reported to be higher than those in Western countries [8]. However, the incidence of gastric cancer in countries located in West South Asia is similar to that in Western countries, even though H. pylori infection is more prevalent in West South Asia than in Western countries [11]. These studies suggest that other factors in addition to infection could play a role in the pathogenesis of H. pylori and the development of pestilential gastric symptoms.
**Materials and Methods**

**H. pylori isolates**

H. pylori strains were isolated at Gyeongsang National University Hospital (Korea), Kosin University Gospel Hospital (Korea), and St. Carollo Hospital (Korea) from 1987 to 2005, as previously described [35-37]. Briefly, biopsy specimens were inoculated onto Mueller-Hinton agar (Merck, USA) plates containing 10% bovine serum (Gibco, USA), vancomycin (Merck, 10 μg/ml), nalidixic acid (Merck, 25 μg/ml), and amphotericin B (Merck, 1 μg/ml). The plates were incubated at 37°C under 10% CO2 and 100% humidity for 7 days. The bacteria were identified as H. pylori on the basis of their morphology, urease test, and 16S rRNA PCR. To amplify the 16S rRNA gene of H. pylori, primers of PV31 (5'-CGGCCCAGACTCCTACGGG-3') and PV32 (5'-TTACCGCGGCTGCTGGGCAC-3') were designed based on the previous publication [38]. The PCR amplification was performed with AccuPower PCR PreMix (Cat. No. K-2016, Bioneer, Korea) plates containing the same composition as Mueller-Hinton agar at 37ºC under 10% CO2 and 100% humidity for 7 days. The PCR condition was as follows: pre-denaturation at 94°C for 5 min; followed by denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 3 min (35 cycles), and a final extension at 72°C for 7 min. PCR-amplified products (180 bp) were detected by agarose gel electrophoresis to identify H. pylori.

All isolates were deposited at the former H. pylori Korean Type Culture Collection (HpKTCC; College of Medicine, Gyeongsang National University, Korea; 2006–2015) with anonymized clinical information. Among the deposited strains, H. pylori strains recovered from patients with chronic gastritis, peptic ulcer, and gastric cancer were selected for analysis. Frozen stocks within 50 subcultures were revitalized and grown on Brucella agar (Accumedia, USA) plates containing the same composition as Mueller-Hinton agar at 37°C under 10% CO2 and 100% humidity.

**Preparation of Genomic DNA from H. pylori**

Genomic DNA was extracted from H. pylori as described previously [39]. Briefly, cultured bacterial colonies were collected and suspended in 1 ml of phosphate-buffered saline (PBS, pH 7.2). Bacterial cells were harvested by centrifugation at 12,700 ×g for 5 min and suspended in 200 μl of Tris-EDTA (TE) buffer (pH 7.0). After adding 600 μg of lysozyme (Genery, China), the cell mixture was incubated at 37°C for 1 h. Then, after adding 20 μl of 10% SDS solution and 10 μg of RNase (RBC, Taiwan), the cell mixture was incubated at 37°C for 1 h, followed by treatment with 120 μg of Protease K (Genet Bio, Korea) and 172 μg of pronase (Merck) at 37°C for 1 h. After adding 1/10 volume of 5% cetyltrimethylammonium bromide (BDH Chemicals Ltd., England)-0.5 M NaCl solution and incubating at 37°C for 1 h, the mixture was chloroform extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution and vortexing. After centrifuging at 12,700 ×g for 10 min, the aqueous layer was collected, mixed with 1/10 volume of 3 M sodium acetate (pH 5.2), and added to an equal volume of isopropanol. After incubating at −70°C for 20 min, the sample was centrifuged at 12,700 ×g for 10 min. DNA pellets were washed with 1 ml of 70% ethanol, dried completely, dissolved in 30 μl of TE buffer, and frozen at −70°C until required.
Extracted DNA (50 ng) was PCR amplified with AccuPower PCR PreMix using 10 pmol of forward and reverse primers listed in Table 1. After pre-denaturation at 94°C for 4 min, PCR was carried out at the annealing temperature for 1 min with extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min. The annealing temperatures and number of cycles are listed in Table 1. Amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized and analyzed with a Fluor-S MultiImager (Bio-Rad, USA).

For nucleotide sequence analysis, PCR was performed using the *pfu* PCR Kit (Cat. No. EBT-11011, ELPIS, Korea), 4 ng of genomic DNA, 10 pM of primers, and 0.5 unit of *pfu* DNA polymerase under the conditions described above. Amplified PCR product was purified using an EZ-Pure PCR Purification Kit (Cat. No. EP201-50N, Enzymomics, Korea). Purified PCR products were sequenced using an Applied Biosystems 3730xl DNA Analyzer (Carlsbad, USA).

**Table 1. Sequences of oligonucleotide primers and PCR conditions for the genotyping of *H. pylori* isolates.**

| Genes | Primer F (5' ⇒ 3') | Primer R (5' ⇒ 3') | Annealing temp | Cycles | Amplified DNA size (bp) | References |
|-------|--------------------|--------------------|----------------|--------|------------------------|------------|
| cagA 5' region | GATAAACGGCGAACATTTTTGCTG | CTGCAAAAGATTTGTTTGGCCAGA | 55°C | 35 | 349 | [16] |
| cagA EPIYA FR | ACCCTAGTCCGTAATGCG | GCAATTATTTGTATATCCCGAGTC | 48°C | 30 | 293–299 | [72] |
| cagA EPIYA ISR | GCAAAGTTTTTGAATACCCACGTAATGCTC | GCTTTAGCTCTGAYAGC | 48°C | 30 | 222 | [72] |
| vacA s1/s2 | ATGGAATAACAAACACACACAC | CTTGCTGAATGCGCCA | 52°C | 35 | 259/286 | [73] |
| vacA s1a | TCTGGCTTTTATAGGAGAC | CTTGCTGAATGCGCCA | 52°C | 35 | 212 | [16] |
| vacA s1b | AGGCGCATACCGCAAGAG | CTTGCTGAATGCGCCA | 55°C | 35 | 187 | [73] |
| vacA s1c | CTCCCTTATGCGG | CTTGCTGAATGCGCCA | 55°C | 35 | 213 | [16] |
| vacA m1/m2 | CAATCTGCTCATAACCGAACGG | GCGCTCTAAATCAAACGAG | 52°C | 35 | 570/645 | [16] |
| vacA i1 | TATAATTTAACGCTGTTTAGGAA | GTTGGGATTGGGGGAGTGC | 53°C | 35 | 426 | [74] |
| vacA i2 | GATCAACGTTTCTGATTTGGA | GTTGGGATTGGGGGAGTGC | 53°C | 35 | 432 | [74] |
| alpA | AGCGTTTCTCTCAATACCC | AACGATATATAACGCAT | 65°C | 40 | 304 | [75] |
| alpB | TGCCCGGGAGCTTTGAGGCAAC | TGGCTAAAGGGCGGCTCCAA | 55°C | 35 | 505 | Designed |
| oipA | CAAGCGCTTAAACAGATAGGC | AAGGCGTCTTTCTGCTGAG | 55°C | 35 | 450 | [76] |
| babA2 | AATCCAAAAAGGGAGAA | ATGTTGCTGAAAGATTAGTC | 50°C | 38 | 180 | [26] |
| hopZ | GCCTGATATGGGGGATG | ATTTGATAGCCCGCTGAT | 50°C | 35 | 493 | [77] |
| sabA | GAGCTATTTGACCAGCTGAAAGC | TAGTTTTGATCTGTTCTGATTA | 50°C | 35 | 447 | [79] |
| iceA1 | TATTTCTGTGGAGCTTCTGGCAACCTGTGAT | GGCTTAAAAGGGCGGCTCCAA | 55°C | 30 | 642 | [79] |
| iceA2 | CGGCTGTAGGCACTAAAGCTA | TCAATCTGATGAACAAATATGCTG | 55°C | 30 | 662 | [79] |
| iceA1Δ94 | TCTCGTGAAAGGAATATTTCTGTTTATC | CACAAACCACCATATCTTCGCCCTCCCCCTCATA | 55°C | 30 | 520 | [79] |
| hrgA | GTGTAAGTCTTTGTTTTGGAAGGTC | TAAGTGGGATTGATATCTTGC | 55°C | 30 | 682 | [80] |
| hpyIIIR | TGTCTGGAGATGGGMTTGGGAAATCT | TCTCTGATTGGATCTGTTG | 55°C | 30 | 443 | [80] |
| hpyIIIM | CTGTTGCTGATGGGATG | TCTCATAGGATCTGTTG | 55°C | 30 | 562 | [80] |
| dupA jhp0917 | TTGCTTTCTACTGACAGG | AAGCTGCTACGGAGCACGCG | 57°C | 30 | 307 | [22] |
| dupA jhp0918 | CCTATATCGTACGCG | AAGCTGCTACGGAGCACGCG | 57°C | 30 | 276 | [22] |
Vacuolation Activity Test

Vacuolation activity was measured using RK-13 (ATCC CCL-37) cells as described previously with slight modifications [40]. Briefly, RK-13 cells were grown overnight in RPMI 1640 medium (Gibco) supplemented with 5% fetal bovine serum (FBS; Gibco), adjusted to 1.5 × 10^4 cells/100 μl, added in 96-well microplates at 100 μl/well and incubated for 4 h. H. pylori strains were grown in a thin-layer liquid culture system [1]. Briefly, bacteria were grown overnight on a Petri dish (100 mm diameter) containing 3 ml of Brucella broth (Accumedia) supplemented with 10% bovine serum (Gibco) at 37°C for 24 h in an atmosphere of 10% CO2 and 100% humidity. Supernatants (50 μl) were harvested, serially (1:2) diluted with the culture medium, added into 96-well plates of RK-13 cells, and incubated at 37°C for 12 h. The degree of vacuolation was observed under a light microscope (Olympus, Japan). Vacuolation activity was defined as the reciprocal of the dilution factor of the culture supernatant in which 10% of cells were vacuolated. Vacuolation activity was defined as the reciprocal of the dilution factor of the culture supernatant in which 10% of cells were vacuolated.

Statistical Analysis

The two-way association between genotypes of virulent factors and clinical entities of gastric disease was examined using the Chi-square test and Fisher’s exact test. The distribution of patient ages among clinical entities of gastric disorder was assessed with an independent two-sample t-test. A P-value of < 0.05 was considered statistically significant. Odds ratios (ORs) adjusted for sex were given with 95% confidence intervals to estimate the risk. All data were statistically analyzed using the Statistical Package for Social Sciences 22 (SPSS Inc., USA).

Results

Patients and H. pylori Isolates

A total of 116 H. pylori strains were recovered from patients diagnosed clinically and pathologically with chronic gastritis (38 strains; male/female, 18/20), peptic ulcers (39 strains; male/female, 32/6), and gastric cancers (39 strains; male/female, 25/14) [35-37]. Patients were from the southern area of the Korean peninsula. The average patient age with standard deviation was 48.6 ± 12.1 years for those with chronic gastritis, 48.4 ± 13 years for those with peptic ulcer, and 52.9 ± 11.1 years for those with gastric cancer. Patient ages did not significantly differ from each other, although the average age of patients with gastric cancer was higher than that of others (Fig. 1). Strains that had been subcultured less than 50 times were chosen for this study to minimize the probability of genomic mutation during in vitro maintenance.

Polymorphism of vacA Alleles

vacA allele polymorphisms are determined by variation in the signal sequence region (s1a, s1b, s1c, and s2), mid-region (m1 and m2), and intermediate region (i1, i2, and i3). In this study, the vacA genotype in the signal sequence region was the s1 type for all 116 H. pylori strains, whereas 109 strains (94%) were grouped as the m1 type in the mid-region, and 110 strains (95%) were grouped as the i1 type in the intermediate region (Table 2). No strains exhibited the s2 type. Only 6% and 2.5% of strains were grouped as m2 and i2 types, respectively. The s1 subtypes s1a and s1c were identified in 37 (32%) and 79 (68%) strains, respectively, whereas the s1b subtype was not detected. Eighteen strains associated with chronic gastritis (47%) were grouped into the s1a subtype, which was more than the number of strains associated with gastric cancer (eight strains, 21%) grouped into this subtype. In contrast, 31 (79%) strains associated with gastric cancer were grouped into the s1c subtype, which was significantly (p = 0.017) higher than the number (20 strains, 53%) of strains associated with chronic gastritis grouped into this subtype. No significant differences were found when vaculating toxin activity was measured, although the average value for the gastric cancer-associated strains was higher than that of those associated with chronic gastritis or peptic ulcer (Fig. 2 and Table 2). There was no significant difference in vaculating toxin activity between the s1a and s1c subtypes. However, 33% of gastric cancer strains were classified into the extremely strong group, which was significantly (p = 0.026) higher than that of chronic gastritis strains or peptic ulcer strains.

Fig. 1. Age distribution of patients with chronic gastritis, peptic ulcer, or gastric cancer.
when toxin activity was classified into the following four groups: extremely strong (>41, more than the average value plus 1 SD); strong (41–21.8, from less than the extremely strong to the average); weak (21.7–2.6, from the average value to the average minus 1 SD); and extremely weak (<2.6, less than the value of the average minus 1 SD) (Table 3).

**Genotypes of cagA**

The cagA gene was found in almost all strains, with the exception of two strains isolated from patients with chronic gastritis. Polymorphisms in the 3′-region of cagA were analyzed by PCR and nucleotide sequencing. Five kinds of repeat patterns of EPIYA were identified in cagA-positive H. pylori isolates, and most H. pylori isolates

| vacA genotypes and Vac activity | Total (n = 116) | Chronic gastritis (n = 38) | Peptic ulcer (n = 39) | Gastric cancer (n = 39) |
|--------------------------------|----------------|---------------------------|-----------------------|------------------------|
| s1                            | 116 (100%)     | 38 (100%)                 | 39 (100%)             | 39 (100%)              |
| s1a                           | 37 (32%)       | 18 (47%)                  | 11 (28%)              | 8 (21%)                |
| s1b                           | 0              | 0                         | 0                     | 0                      |
| s1c                           | 79 (68%)       | 20 (53%)                  | 28 (72%)             | 31 (79%)              |
| s2                            | 0              | 0                         | 0                     | 0                      |
| m1                            | 109 (94%)      | 36 (95%)                  | 37 (95%)             | 36 (92%)              |
| m2                            | 7 (6%)         | 2 (5%)                    | 2 (5%)                | 3 (8%)                |
| i1                            | 110 (95%)      | 35 (89%)                  | 38 (97%)             | 37 (95%)              |
| i2                            | 3 (2.5%)       | 1 (3%)                    | 0                     | 2 (5%)                |
| i1,i2(-)                      | 3 (2.5%)       | 2 (5%)                    | 1 (3%)                | 0                     |

Vacuolating toxin activity 21.8±19.2 20.3±17.2 18.7±18.0 26.3±21.2

*p = 0.103 and p = 0.017 compared with that in chronic gastritis with Fisher’s exact test.

### Table 2. vacA genotypes and vacuolating toxin activity of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| Symptom             | No   | Extremely strong (>41) | Strong (41–21.8) | Moderate (21.8–2.6) | Weak (<2.6) |
|---------------------|------|------------------------|------------------|----------------------|-------------|
| Chronic gastritis   | 38   | 5 (13%)                | 8 (21%)          | 20 (53%)             | 5 (13%)     |
| Peptic ulcer        | 39   | 4 (10%)                | 7 (18%)          | 19 (49%)             | 9 (23%)     |
| Gastric cancer      | 39   | 13 (33%)*              | 6 (15%)          | 17 (44%)             | 3 (8%)      |
| Total               | 116  | 22 (19%)               | 21 (18%)         | 56 (48%)             | 17 (15%)    |

Parentheses indicate percentage of each group classified by vacuolating cytotoxin activity. *p = 0.026 compared with groups of chronic gastritis or peptic ulcer using Fisher’s exact test.

when toxin activity was classified into the following four groups: extremely strong (>41, more than the average value plus 1 SD); strong (41–21.8, from less than the extremely strong to the average); weak (21.7–2.6, from the average value to the average minus 1 SD); and extremely weak (<2.6, less than the value of the average minus 1 SD) (Table 3).

### Table 3. Vacuolating toxin activity of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| Symptoms | No   | Extremely strong (>41) | Strong (41–21.8) | Moderate (21.8–2.6) | Weak (<2.6) |
|----------|------|------------------------|------------------|----------------------|-------------|
| Chronic gastritis | 38   | 5 (13%)                | 8 (21%)          | 20 (53%)             | 5 (13%)     |
| Peptic ulcer     | 39   | 4 (10%)                | 7 (18%)          | 19 (49%)             | 9 (23%)     |
| Gastric cancer   | 39   | 13 (33%)*              | 6 (15%)          | 17 (44%)             | 3 (8%)      |
| Total            | 116  | 22 (19%)               | 21 (18%)         | 56 (48%)             | 17 (15%)    |

### Table 4. Repeat patterns of EPIYA in the 3′-region of cagA of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| CagA EPIYA types a | Chronic gastritis (n = 38) | Peptic ulcer (n = 39) | Gastric cancer (n = 39) |
|--------------------|---------------------------|-----------------------|-------------------------|
| ABD or A′bD        | 35 (92.0%)                | 38 (97.4%)            | 35 (89.7%)              |
| ABD/bD or A′bD/bD  | 0                         | 1 (2.6%)              | 3 (7.7%)                |
| ABABD              | 1 (2.7%)                  | 0                     | 1 (2.6%)                |
| CagA negative      | 2 (5.3%)                  | 0                     | 0                       |

a, A, B, and D indicate typical EPIYA segment, b notes the EPIYA-B segment deleted in the 5′-end region including EPIYA motif, and A′ and D′ indicate the segment deleted in the downstream region of EPIYA motif. ABD segments produced DNA fragments of 293–299 bp with cagA EPIYA FR primers and 222 bp with EPIYA JSR primers. When amplified DNAs of different sizes were produced, amplified DNAs were subjected to nucleotide sequence analysis.
grouped into the EPIYA-ABD pattern, which has been frequently isolated in Asia. Four patterns of variant EPIYA-ABDs were found in six strains by PCR and nucleotide sequencing (Table 4). There was no EPIYA motif in the EPIYA-B segment or deletion of amino acid residues in the downstream region of EPIYA-A and/or -D segments. Multiple EPIYA-AB motifs were found in one chronic gastritis strain and one gastric cancer strain. Multiple EPIYA-D segments (EPIYA-ABD′bD and A′bD′bD) were present in one peptic ulcer strain and in three gastric cancer strains.

Genotypes of Adhesion Genes

The genotypes of the adhesion genes *alpA*, *alpB*, *babA2*, *hopZ*, *oipA*, and *sabA* were examined by PCR. Among the 116 strains, *alpA*, *alpB*, *babA2*, *hopZ*, *oipA*, and *sabA* were detected in 88 (76%), 113 (97%), 91 (79%), 96 (83%), 112 (97%), and 70 (60%) strains, respectively (Table 5). The prevalence of *alpA* in peptic ulcer strains (82%) and gastric cancer strains (87%) was significantly (*p* = 0.026 and 0.005, respectively) higher than that in chronic gastritis strains (58%), and the prevalence of *babA2* in peptic ulcer (95%) or gastric cancer strains (95%) was also significantly (*p* = 0.000) higher than that in chronic gastritis strains (45%). The prevalence of *hopZ* was significantly (*p* = 0.036) higher in the gastric cancer strain (92%) than that in the chronic gastritis strains (73%).

The prevalence of *alpB*, *oipA*, and *sabA* did not differ significantly among the three clinical entities of gastric diseases (Table 5).

Genotypes of Genes in the Endonuclease Restriction/Modification Systems

Genes in the endonuclease restriction/modification systems were used for genotyping *H. pylori* isolates, including *hrgA*, *hpgIII*, and *iceA*. Among the 116 strains, *hrgA*, *hpgIII*, and *hpgIIIM* were detected in 49 (35%), 79 (69%), and 116 (100%) strains, respectively. The *iceA1* and *iceA2* genotypes were found in 98 (84%) and 15 (13%) strains, respectively. The prevalence of *hrgA* in chronic gastritis (44%) strains was higher than that in peptic ulcer (21%) or gastric cancer (33%), although there was no significant difference among the three groups. The prevalence of *hpgIII* and *iceA* was not significantly associated with clinical outcomes (Table 6).

Genotypes of *dupA* Genes in CagPAI

Genotyping of *dupA* genes was performed by PCR-based identification of *jhp0917* and *jhp0918; dupA* was detected in 28 (24%) strains while *jhp0918* was found in 2 (2%) strains. The prevalence of *dupA* in peptic ulcer strains (26%) and gastric cancer strains (28%) was higher than that in chronic gastritis strains (18%), although this lacked statistical significance (Table 7).

### Table 5. Genotypes of adhesion proteins of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| Genotypes | Total (n = 116) | Chronic gastritis (n = 38) | Peptic ulcer (n = 39) | Gastric cancer (n = 39) |
|-----------|----------------|---------------------------|----------------------|------------------------|
| *alpA*    | 88 (76%)       | 22 (58%)                  | 32 (82%)             | 34 (87%)               |
| *alpB*    | 113 (97%)      | 35 (92%)                  | 39 (100%)            | 39 (100%)              |
| *babA2*   | 91 (79%)       | 17 (45%)                  | 37 (95%)             | 37 (95%)               |
| *hopZ*    | 96 (83%)       | 28 (73%)                  | 32 (82%)             | 36 (92%)               |
| *oipA*    | 112 (97%)      | 35 (92%)                  | 38 (97%)             | 39 (100%)              |
| *sabA*    | 70 (60%)       | 25 (66%)                  | 23 (59%)             | 22 (56%)               |

*p* = 0.026, †*p* = 0.005, ‡*p* = 0.000, §*p* = 0.000, and ¶*p* = 0.036 compared with those with chronic gastritis using Fisher’s exact test.

### Table 6. Genotypes of endonuclease enzyme genes of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| Genes     | Total (n = 116) | Chronic gastritis (n = 38) | Peptic ulcer (n = 39) | Gastric cancer (n = 39) |
|-----------|----------------|---------------------------|----------------------|------------------------|
| *hrgA*    | 36 (35%)       | 16 (44%)                  | 8 (21%)              | 12 (30%)               |
| *hpgIII*  | 80 (70%)       | 22 (56%)                  | 30 (77%)             | 27 (70%)               |
| *hpgIIIM* | 116 (100%)     | 38 (100%)                 | 39 (100%)            | 39 (100%)              |
| *iceA1*   | 98 (84%)       | 30 (78%)                  | 34 (87%)             | 33 (85%)               |
| *iceA1.494* | 2 (2%)   | 1 (3%)                    | 0                    | 1 (3%)                 |
| *iceA2*   | 15 (13%)       | 6 (16%)                   | 4 (10%)              | 5 (13%)                |

### Table 7. Genotypes of *dupA* in CagPAI of *H. pylori* isolates recovered from patients with chronic gastritis, peptic ulcer, or gastric cancer.

| Genes     | Total (n = 116) | Chronic gastritis (n = 38) | Peptic ulcer (n = 39) | Gastric cancer (n = 39) |
|-----------|----------------|---------------------------|----------------------|------------------------|
| *jhp0918* | 2 (2%)         | 2 (5%)                    | 0                    | 0                      |
| *jhp0917−0918* | 28 (25%) | 7 (18%)                   | 10 (26%)             | 11 (28%)               |
Table 8. Analysis of the contributory role of genotyping factors and vacuolating toxicity in the development of gastric cancer and peptic ulcer.

| Genotypes          | Peptic ulcer OR (95% CI) | Gastric cancer OR (95% CI) | Peptic ulcer OR (95% CI) | Gastric cancer OR (95% CI) | Peptic ulcer OR (95% CI) | Gastric cancer OR (95% CI) | Peptic ulcer OR (95% CI) | Gastric cancer OR (95% CI) | Extremely strong vacuolating toxin |
|--------------------|--------------------------|---------------------------|--------------------------|---------------------------|--------------------------|---------------------------|--------------------------|---------------------------|---------------------------------|
| vacA1c             | 1.7 (0.60–5.2)           | 3.2 (1.1–9.8)             | 7.0 (2.0–24.9)           | 4.5 (1.2–17.6)           | 27.5 (5.0–151.2)         | 21.7 (4.4–106.3)          | 0.7–8.8 (0.07–8.8)         | 0.000 (0.000)               | 1.4 (0.3–6.0)                  |
| alpA               | 0.103 (0.017)            |                           | 0.026 (0.005)            |                           | 0.000 (0.000)            |                           | 0.421 (0.036)             |                           | 0.702 (0.009)                 |
| hopZ               |                          |                           |                          |                           |                          |                           |                          |                           | 1.3 (0.1–9.8)                |
| babA2              |                          |                           |                          |                           |                          |                           |                          |                           | 1.1–11.2                      |

Contribution of Genotyping Factors and Vacuolating Toxicity to the Development of Severe Gastric Symptoms

Among the bacterial factors analyzed in this study, the prevalence of genotypes vacA1c, alpA, babA2, and hopZ and the extremely strong vacuolating toxicity showed significant differences between strains associated with chronic gastritis strains and those associated with severe symptoms (peptic ulcer and gastric cancer). The presence of the babA2 genotype contributed the most to the development of peptic ulcer (OR: 27.5; 95% CI: 5.0–151.2; p = 0.000) and gastric cancer (OR: 21.7; 95% CI: 4.4–106.3; p = 0.000) (Table 8). The presence of alpA also contributed to the development of peptic ulcer (OR: 7.0; 95% CI: 2.0–24.9; p = 0.026) and gastric cancer (OR: 4.5; 95% CI: 1.2–17.6; p = 0.005). Genotypes of vacA1c and hopZ also showed significant associations with the development of gastric cancer. Extremely strong vacuolating toxicity was significantly associated with the development of gastric cancer compared with that of chronic gastritis (OR: 3.5; 95% CI: 1.1–11.2; p = 0.009) (Table 8).

Combination of Genotypes Predicting the Development of Peptic Ulcer and Gastric Cancer

A total of 11 combinations generated by double, triple, and quadruple genotypes of vacA1c, alpA, babA2, and hopZ were significantly associated with the development of severe symptoms (Table 9). Among these combinations, babA2/hopZ was present at the highest proportion (82.1%) in severe symptom strains, followed by alpA/babA2 (79.5%), alpA/hopZ (75.6%), vacA1c/babA2 (71.8%), and alpA/babA2/hopZ (70.5%). The combination of vacA1c/alpA/babA2 was the most predictable determinant for the development of severe symptoms (OR: 15.8; 95% CI: 4.6–53.8; p = 0.000), followed by vacA1c/alpA/hopZ (OR: 11.6; 95% CI: 3.4–39.6; p = 0.000), alpA/babA2 (OR: 10.9; 95% CI: 3.9–30.5; p = 0.000), vacA1c/babA2 (OR: 8.4; 95% CI: 3.0–23.6; p = 0.000), and vacA1c/babA2/hopZ (OR: 8.6; 95% CI: 3.0–25.0; p = 0.000).

Among the four genotype markers, the OR average of double and triple combination groups containing babA2 showed the highest value when compared with combinations containing other genotype markers, demonstrating that babA2 might be the most critical determinant in the development of severe symptoms of gastric diseases (Tables 8 and 9).

Discussion

Toxigenic strains of H. pylori carrying cagA and vacA are known to be closely associated with the development of gastric diseases such as peptic ulcer and gastric cancer [2, 10, 11, 14, 15]. Notably, although infections of H. pylori bearing cagA and vacA cause chronic gastritis in many cases, they are not always associated with peptic ulcer or gastric cancer [3]. Only approximately 10–15% of infected individuals were reported to develop peptic ulcer, gastric carcinoma, and MALT lymphoma [6, 19]. In Korea, most people are infected early in life with toxigenic H. pylori strains bearing cagA and vacA [20–22], but only a small proportion of these individuals have

Table 9. The multiple correspondence analysis of the genotypes vacA1c, alpA, babA2, and hopZ with gastric symptoms.

| Genotypes          | Double (95% CI) | Triple (95% CI) | Quadruple (95% CI) |
|--------------------|----------------|----------------|-------------------|
| VacA1c             | +              | +              | +                 |
| AlpA               | +              | +              | +                 |
| BabA2              | +              | +              | +                 |
| HopZ               | +              | +              | +                 |

No. (% in 38 chronic gastritis strains) | 10 (26.3) | 8 (21.1) | 15 (39.5) | 11 (29.0) | 18 (47.4) | 13 (34.2) | 4 (10.5) | 6 (15.8) | 8 (21.1) | 10 (26.3) | 4 (10.5) |

No. (% in 78 severe symptom strains) | 52 (66.7) | 56 (71.8) | 52 (66.7) | 62 (79.3) | 59 (75.6) | 64 (82.1) | 49 (62.8) | 49 (62.8) | 46 (59.0) | 55 (70.5) | 43 (55.1) |

Odds ratio | 7.0 | 8.4 | 3.3 | 10.9 | 4.0 | 8.9 | 15.8 | 8.6 | 6.5 | 7.4 | 11.6 |

95% CI | 2.7–18.0 | 3.0–23.6 | 1.4–8.0 | 3.9–30.5 | 1.5–10.6 | 3.4–23.2 | 4.6–53.8 | 3.0–25.0 | 2.4–17.1 | 2.8–19.3 | 3.4–39.6 |

p-value | 0.000 | 0.000 | 0.000 | 0.000 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
complained of severe gastric maladies [21-23]. These studies suggest that, along with \(cagA\) and \(vacA\), other virulence factors of \(H. pylori\) are involved in disease outcomes.

A variety of candidate virulence factors have been identified for their role in provoking gastric pathogenesis. Unfortunately, their relevance to severe gastric disorders has proved epidemiologically controversial [3].

The reported discrepancies in the relationship between virulence factors and disease outcomes may be due to differences in prevalence rates and the types of \(cagA\) and \(vacA\) virulence factors present in different regions [3, 5, 26-28, 41, 42]. In contrast, most Korean \(H. pylori\) isolates contain \(cagA\) and \(vacA\), thereby simplifying the analysis of the influence of other virulence factors and the understanding of their roles in the development of severe gastric symptoms.

The risk prediction of severe gastric symptoms according to virulence factors is critical in the management and treatment of \(H. pylori\) infections. Therefore, in this study we investigated the relevance of virulence factors to clinical symptoms and sought to establish risk rates for the development of gastric diseases caused by \(H. pylori\) infections based on previously identified virulence factors.

**Difference in vacuolating ability of \(H. pylori\)**: The vacuolating ability of \(H. pylori\) was examined using a vacuolating cytotoxin assay. The vacuolating cytotoxin activities of all 116 strains were significantly different between peptic ulcer strains (72%) and gastric cancer strains (79%) than those (28%) in peptic ulcer strains and 21% in gastric cancer strains) of symptom groups (Table 2). However, when analyzed based on the expressed toxicity were analyzed. Values for the vacuolating cytotoxin activities of all 116 strains have been reported to be distributed from 0 to 191 with no significant difference among gastric symptoms (Table 2). However, when analyzed based on the expressed toxicity were analyzed. Values for the vacuolating cytotoxin activites of all 116 strains were distributed from 0 to 191 with no significant difference among gastric symptoms (Table 2).

**Development of vacA**: In this study, the presence of \(alpA\), \(babA2\), and \(hopZ\) in \(H. pylori\) strains with severe gastric symptoms was significantly higher than that in chronic gastritis strains (Table 5). \(alpA\) is known as a virulence factor involved in signal transduction of host epithelial cells during \(H. pylori\) infection [4, 48]. Previous studies also demonstrated that \(alpA\) and \(babA2\) play an important role in bacterial colonization [24, 25, 48]. AlpA and AlpB have not yet been clearly identified as a virulence factor in humans and their role remains controversial [4]; however, in this study, we confirmed that the presence of \(alpA\) was significantly correlated with the development of gastric cancer in \(H. pylori\) infection (Table 5, \(p < 0.05\)).

**Previous studies showed that the presence of \(babA2\)**: The presence of \(babA2\) is known as a virulence factor involved in signal transduction of host epithelial cells during \(H. pylori\) infection [4, 48]. Previous studies also demonstrated that \(babA2\) and \(hopZ\) have a strong correlation to disease outcome [29, 50]. Although there is still controversy as to whether \(babA2\) is related to the development of severe gastric symptoms, we observed the presence of \(babA2\) with high frequency in patients with peptic ulcer and gastric cancer in this study (Table 5, \(p < 0.05\)).

\(hopZ\) was found to be regulated at the transcriptional level according to pH change [51], and \(hopZ\) expression depends on an on/off switch during early bacterial colonization [29]. These results indicate that \(hopZ\) has a strong selective advantage in vivo, which may be an important role in adaptation to the host environment and colonization of the gastric mucosa during early infection [29]. Nevertheless, it is unclear whether \(hopZ\) is related to other virulence factors or is linked to other clinical diseases [29, 53]. However, we confirmed that the presence of...
the hop2 gene had a significant correlation with the disease outcome of gastric cancer (Table 5, p < 0.05).

Unlike alpA, babA2, and hop2, the difference in gene prevalence of alpB, oipA, and sabA was difficult to determine according to the clinical outcomes. alpB has high homogeneity with alpA and is known to play a similar important role in adhering to gastric tissues and colonization [24, 25, 48]. In this study, most isolates from each clinical symptom group were found to have alpB (97%), and therefore alpB may not be a useful marker for predicting the clinical outcome of H. pylori infection. Previous studies reported that oipA is closely linked to the expression of cagA [31, 54], which was also confirmed in this study with only 2 out of 112 oipA (+) strains being cagA (-) strains. Yamaoka Y. et al. reported that oipA can be functionally turned “on” or “off” by a slipped strand mispairing mechanism [6]. Several studies of prevalence and meta-analysis demonstrated that the oipA “on” function increased the risk of development of peptic ulcer and gastric cancer [55]. Conversely, an induced oipA “on” status is reported to have no relation to the risk of severe gastric symptoms [27, 54, 56]. This study focused on discovering virulence factors of H. pylori that could be detected by PCR and then used as markers of development of gastric symptoms, which would be more useful in clinical approaches than sequencing to identify the functional status of oipA. This limitation made it more difficult to find any link between oipA prevalence and disease outcome in this study. Moreover, alpA does not appear to be a useful marker for predicting the clinical outcome of H. pylori infection because most H. pylori isolates in Korea were identified as virulent strains [56].

sabA is known to be associated with chronic infection establishment of H. pylori [57]. The proportion of the sabA gene was slightly higher in chronic gastritis strains than in others, although this lacked statistical significance (Table 5). A survey in Japan reported that sabA was linked to gastric cancer [58]. However, although severe neutrophil infiltration and epithelial atrophy are associated with sabA, there are reports that sabA does not affect clinical outcome, and therefore the relationship between sabA and clinical diseases remains controversial [58, 59].

Alleles of restriction and modification systems, including iceA and hrgA, are reportedly predictive of gastric symptoms in East Asia [14, 33, 60]. However, the relatiobship between H. pylori iceA/hrgA and clinical outcomes is still controversial [5, 13, 61]. There are two main allelic variants, iceA1 and iceA2, of which iceA1 is reported to be predominant in East Asia [62]. Several studies have suggested that iceA1 is frequently found in strains isolated from patients with peptic ulcer and gastric cancer [33, 60, 62], whereas others have shown different findings [16, 63], even though iceA1 is presumed to facilitate neutrophil filtration and inflammation [62, 64]. In this study, the prevalence of iceA1 was higher in peptic ulcer- and gastric cancer-associated strains in comparison with that of iceA2, and significant discrepancies were not found. H. pylori has a highly heterogeneous and variable type II R-M system, whereas the hypIII R-M system contains two genes, hypIII R and hypIII M [12]. The hrgA gene, which is considered an important virulence determinant in H. pylori-associated gastric diseases, can replace hypIII R [14]. Therefore, the correlation between hrgA/hypIII R status and clinical outcome has been used to determine H. pylori toxicity [14]. Subgroup analysis of possible correlations between clinical outcome and hrgA/hypIII R status suggested that the prevalence of the hrgA gene was increased among gastric cancer patients (42%) in East Asian countries compared with that in patients without gastric cancer (17%) [14]. In contrast, another study reported that hrgA/hypIII R status and iceA genotypes are not related to gastric outcomes, although regional differences in the prevalence between Asia and the West were observed [61]. In addition, these authors suggested that the prevalence of the hrgA gene was not related to other putative virulence factors, such as cagA, vacA, or iceA, in either East Asia or Western countries [61]. In this study, the prevalence of hrgA gene was higher, though not significantly, in chronic gastritis strains than in other strains, showing different results from the previous study. Thus, the results of studies on hrgA are contradictory. This may be because hrgA prevalence was not related to other putative virulence factors (cagA, vacA, or iceA), which play an important role in the development of peptic ulcer disease caused by H. pylori infection [22, 61].

Members of the pathogenic island region (CagPAI) of H. pylori containing the type IV secretion system have been proposed as playing a role in the pathogenesis of gastric diseases [12, 19]. dupA is a component gene of the type IV secretion system, which encompasses both jhp0917 and jhp0918. dupA is the first identified disease-specific H. pylori virulence factor that induces duodenal ulcer and has a suppressive action on gastric cancer [22]. Despite the reported gastric cancer inhibitory function, a pooled analysis of data from three Western countries (the USA, Belgium, and South Africa) linked the presence of the dupA gene to peptic ulcer and gastric cancer [65]. In addition, although there is a large regional difference in prevalence of the dupA gene, this may be a risk factor for gastric cancer along with duodenal ulcer [66]. However, a meta-analysis using 11–12 studies on dupA (9 countries) reported that dupA had no correlation with the development of peptic ulcer and gastric cancer [67]. Other studies also reported no correlation between dupA and gastric disease outcome, and this association remains controversial [68, 69]. In this study, the prevalence of dupA (jhp0917) was higher, but not significantly, in peptic ulcer- and gastric cancer-associated strains than that in chronic gastritis-associated strains. The dupA gene may be mutated and protein expression may be inhibited. Therefore, a study based on DupA protein expression should be considered to accurately analyze the correlation between dupA expression and clinical outcome [6].

Prediction of the severe gastric disease outcome of H. pylori using one virulence factor is difficult. Many virulence factors have been correlated with each other. The oipA “on” status has been found to be associated with cagA and vacA [27, 30], and other major virulence factors have also been reported to be correlated [3]. Therefore, severe symptoms may originate from the complex results of several linked virulence factors, rather than the function of a single factor [3, 52, 53, 57]. Multiple correspondence analysis has been shown to serve as a better approach for the prediction of peptic ulcer, gastric cancer, and MALT lymphoma [26, 57]. Therefore, this study was conducted using the four identified virulence factors, vacAs1c, alpA, babA2, and hopZ, that showed significant discrepancies in their prevalence between chronic gastritis strains and severe symptoms, to investigate whether clinical outcome could be predicted with each combination. Eleven combinations of genotypes using the
four virulence factors were generated (Table 9), all of which showed an OR value of 3.3 or higher (p < 0.001). These results confirmed that all four factors could affect gastric symptoms and be used as markers for disease outcome. Among the combinations, the triple genotype vacAs1c/-alpA/babA2 was the most predictable for the development of severe symptoms (EOD: 15.8). The average OR of double and triple combination groups containing babA2 was significantly higher than that of the other groups. Taken together, these data suggest that the genotype factor babA2 might contribute more to the development of severe gastric symptoms than others.

Various other factors, as well as genotype factors, play an important role in the development of severe clinical symptoms, including age, sex, genetic characteristics, diet conditions, and underlying diseases [3, 54]. The link between gene presence and functional protein expression must also be considered [28]. In addition, the possibility of complex *H. pylori* infections in which different genotype combinations coexist cannot be ruled out [70]. In this case, only one strain of *H. pylori* was isolated from each patient, making it difficult to analyze the correlation between virulence factors and disease outcome. The large regional differences in the prevalence of virulence factors also impose limitations, causing bias in the analysis results. In this study, 116 strains of *H. pylori* isolates from patients with no significant age difference were analyzed (Fig. 1): all strains were vacA*G* genopositive while 114 strains were cagA genopositive. The OR value for the development of gastric symptoms and the statistical significance in the relevance of genotype factors to gastric symptoms were calculated by the adjustment of sex using the Mantel-Haenszel method [71] to overcome gender imbalance. However, other limiting factors in this study remain a challenge.

This study attempted to identify candidate virulence factors of *H. pylori* that can predict severe gastric symptoms via PCR screening. Since this study analyzed *H. pylori* isolated over a limited period of time, it could not provide information on the current prevalence of the virulence factors. However, we found that the presence of vacAs1c, alpA, babA2, and hopZ genes could increase the risk of disease outcome in infections with toxigenic *H. pylori* that also harbor the cagA and vacA genes. Moreover, we confirmed that severe gastric symptoms could be predicted at a high level of possibility through multiple correspondence analysis using a combination of these four genes. This could improve our understanding of the role of these virulence factors of *H. pylori*, which will assist in early prediction of disease outcome through the simple PCR method and thereby enable appropriate treatment.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**

1. Joo JS, Park KC, Song JY, Kim DH, Lee KJ, Kwon YC, et al. 2010. A thin-layer liquid culture technique for the growth of *Helicobacter pylori*. Helicobacter 15: 295–302.
2. Blaser MJ, Atherton JC. 2004. *Helicobacter pylori* persistence: biology and disease. J. Clin. Investig. 113: 321–333.
3. Sterberg A, Arc E, Poljak M, Homan M. 2019. *Helicobacter pylori* virulence genes. World J. Gastroenterol. 25: 4870–4884.
4. Matsui Y, Kido Y, Yamaoka Y. 2017. *Helicobacter pylori* outer membrane protein-related pathogenesis. Toxins (Basel) 9: 101.
5. Yamaoka Y, Graham DY. 2014. *Helicobacter pylori* virulence and cancer pathogenesis. Future Oncol. 10: 1487–1500.
6. Yamaoka Y. 2010. Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat. Rev. Gastroenterol. Hepatol. 7: 629–641.
7. Malaty HM. 2007. Epidemiology of *Helicobacter pylori* infection. Best Pract. Res. Clin. Gastroenterol. 21: 205–214.
8. Youn HS, Bai C, Cho YK, Woe HO, Ahn YO, Kim K, et al. 1998. Comparison of *Helicobacter pylori* infection between Fukushima, Japan and Chinju, Korea. Helicobacter 3: 9–14.
9. Shin A, Kim J, Park S. 2011. Gastric cancer epidemiology in Korea. J. Gastric Cancer 11: 135–140.
10. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer 127: 2893–2917.
11. Fock KM, Ang TL. 2010. Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. J. Gastroenterol. Hepatol. 25: 479–486.
12. Alam RA. 1999. Analysis of the genetic diversity of *Helicobacter pylori*: the tale of two genomes. J. Mol. Med. 77: 834–846.
13. Shiotani S, Suzuki R, Yamaoka Y. 2013. The significance of virulence factors in *Helicobacter pylori*. J. Dig. Dis. 14: 341–349.
14. Año T, Wassenaar TM, Pende RM, Aras RA, Tishum AI, van Doorn LJ, et al. 2002. A *Helicobacter pylori* restriction endonuclease-replacing gene, hrgA, is associated with gastric cancer in Asian strains. Cancer Res. 62: 2385–2389.
15. Cho YE, Kim PS, Lee DH, Kim HK, Kim YS, Shin YW, et al. 2002. Diverse vacA allelic types of *Helicobacter pylori* in Korea and clinical correlation. Yonsei Med. J. 43: 351–356.
16. Yamaoka Y, Kosada T, Gutierrez O, Kim IG, Kashima K, Graham DY. 1999. Relationship between *Helicobacter pylori* vacA, cagA, and vacA status and clinical outcome: studies in four different countries. J. Clin. Microbiol. 37: 2274–2279.
17. Kao CY, Sheu BS, Wu JF. 2016. *Helicobacter pylori* infection: An overview of bacterial virulence factors and pathogenesis. Biomed. J. 39: 14–23.
18. McClain MS, Beckett AC, Cover TL. 2017. *Helicobacter pylori* vacuolating toxin and gastric cancer. Toxins (Basel) 9: 316.
19. Kalali B, Mejias Luque R, Javaheri A, Gerhard M. 2014. *H. pylori* virulence factors: influence on immune system and pathology. Mediators Inflamm. 2014: ID 426309.
20. Rhee KH. 1990. Prevalence of *Helicobacter pylori* infection in Korea. J. Korean Soc. Microbiol. 25: 475–490.
21. Ko JS, Kim KM, Oh YL, Seo JK. 2008. cagA, vacA, and iceA genotypes of *Helicobacter pylori* in Korean children. Pediatr. Int. 50: 628–631.
22. Lu H, Hsu PI, Graham DY, Yamaoka Y. 2005. Duodenal ulcer promoting gene of *Helicobacter pylori*. Gastroenterology 128: 833–848.
23. Kim N, Park KY, Cho SI, Lim SH, Lee KH, Lee W, et al. 2008. *Helicobacter pylori* infection and development of gastric cancer in Korea: long-term follow-up. J. Clin. Gastroenterol. 42: 448–454.
24. Senkovich OA, Yin J, Eksbryan V, Conant C, Traylor J, Adegboyega R, et al. 2011. Helicobacter pylori AlpA and AlpB bind host laminin and influence gastric inflammation in gerbils. Infect. Immun. 79: 3166-3161.

25. Diep R, Duarte AH, Rijkema S, Kuipers EJ, van Vliet AH, Kusters JG. 2004. Role of the Helicobacter pylori outer membrane protein AlpA and AlpB in colonization of the guinea pig stomach. J. Med. Microbiol. 53: 375-379.

26. Gerhard M, Luhn N, Neumayer N, Boren T, Rada R, Specht W, et al. 1999. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. Proc. Natl. Acad. Sci. USA 96: 12778-12783.

27. Zamboni C, Navaglia F, Rasso D, Rugge M, Plebani M. 2003. Helicobacter pylori bAb2A, CagA, and s1 vacA genes work synergistically in causing intestinal metaplasia. J. Clin. Pathol. 56: 287-291.

28. Fujimoto S, Ojo AO, Amsel R, Wu JY, Odenbreit S, Haas R, et al. 2007. Helicobacter pylori BabA expression, gastric mucosal injury, and clinical outcome. Clin. Gastroenterol. Hepatol. 5: 49-58.

29. Kennefick L, Brenneke B, Andrews S, Engravid L, Meyer TF, Hebischker T, et al. 2012. In vivo sequence variation in HopZ, a phase-variable outer membrane protein of Helicobacter pylori. Infect. Immun. 80: 4364-4373.

30. Dossumbekova A, Prinz C, Mages J, Lang R, Kusters JG, Van Vliet AH, et al. 2006. Helicobacter pylori HopH (OipA) and bacterial pathogenicity: genetic and functional genomic analysis of hopH gene polymorphisms. J. Infect. Dis. 194: 1346-1355.

31. Horridge DN, Begley AA, Kim J, Aravindan N, Fan K, Forsyth MH. 2017. 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. 75: ftx113.

32. Odenbreit S. 2005. Adherence properties of Helicobacter pylori impact on pathogenesis and adaptation to the host. Int. J. Med. Microbiol. 295: 317-324.

33. Peak JR, Thompson SA, Donahue JP, Tham KT, Atherton JC, Blaser MJ, et al. 1998. Adherence to gastric epithelial cells induces expression of a Helicobacter pylori gene, iceA, that is associated with clinical outcome. Proc. Assoc. Am. Physicians 110: 531-544.

34. Kim SY, Woo CW, Lee YM, Son BR, Kim JW, Chee HB, et al. 2001. Genotyping CagA, VacA subtype, IcecA1, and BabA of Helicobacter pylori isolates from Korean patients, and their association with gastro-duodenal diseases. J. Korean Med. Sci. 16: 579-584.

35. Baik SC, Youn HS, Chung MH, Lee WK, Cho MJ, Ko GH, et al. 1996. Increased oxidative DNA damage in Helicobacter pylori-infected human gastric mucosa. Cancer Res. 56: 1279-1282.

36. Song GY, Chang MW. 1999. Antibiotic susceptibility of Helicobacter pylori and the combination effect of antibiotics on the antibiotic-resistant H. pylori strains. J. Korean Soc. Microbiol. 35: 455-462.

37. Baik SC, Youn HS, Chung MH, Lee WK, Cho MJ, Ko GH, et al. 1996. Increased oxidative DNA damage in Helicobacter pylori-infected human gastric mucosa. Cancer Res. 56: 1279-1282.

38. Taylor NS, Fox JK, Akopyants NS, Berg DE, Thompson N, Shames B, et al. 1995. Long-term colonization with single and multiple strains of Helicobacter pylori assessed by DNA fingerprinting. J. Clin. Microbiol. 33: 918-923.

39. Ohta-Tada U, Takagi A, Koga Y, Kamiya S, Miwa T. 1997. Flagellin gene diversity among Helicobacter pylori strains and relationship to clinically significant disease. J. Gastroenterol. Hepatol. 5: 49-58.

40. Ohta-Tada U, Takagi A, Koga Y, Kamiya S, Miwa T. 1997. Flagellin gene diversity among Helicobacter pylori strains and relationship to clinically significant disease. J. Gastroenterol. Hepatol. 5: 49-58.

41. Park JY, Forman D, Waskito LA, Yamoka Y, Crabtree JE. 2018. Epidemiology of Helicobacter pylori and CagA-positive infections and global variations in gastric cancer. Toxins (Basel) 10: 163.

42. Berthetnet E, Tahara K, Thorell K, Pascoe B, Meric G, Mikhail JM, et al. 2018. A GWAS on Helicobacter pylori strains points to genetic variants associated with gastric cancer risk. BMC Biol. 16: 84.

43. Soyfoo DM, Doonah YM, Xu D, Zhang CR, Pan H, Lu JY, et al. 2021. New genotypes of Helicobacter pylori VacA A-region identified from global strains. BMC Mol. Cell. Biol. 22: 4.

44. Aiydin F, Kallikkaya N, Ogur O, Cubukcu K, Kilic A, Tosun I, et al. 2004. Distribution of vacA alleles and cagA status of Helicobacter pylori in peptic ulcer disease and non-ulcer dyspepsia. Clin. Microbiol. Infect. 10: 1102-1104.

45. Hocker M, Hohenberger P. 2003. Helicobacter pylori virulence factors—one part of a big picture. Lancet 362: 1231-1233.

46. Homan M, Luzar B, Kocjan BJ, Mocilnik T, Shrestha M, Kveder M, et al. 2005. Prevalence and clinical relevance of cagA, vacA, and iceA genotypes of Helicobacter pylori isolated from Slovenian children. J. Pediatr. Gastroenterol. Nutr. 45: 289-296.

47. Posse G, Backet R, Wesseler S. 2013. The functional interplay of Helicobacter pylori factors with gastric epithelial cells induces a multi-step process in pathogenesis. Cell Commun. Signal. 11: 77.

48. Lu H, Wu JY, Beswick EL, Onno T, Odenbreit S, Haas R, et al. 2007. Functional and intracellular signaling differences associated with the Helicobacter pylori AlpB AB adhesin from Western and East Asian strains. J. Biol. Chem. 282: 6242-6254.

49. Chen MY, He CY, Meng X, Yuan Y. 2013. Association of Helicobacter pylori babA2 with peptic ulcer disease and gastric cancer. World J. Gastroenterol. 19: 4242-4251.

50. Homan M, Sterbec B, Kocjan BJ, Luzar B, Zidar N, Poljak M. 2014. Prevalence of the Helicobacter pylori babA2 gene and correlation with the degree of gastritis in infected Slovenian children. Antonie Van Leeuwenhoek. 106: 637-645.

51. Merrell DS, Goodrich ML, Otto G, Tompkins LS, Falkow S. 2003. pH-regulated gene expression of the gastric pathogen Helicobacter pylori. Infect. Immun. 71: 3529-3539.

52. Xu C, Soyfoo DM, Wu Y, Xu S. 2020. Virulence of Helicobacter pylori outer membrane proteins: An updated review. Eur. J. Clin. Microbiol. Infect. Dis. 39: 1821-1830.

53. Servetas SL, Kim A, Su H, Cha KH, Merrell DS. 2018. Comparative analysis of the Hom family of outer membrane proteins in isolates from two geographically distinct regions: the United States and South Korea. Helicobacter 23: e12461.

54. Farzí N, Yadegar A, Aghdaie HA, Yamaoka Y, Zali MR. 2018. Genetic diversity and functional analysis of cagA gene in association with other virulence factors among Helicobacter pylori isolates from Iranian patients with different gastric diseases. Infect. Genet. Evol. 60: 26-34.

55. Sallas ML, Dos Santos MP, Orcini WA, David EB, Peruquetti RL, Payão SLM, et al. 2019. Status (on/off) of cagA gene: their associations with gastritis and gastric cancer and geographic origins. Arch. Microbiol. 201: 93-97.

56. Torres KV, Valladerrama E, Saeys M, Ramirez JL, Churillo MA. 2014. Study of the cag/pvAg diversity and EPITAXY motif patterns in cag/pvAg positive Helicobacter pylori strains from Venezuelan patients with chronic gastritis. Microb. Pathog. 76: 26-32.

57. Lehours P, Ménard A, Dupouy S, Bergey B, Richy E, Zerbib F, et al. 2004. Evaluation of the association of nine Helicobacter pylori virulence factors with strains involved in low-grade gastric mucosa-associated lymphoid tissue lymphoma. Infect. Immun. 72: 880-888.

58. Yamaoka Y, Ojo O, Fujimoto S, Odenbreit S, Haas R, Gutiérrez O, et al. 2006. Helicobacter pylori outer membrane proteins and gastrointestinal disease. Gut 55: 775-781.

59. Yanai A, Maeda S, Hikiba Y, Shibata W, Ohmae T, Hiraata Y, et al. 2007. Clinical relevance of Helicobacter pylori sabA genotype in Japanese clinical isolates. J. Gastroenterol. Hepatol. 22: 2228-2232.

60. J. Microbiol. Biotechnol. 378 Lee et al.
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62. Yakoob I, Abbas Z, Khan R, Salim SA, Abrar A, Awan S, et al. 2015. Helicobacter pylori: correlation of the virulence marker iceA allele with clinical outcome in a high prevalence area. Br. J. Biomed. Sci. 72:67-73.
63. Ahsne AAR, Collares GB, Mendes EN, de Gusmão VR, de Magalhães Queiroz DM, Magalhães PP, et al. 2001. iceA genotypes of Helicobacter pylori strains isolated from Brazilian children and adults. J. Clin. Microbiol. 39:1746-1750.
64. Sgouras DN, Trang TTH, Yamaoka Y. 2015. Pathogenesis of Helicobacter pylori infection. Helicobacter 20:8-16.
65. Argent RH, Burette A, Mendsje Deyi YY, Atherton JC. 2007. The presence of dupA in Helicobacter pylori is not significantly associated with duodenal ulceration in Belgium, South Africa, China, or North America. Clin. Infect. Dis. 45:1204-1206.
66. Hussein N. 2010. The association of dupA and Helicobacter pylori-related gastroduodenal diseases. Eur. J. Clin. Microbiol. Infect. Dis. 29:817-821.
67. Shiota S, Matsunari O, Watada M, Hanada K, Yamaoka Y. 2010. Systematic review and meta-analysis: the relationship between the Helicobacter pylori dupA gene and clinical outcomes. Gut Pathog. 2:13.
68. Pacheco A, Proença-Môdena J, Sales A, Fukuhara Y, Da Silveira W, Pimenta-Môdena J, et al. 2008. Involvement of the Helicobacter pylori plasticity region and cag pathogenicity island genes in the development of gastroduodenal diseases. Eur. J. Clin. Microbiol. Infect. Dis. 27:1053-1059.
69. Nguyen L, Uchida T, Tsukamoto Y, Kuroda A, Okimoto T, Kodama M, et al. 2010. Helicobacter pylori dupA gene is not associated with clinical outcomes in the Japanese population. Clin. Microbiol. Infect. 16:1264-1269.
70. Kim JW, Kim JG, Chae SL, Cha YJ, Park SM. 2004. High prevalence of multiple strain colonization of Helicobacter pylori in Korean patients: DNA diversity among clinical isolates from the gastric corpus, antrum and duodenum. Korean J. Intern. Med. 19:1-9.
71. dos Santos Silva I. 1999. Cancer epidemiology: principles and methods, pp. 305-331. Renouf Pub Co Ltd, Lyon, France.
72. Yamaoka Y, El-Zimaity HMT, Gutierrez O, Figura N, Kodama T, et al. 1999. Relationship between the cagA3 repeat region of Helicobacter pylori, gastric histology, and susceptibility to low pH. Gastroenterology 117:342-349.
73. Atherton JC, Caop P, Pek RM, Tummurom KMR, Blaser MJ, Cover TL. 1995. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori: association of specific vacA types with cytotoxin production and peptic ulceration. J. Biol. Chem. 270:17771-17777.
74. Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Hosseini ME, et al. 2007. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 133:926-936.
75. Rokbi B, Seguin D, Guy B, Mazarin V, Vidor E, Mion F, et al. 2001. Assessment of Helicobacter pylori gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. Infect. Immun. 69:4759-4766.
76. Yamaoka Y, Iwamoto D, Graham DY. 2000. a-M, 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. Proc. Natl. Acad. Sci. USA 97:7533-7538.
77. Peck B, Ottkamp M, Dohil KD, Hundt E, Knapp B. 1999. Conservation, localization and expression of HopZ, a protein involved in adhesion of Helicobacter pylori. Nucleic Acids Res. 27:3325-3333.
78. De Jonge R, Pot RGL, Loffeld RLJF, Van Vliet AHM, Kuipers EJ, Kusters JG. 2004. The functional status of the Helicobacter pylori sabB adhesin gene as a putative marker for disease outcome. Helicobacter 9:158-164.
79. Mukhopadhyay AK, Kersulyte D, Jeong JY, Datta S, Ito Y, Chowdhury A, et al. 2000. Distinctiveness of genotypes of Helicobacter pylori in Calcutta, India. J. Bacteriol. 182:3219-3227.
80. Ando T, Wassenaar TM, Peek RM, Aras RA, Tsuchim AI, van Doorn L-J, et al. 2002. A Helicobacter pylori restriction endonuclease-replacing gene, hrgA, is associated with gastric cancer in Asian strains. Cancer Res. 62:2385-2389.