Genotyping CagA, VacA Subtype, IceA1, and BabA of Helicobacter pylori Isolates From Korean Patients, and Their Association with Gastroduodenal Diseases

INTRODUCTION

Although more than 50% of the world’s population is infected with Helicobacter pylori, only a minority of carriers develop serious gastroduodenal diseases (1). There is increasing evidence that the genetic variability of H. pylori may have a clinical importance. Several genes have been identified that may play a role in the pathogenesis of H. pylori, such as cagA, vacA s1/m1, iceA1, and babA.

The cytotoxin-associated gene (cagA) is considered to be a marker for a genomic pathogenicity island (2). Several genes of this cag island encode proteins that enhance the inflammatory responses such as interleukin (IL)-8 production in gastric epithelial cells (3). The vacuolating cytotoxin gene (vacA) is present in all H. pylori strains (4). vacA genotype is composed of a hypervariable signal sequence and a mid-region allele. The vacA subtypes are determined by the combination of s1a, s1b, s1c, and s2, and m1, m2a, and m2b (5). Cytotoxin production and virulence are higher in the s1/m1 subtypes than in the s1/m2 subtype, and lower still in the s2/m2 subtype (6). Previous studies have shown that the vacA subtypes show regional and racial differences, and that the vacA s1a/m1 is associated with peptic ulcer disease (7). Because of the clinical importance of these virulence factors, H. pylori strains are classified as type 1 (VacA-positive and CagA-positive) and type 2 (VacA-negative and CagA-negative) (8), but this classification alone does not allow a proper clinical distinction between pathogenic and non-pathogenic strains, because of the high prevalence of type 1 strains in Korean populations (9).

A novel gene has recently been discovered, designated iceA (induced by contact with epithelium) (10). There are two main allelic variants of the gene, iceA1 and iceA2. The expression of iceA1 is up-regulated on contact between H. pylori and human epithelial cells, and may be associated with peptic ulcer disease (11). Ilver et al. (12) identified the H. pylori blood group antigen-binding adhesion gene, babA, involved in the binding activity between bacterial adhesion and human Lewis blood group antigens on gastric epithelial cells. Several lines of evidence suggest that the presence of babA is
related to the occurrence of peptic ulcer (13, 14).

H. pylori strains may differ in various geographical regions (7), and studies of different populations may clarify the importance and universality of putative virulence factors. In the present study, the prevalence of cagA, iceA, and babA genes, and the subtyping of the vacA gene were investigated in 76 H. pylori isolates recovered from patients with peptic ulcer and gastritis in Korea. The correlation between the genetic status of the isolates and the occurrence of peptic ulcer was assessed.

## MATERIALS AND METHODS

H. pylori was isolated from biopsy specimens sampled from the antrum and the corpus of 41 patients (26 men, 15 women; median age, 52 yr; age range, 20 to 77 yr), in Chungbuk National University Hospital, Cheongju, Korea. Informed consent was obtained from all patients, and research protocols were approved by the Ethics Committee of Chungbuk National University Hospital. No patient had taken bismuth, antibiotics, or omeprazole within the 6 weeks before sampling. The final diagnoses were duodenal ulcer in 15 patients, peptic ulcer in 19 (46.3%) patients. In six gastritis patients, the status of the isolates and the occurrence of peptic ulcer was assessed.

### DNA Isolation

H. pylori DNA was isolated using GeneReleaser (GR; Bio Ventures, Inc., Murfreesboro, Tenn., U.S.A.) (15). One loop of each isolated bacterial strain was added to 20 μL of GR in the thermocycle tube. Samples with GR were denatured (65 ºC for 30 sec, 87 ºC for 30 sec, 65 ºC for 90 sec, 97 ºC for 180 sec, 87 ºC for 60 sec, 65 ºC for 180 sec, 97 ºC for 60 sec, and 65 ºC for 60 sec) in the thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, Conn., U.S.A.).

### Polymerase Chain Reaction for cagA, vacA, iceA1, and babA genes

For the detection of cagA, vacA, iceA1, and babA, polymerase chain reactions (PCRs) were performed in a volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 2 μL of genomic DNA released by GR, 2.5 U of Taq DNA polymerase, and 25 pmol of specific primer sets (Table 1). The PCR primers for cagA, vacA, iceA1 were synthesized as described (5, 10, 16). The babA primers were designed on the basis of the recently published signal sequence of the babA gene (12). The PCR program for cagA comprised 32 cycles of 1 min at 94 ºC, 1 min 30 sec at 55 ºC, and 2 min at 72 ºC (349-bp cagA fragment) or 1 min at 94 ºC, 1 min 30 sec at 48 ºC, and 2 min at 72 ºC (208-bp cagA fragment). The PCR program for vacA was 40 cycles of 30 sec at 94 ºC, 30 sec at 50 ºC, and 30 sec at 72 ºC and that for iceA1 comprised 40 cycles of 30 sec at 94 ºC, 45 sec at 50 ºC, and 45 sec at 72 ºC. The PCR program for babA comprised 30 cycles of 1 min at 94 ºC, 1 min at 38 ºC, and 1 min at 72 ºC. After amplification 10 μL of PCR product was electrophoresed on 1.7% agarose gel and examined under UV illumination.

### Table 1. PCR primers for amplification of cagA, iceA1, babA, and s and m sequences of vacA

| Gene and segment | Primer designation | Primer Sequence (5′→3′) |
|------------------|--------------------|------------------------|
| cagA-349 bp      | Ch1                | GATAACAGCGAAGCTTTTGGGA |
|                  | Cr1                | CTGCAGAGATTTGGTCCGAGA |
| cagA-208 bp      | C1                 | GTATCGATTATTTCAGAC     |
|                  | C2                 | GGGTATGATATATTTCCTT   |
| iceA-557 bp      | ice1f              | GTTGGTAAAGCTTACAGAATTT |
|                  | ice1r              | CATTGATATCCATCTTACAAG |
| babA-190 bp      | LBf                | AATCCATAATTAACCAA     |
|                  | Lbr                | ATAGGTCTGTTGAAGGAT    |
| vacA S           | VA1F               | Bio*-ATGAGATAACAAAACACAAC |
| vacA S           | VA1XR              | Bio-CCTGAPACCTTCTCAGACO |
| vacA M           | MF1.1              | Bio-GTGGATACCTCGATCCTGA |
| vacA M           | MF1.2              | Bio-GTGGATGTCTCATAACGCTWA |
| vacA M           | MF1.3              | Bio-GTGGATCTGCTACATGCTAA |
| vacA M           | MF1.4              | Bio-GCCGCGCTCAGTCTGCGA |
| vacA M           | MR1                | Bio-RTGAGCCTTGTTAGTATGGAC |

R*=G or A, W*=A or C, Bio*=biotin

### Table 2. Allele-specific probes for vacA s and m regions used for reverse hybridization

| Gene segment and probe designation | Probe sequence (5′→3′) | Specificity |
|-----------------------------------|------------------------|-------------|
| vacA-s                            | P1S1                  | GAGGCRTTRGTCAGCATCAC | s1a |
|                                   | P22S1a                | GCTTTAGAGAGGCACTTGC | s1a |
|                                   | P1S1b                 | GAGCCGTTAGATTAGKCCATC | sbb |
|                                   | P2S1b                | GTTTTAGCAGGAGGCTGGA | sbb |
|                                   | P3S1                 | GGGYATTGAGYAGCTAC | scc |
|                                   | P4S1                 | GCTATAGTGGATTTGTT | scc |
|                                   | P1S2                 | GTCAAYGCAGCATAACCATCCC | s2 |
|                                   | P2S2                 | GATCCACGACAGGAGGAG | s2 |
| vacA-m                            | P1M1                 | TTGATACGAGGTAATGAGG | m1 |
|                                   | P2M1                 | GGGTAATGGTGTTTACACAA | m1 |
|                                   | P1M2a                | ACAGAACTTACAGGCTAAGG | m2a |
|                                   | P2M2a                | AGAGGAATAAATGGATGGACA | m2a |

R*=G or A, Y*=C or T, K*=G or T
Reverse hybridization-line probe assay (LiPA) for vacA subtypes

PCR products from the vacA s and m regions were analyzed by reverse hybridization on a line probe assay. A nitrocellulose strip that contains a number of oligonucleotide probe for type-specific detection of H. pylori genotypes was prepared as described earlier (5) (Table 2). Ten μL of each of the PCR products was placed in a plastic trough, and 30 μL of 400 mmol/L NaOH and 10 mmol/L ethylene diamine tetraacetic acid was added to denature the DNA. After 5 min, 1 mL of preheated hybridization buffer (2X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM Tris-HCl [pH 7.5], 0.1% sodium dodecyl sulfate [SDS]) was added, and an LiPA strip was submerged into the solution, followed by incubation in a shaking water bath at 50 ± 0.5 °C for 1 hr. The strips were washed with 1 ml of 2X SSC-0.1% SDS for 30 min at 50 °C. Subsequently, the strips were rinsed three times in phosphate buffer, and streptavidin-alkaline phophatase was added. After 30 min of incubation at room temperature, the strips were rinsed again and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate substrate was added. Positive hybridization was visible as purple probe lines. Interpretation of the results was performed visually.

Statistical analysis

Prevalences were compared using two tailed Fisher’s exact test (SPSS 9.0, Chicago, U.S.A.). A p value <0.05 was considered statistically significant.

Table 3. Genotypes of cagA, iceA1, babA, and vacA genes of 76 H. pylori strains isolated from 41 patients

| Genotype | Peptic ulcer (n=44) | Gastritis (n=32) | Total |
|----------|---------------------|------------------|-------|
| cagA (+) | 43                  | 31               | 74*   (97.4%) |
| 349 bp   | 1                   | 1                | 2 (2.6%) |
| cagA (+) | 44                  | 29               | 73 (96.1%) |
| 208 bp   | 0                   | 3                | 3 (3.9%) |
| iceA1 (+) | 40                | 24               | 64 (84.2%) |
| babA (+) | 12                  | 14               | 26 (36.1%) |
| vacA slc/m1 | 5                    | 4               | 9 (11.8%) |
| not determined | 4             | 0               | 4 (2.1%) |
| vacA s2a/m1 | 0                  | 1              | 1 (1.3%) |
| multiple | 9                   | 5                | 14 (18.4%) |

n*, number of strain (%)
Table 4. Fourteen isolates with multiple vacA subtypes from single biopsy specimen

| Number | Diagnosis | vacA (s) | vacA (m) |
|--------|-----------|----------|----------|
| 1 | DU* | slc | m1+m2a |
| 2 | DU | slc | m1+m2a |
| 3 | DU | sla+slc | m2a |
| 4 | DU | slc | m1+m2a |
| 5 | DU | slc | m1+m2a |
| 6 | DU | slc | m1+m2a |
| 7 | DU | slc | m1+m2a |
| 8 | GU | slc | m1+m2a |
| 9 | GU | slc | m1+m2a |
| 10 | Gastritis | slc | m1+m2a |
| 11 | Gastritis | slc | m1+m2a |
| 12 | Gastritis | slb+slc | m2a |
| 13 | Gastritis | slc | m1+m2a |
| 14 | Gastritis | slc | m1+m2a |

DU*, duodenal ulcer; GU*, gastric ulcer

ied in 0, 2, 2, and 9 patients, respectively. Overall, in 21 (60%) of the 35 strain pairs, the genetic status of cagA, iceA1, babA, and the vacA subtype varied between the isolates taken from the different stomach locations. The four pathogenicity-related genes, cagA, vacA s1c/m1, iceA1, and babA, did not correlate with other genes (p > 0.05).

Relationship between the genetic status (cagA, vacA subtype, iceA1, and babA) and peptic ulcer

The incidence of cagA, vacA s1l/m1, iceA1, and babA genes relative to clinical outcome is described in Table 5. We compared the genetic status of H. pylori isolates taken from the antrum, in a comparison of 22 peptic ulcer patients and 19 gastritis patients. In the cases of mixed vacA subtypes, the presence or absence of s1/m1 in the multiple strains was identified. All isolates from the antrum possessed more than one fragment of the cagA gene. There was no significant difference in the presence of the iceA1 and babA genes in H. pylori isolates from peptic ulcer and gastritis patients (90.9% vs 73.7%, p=0.148, and 27.3% vs 26.3%, p=0.578, respectively). There was no statistical difference in the presence of vacA s1/m1 between peptic ulcer and gastritis groups (95.5% vs 94.7%, p=0.718).

DISCUSSION

The present study demonstrates that the genetic status of H. pylori isolates in Korea is characterized by a high incidence of cagA, vacA s1c/m1, and iceA1, but a relatively low incidence of the babA gene, and that these virulence-associated determinant genes are not related to clinical outcome.

Our result that all strains contained more than one fragment of the cagA gene would seem to resemble the other Asian countries, where a higher percentage of cagA-positive isolates are found (17, 18). This finding suggests that cagA is not a useful single marker for the discrimination between pathogenic and non-pathogenic strains of H. pylori in the Korean population.

All strains can be classified into vacA subtypes, and seven vacA subtypes (s1a/m1, s1b/m1, s1c/m1, s2/m1, s1a/m2a, s1b/m2a, and s1c/m2a) were identified in the present study. van Doorn et al. (5) expanded the allelic diversity of H. pylori vacA by further discriminating the m1, m2a, and m2b subtypes. They found the m2b variant in eight of 70 Asian, s1c strains (7). However, we did not find m2b subtype in the 76 isolates from Korean patients in the present study. The distribution of vacA subtypes differs between races, or over geographic locations (7, 18). van Doorn et al. (7) reported that s1c was predominant in Asian, and m1 and m2a were equally represented. In our study, the s1c and m1 variants were predominant among the vacA subtypes, and the presence of s1/m1 was not associated with peptic ulcer. The relation between s1/m1 and peptic ulcer has been controversial, because studies reported in western countries have shown that the vacA s1 or m1 genotypes correlate with clinical outcomes (7, 19). However, data from Asian including our study suggest various genotypes in H. pylori isolates are not associated with peptic ulcer (20, 21).

The iceA1 gene, which may be related to the onset of peptic ulcers (10, 11), was also frequently detected in the present study (64 of 76 strains). Consistent with this, recent reports from Japan (18, 22) and Singapore (21) have shown that the incidence of iceA1 did not correlate with clinical outcomes.

BabA-mediated adherence of H. pylori to the gastric epithelium plays a critical role in the efficient delivery of bacterial virulence factors that damage host tissue (12). Gerhard et al. (13) reported that the presence of babA 2 could be regarded as a good indicator of the ability of strains to express the Lewis antigen-binding adhesin, and that babA 2 is signifi-
cantly associated with duodenal ulcer in H. pylori isolated from a German population. The incidence of the babA2 genotype was about 72% in their study (duodenal ulcer 100%, gastric cancer 77.8%, and gastritis 51.4%). However, in our study, the incidence of babA was low, and was not related to peptic ulcer disease. This may result from the possibility that Korean strains have sequence variations in the babA PCR primer regions and this affect the low incidence of babA. But, according to the reports (12, 23, 24) about variations within bab genes, the 5′ and 3′ regions are well conserved and it also suggests that there may be rarely any well conserved in our babA PCR primer regions located in the 5′ region of babA gene.

The pathogenicity-related genes tend to be coexpressed with other genes. Ilver et al. (12) found expression of both cagA and babA in 70% of subjects. van Doorn et al. (11) showed that both cagA and vacA s1 were strongly associated. Gerhard et al. (13) showed that the vacA s1 genotype was also significantly associated with the presence of babA, and that Lewis-antigen-binding activity strongly correlated with the presence of the cag pathogenicity-associated islands. In contrast to these results, we did not find associations among virulence-related markers.

As H. pylori carries only a single copy of vacA (19), detection of multiple genotypes implies the presence of multiple strains in a clinical sample. The frequency of multiple genotypes of vacA in a single biopsy specimen in our study was 18.4%. Comparing the genetic differences in 70 isolates of H. pylori from the antrum and the corpus of 35 patients, 21 patients were infected with at least two strains of different cagA, vacA, babA, and vacA genotypes. H. pylori infection with multiple genotypes is more common in Korea than in western countries (5, 25, 26). This may be related to the high prevalence of H. pylori and higher rates of childhood infection in Korea (27). In Korea, approximately 71.5-91% of adults (27, 28) and 50% of children (29) are infected, so coinfection or superinfection with different strains may be common (30, 31). And H. pylori may also have the opportunities to evolve genetic variations during the long-term colonization from childhood (32). The prevalence of multiple-strain colonization should be considered when planning therapeutic strategies, as well as in any study of the pathogenesis of H. pylori infection.

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