Relationship between *Helicobacter pylori* iceA, cagA, and vacA Status and Clinical Outcome: Studies in Four Different Countries

YOSHIKO YAMAOKA,1,2* TADASHI KODAMA,3 OSCAR GUTIERREZ,3 JONG G. KIM,4 KEI KASHIMA,2 AND DAVID Y. GRAHAM1

Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas1; Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan2; Universidad Nacional de Colombia, Bogota, Colombia3; and Guro Hospital, Korea University College of Medicine, Seoul, Korea4

Received 24 November 1998/Returned for modification 4 March 1999/Accepted 16 April 1999

There is continuing interest in identifying *Helicobacter pylori* virulence factors that might predict the risk for symptomatic clinical outcomes. It has been proposed that iceA and cagA genes are such markers and can identify patients with peptic ulcers. We compared *H. pylori* isolates from four countries, looking at the cagA and vacA genotypes, iceA alleles, and presentation of the infection. We used PCR to examine iceA, vacA, and cagA status of 424 *H. pylori* isolates obtained from patients with different clinical presentations (peptic ulcer, gastric cancer, and atrophic gastritis). The *H. pylori* isolates examined included 107 strains from Bogota, Colombia, 70 from Houston, Tex., 135 from Seoul, Korea, and 112 from Kyoto, Japan. The predominant genotype differed among countries: the cagA-positive iceA1 vacA s1c-m1 genotype was predominant in Japan and Korea, the cagA-positive iceA2 vacA s1b-m1 genotype was predominant in the United States, and the cagA-positive iceA2 vacA s1a-m1 genotype was predominant in Colombia. There was no association between the iceA, vacA, or cagA status and clinical outcome in patients in the countries studied. iceA1 status shows considerable geographic differences, and neither iceA1 nor combinations of iceA, vacA, and cagA were helpful in predicting the clinical presentation of an *H. pylori* infection.

*Helicobacter pylori* is the major cause of chronic gastritis and plays an important role in the pathogenesis of peptic ulcer, gastric carcinoma, and primary B-cell gastric lymphoma (7–9, 13, 15). Histological gastritis is essentially universal among *H. pylori*-infected individuals, but only a minority develop a clinically significant outcome, such as peptic ulcer disease or gastric cancer. Experience with other bacterial pathogens suggests that *H. pylori* strain-specific factors may influence the pathogenicity of different *H. pylori* isolates. *H. pylori* studies have primarily focused on two groups of putative bacterial virulence factors, the cag pathogenicity island (for which cagA is a marker) and the vacuolating cytotoxin VacA (4, 24). The presence of an intact cag pathogenicity island is associated with increased interleukin-8 production and mucosal inflammation (4). Overall, the data support the notion that infection with a cagA-positive isolate increases the risk but does not predict the presence of a clinically significant outcome (8, 25, 26). Differences in the vacA gene (the mosaic combination of signal [s] regions and middle [m] region allelic types) have been identified, and attempts have been made to associate specific vacA genotypes (especially s1-m1 type) with different outcomes, especially with duodenal ulcer (DU) disease (1, 2).

In East Asia, the predominant genotype of the circulating *H. pylori* is cagA positive vacA genotype s1-m1 irrespective of outcome (10, 11, 14, 19, 23, 28). Recently, a new candidate gene designated iceA (for induced by contact with epithelium) was suggested to have an association with peptic ulcer (17, 18). The iceA gene has two main allelic variants, iceA1 and iceA2. van Doorn et al. (20) reported that the iceA allelic type was independent of the cagA and vacA status, and there was a significant association between the presence of the iceA1 allele and peptic ulcer disease. Those researchers proposed that genotyping of iceA and cagA might offer an effective combination for identification of patients with peptic ulcers. Their results were obtained from patients in The Netherlands, and the search for virulence factors related to outcome of infection has been hampered by the fact that there appear to be differences in the predominant strain in circulation in different geographic regions (6, 12). Thus, conclusions derived from data from a single geographic region may not be true for other geographic regions.

In this study, we examined the iceA allelic type in strains from four different countries and its relation with cagA status and vacA genotypes and clinical outcome.

MATERIALS AND METHODS

Patients and *H. pylori* isolates. We examined 424 *H. pylori* isolates; 107 strains from Bogota, Colombia (46 with gastric cancer, 27 with DU, and 34 with histological gastritis only [gastritis]), 70 from Houston, Tex. (16 with gastric cancer, 28 with DU, and 26 with gastritis), 135 from Seoul, Korea (60 with gastric cancer, 53 with DU, and 22 with gastritis), and 112 from Kyoto, Japan (34 with gastric cancer, 48 with DU, and 30 with gastritis). DU5s were identified endoscopically. We excluded the DU cases with gastric ulcer. Gastritis was defined as histological gastritis with no peptic ulcers, gastric cancer, or any esophageal diseases (e.g., gastroesophageal reflux disease and esophageal cancer). Histologically, biopsy specimens were embedded in paraffin, stained with Giemsa stain (Korea, Colombia, and the United States) or modified Giemsa stain (Japan), and examined in a blind test (the patient’s clinical diagnosis and the characteristics of the *H. pylori* strain not known to the individual examining the slide) as described previously (5).

Fifty-nine men and 48 women (mean age, 52.0 years) in Colombia, 48 men and 22 women (mean age, 51.9 years) in the United States, 76 men and 59 women (mean age, 51.8 years) in Korea, and 62 men and 50 women (mean age, 51.8 years) in Japan were studied. For the Korean patients, the mean age of patients with gastric cancer (55.4 years) was significantly higher than that of patients with DU (41.6 years) or gastritis (42.7 years); there were no such age differences for the other groups. No subjects had received treatment for *H. pylori* infection. Informed consent was obtained from all patients, and the protocol was approved by the local ethics committee.

Preparation of *H. pylori* genomic DNA. Gastric biopsy specimens were obtained for isolation of *H. pylori* by previously described culture methods (25, 26,
28). All stock cultures were maintained at ~80°C in brucella broth (Difco, Detroit, Mich.) supplemented with 20% glycerol (Sigma Chemical Co., St. Louis, Mo.). The strains used in this study were passaged three times on average in each country. *H. pylori* strains were grown at 37°C on brain heart infusion (BHI) (Difco) plates containing 5% horse blood (Cocalico Biological, Inc. Reamstown, Pa.) in a 12% CO₂ incubator with 100% relative humidity. The organisms were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. Multiple isolates on the plates were pooled to ensure full extension of the PCR products.

Analysis of vacA s, cagA, and iceA by PCR. PCR amplification was performed as previously described (28) for 35 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C. The final cycle included a 7-min extension step to ensure full extension of the PCR products.

All primers used in this study were presented in Table 1. For detection of the iceA gene, primers CAGAF and CAGAR which yield a fragment of 349 bp from the middle conservative region of the gene were used.

For analysis of the vacA s region, primers VAG-F and VAG-R yielded a fragment of 259 bp for s1 variants and a fragment of 286 bp for s2 variants. Each isolate was typed s1b or s1c subtypes, respectively, and each isolate was typed as s1a or s1c by performing PCR with primers S1A-F–VA1-R and S1C-F–VA1-R, respectively. It was previously described (28) for 35 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The final cycle included a 10-min extension step to ensure full extension of the PCR products.

The strains used in this study were passaged three times on average in each country. *H. pylori* strains were grown at 37°C on brain heart infusion (BHI) (Difco) plates containing 5% horse blood (Cocalico Biological, Inc. Reamstown, Pa.) in a 12% CO₂ incubator with 100% relative humidity. The organisms were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. Multiple isolates on the plates were pooled to ensure full extension of the PCR products.

### Table 1. PCR primers for amplification of cagA, vacA, and iceA sequences

<table>
<thead>
<tr>
<th>Gene and DNA region amplified</th>
<th>Primer</th>
<th>Primer sequence (5′→3′)</th>
<th>Size (bp) of PCR product (location)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cagA</em></td>
<td>CAGAF</td>
<td>GATAACAGGCGAATTTTGAGG</td>
<td>349 (1228–1576)</td>
</tr>
<tr>
<td><em>cagA</em></td>
<td>CAGAR</td>
<td>CTGCAAAGATTGTTTGCAGA</td>
<td>259 (797–1055)</td>
</tr>
<tr>
<td>vacA s1</td>
<td>VAI-F</td>
<td>ATGGAAATACAACAAACAC</td>
<td>286 (284–506)</td>
</tr>
<tr>
<td>vacA s2</td>
<td>VAI-R</td>
<td>ATGGAAATACAACAAACAC</td>
<td>212 (844–1055)</td>
</tr>
<tr>
<td>vacA s1a</td>
<td>SIA-F</td>
<td>CTYGTCTTTIATGAGGC</td>
<td>187f</td>
</tr>
<tr>
<td>vacA s1b</td>
<td>SS3-F</td>
<td>AGGCCATACCCGGAAG</td>
<td>570 (2071–2640)</td>
</tr>
<tr>
<td>vacA s1c</td>
<td>SIC-F</td>
<td>CTRATAGCASTYCTITCAG</td>
<td>570 (2071–2640)</td>
</tr>
<tr>
<td>vacA m1</td>
<td>VAG-F</td>
<td>CAATCTGTCCATACAGGAG</td>
<td>645 (639–1283)</td>
</tr>
<tr>
<td>vacA m2</td>
<td>VAG-R</td>
<td>GCGTCTAAATATCCAAAGG</td>
<td>645 (639–1283)</td>
</tr>
<tr>
<td>iceA1</td>
<td>iceA1F</td>
<td>GTTGTITTAACACAAATAGG</td>
<td>247 (844–1055)</td>
</tr>
<tr>
<td>iceA1</td>
<td>iceA1R</td>
<td>CTGCTTGAATGCGCCAAAC</td>
<td>247 (844–1055)</td>
</tr>
<tr>
<td>iceA2</td>
<td>iceA2F</td>
<td>GATGGAATACAACAAACAC</td>
<td>229 (230)</td>
</tr>
<tr>
<td>iceA2</td>
<td>iceA2R</td>
<td>CTRATAGCASTYCTITCAG</td>
<td>229 (230)</td>
</tr>
</tbody>
</table>

**Notes:**
- Y is C or T, M is A or C, S is C or G, and R is A or G.
- a: Nucleotide positions in the *cagA* gene of *H. pylori* ATCC 53726 (GenBank accession no. L117714).
- b: Nucleotide positions in the *vacA* gene of *H. pylori* 60190 (GenBank accession no. U05676).
- c: Nucleotide positions in the *vacA* gene of *H. pylori* Tx30a (GenBank accession no. U29401).
- d: Used with primers VAI-R.
- e: No published coordinates for genes in strains of these types.
- f: Nucleotide positions in the *iceA* gene of *H. pylori* 60190 (GenBank accession no. U43917).
assessed among the strains with a single iceA allelic type. The prevalence of iceA1 was significantly higher in Korea and Japan than in the United States and Colombia (Korea or Japan versus the United States or Colombia; \( P < 0.0001 \) for each) (Table 2). However, in the four countries, there was no association of the iceA genotype and either the cagA status (\( P > 0.6 \)) or the vacA genotype (\( P > 0.7 \)).

As previously reported (20), most isolates with the iceA2 allele (212 of 214 [99%]) could be divided into two types according to the presence of repeated sequences of 105 nucleotides and whether PCR products were 229 bp (iceA2-1) or 334 bp (iceA2-2) long. Only two isolates (one Korean gastritis case and one U.S. gastritis case) had the PCR product of about 124 bp, possibly due to the lack of a 105-bp repeat region. Eighteen isolates (8.4%) had both the iceA2-1 and iceA2-2 alleles.

In Korea, Japan, and the United States, the iceA2-1 allele was predominant irrespective of the clinical outcome (data not shown). In Colombia, the iceA2-1 allele was predominant in gastritis cases (10 of 17 [59%]) and DU cases (7 of 11 [63%]) and the iceA2-2 allele was predominant in gastric cancer cases (14 of 20 [70%]); however, none of these differences were statistically significant (\( P < 0.10 \)).

vacA genotyping and cagA status. The vacA genotype was significantly different in each country (Fig. 1), precluding an association between vacA genotype and clinical outcome. The vacA genotype s1c-m1 was predominant in Japan and Korea,
genotype s1b-m1 was predominant in the United States, and genotype s1a-m1 was predominant in Colombia, irrespective of the clinical outcome for patients from each country (Fig. 1).

In this study, cagA status was determined by PCR using one set of primers. To avoid false-negative results, cagA-negative status was confirmed by immunoblotting in cases yielding no PCR product using cagA-specific primers, as previously described (27). As a result, all cases with cagA gene-negative results by PCR were also CagA protein negative by immunoblotting. The cagA gene-positive isolates were predominant in every country, with no association between cagA status and clinical outcome (Fig. 1). The vacA genotype s1 was almost always associated with the presence of the cagA gene irrespective of the country (P < 0.0001 for United States and Colombia). In Japan and Korea, the predominant strain had the vacA s1 genotype irrespective of the cagA status. For example, of 10 cagA-negative strains, 8 had the vacA s1c genotype and 2 had the vacA s1a genotype.

Combination of iceA, vacA, and cagA genotypes. By using the method of van Doorn et al. (20), we examined eight different combinations based on analysis of the vacA s region (s1 and s2), cagA (positive [+] and negative [−]) and the iceA type (iceA1 and iceA2) in patients with a single genotype (Fig. 2). We were unable to identify an association between these genotypes and clinical outcome. For example, the cagA-positive iceA1 vacA s1 genotype was predominant in Japan and Korea and the cagA-positive iceA2 vacA s1 genotype was predominant in the United States irrespective of the clinical outcome.
van Doorn et al. (20) examined 94 gastric biopsy specimens from patients in The Netherlands and reported a strong association between the iceA1 allele and peptic ulcer disease. They also reported that cagA positivity and vacA s1 genotype were also associated with peptic ulcer disease. Overall, our data are consistent with other recent reports that cagA status and vacA genotype do not predict clinical outcome (6, 8, 10–12, 14, 19, 23, 25, 26, 28). van Doorn et al. suggested that the addition of iceA genotyping might provide a better discrimination. We were unable to confirm an association between the iceA1 allele and clinical outcome. As a general rule, important disease-associated bacterial toxins are tightly associated with their respective diseases and the absence of the factor corresponds with the absence of the diseases in different geographic regions (e.g., cholera toxin and cholerla or diphtheria toxin and diphtheria). The fact that predictions based on the cagA, vacA, and iceA genotypes were not confirmed in different populations suggests that region-associated observations are possibly being construed as disease-specific associations. This problem continues to plague work on H. pylori such that, in the future, it may be prudent to confirm genotypic or phenotypic H. pylori-disease associations in several different geographic regions prior to making any claims.

The fact there were geographic differences in both the vacA and iceA genotypes is interesting. The iceA1 allele was predominant in Japan and Korea, and the iceA2 allele was predominant in the United States and Colombia. In a study of the geographic distributions of the vacA genotype (21), the s1c allele was observed exclusively in isolates from East Asia, which is in agreement with the results of this study. We found that the vacA s1a genotype was dominant in Colombia (72 cases). In contrast, a recent report of strains from Central and South America (Brazil, Costa Rica, Peru, and Colombia) suggested that the vacA s1b genotype was predominant (22). They evaluated only six Colombian isolates, but the results, if confirmed, suggest that there may be marked variation within broad geographic areas. This is also consistent with the fact that when van Doorn et al. examined 60 U.S. (Nashville, Tenn.) and 13 Canadian strains, they reported that the prevalence of s1a and s1b genotypes was identical (22). In contrast, in Houston, Tex., the s1b strains were predominant. Similar marked differences in the prevalence of cagA in Nashville and Houston (1, 2, 6, 12, 16) confirm that regional variations in the dominant circulating strain occur, and failure to take this into account this has repeatedly lead to conclusions that are not true for other geographic regions. It is interesting to note that although H. pylori from Korea and Japan had very similar patterns in cagA, iceA, and vacA status (the cagA-positive iceA1 vacA s1c-m1 genotype was predominant), preliminary data suggest that in Taiwan, vacA m2 is dominant.

In summary, we were unable to confirm the reports of association of iceA status and clinical outcome. iceA shows considerable geographic differences, and neither iceA nor combinations of iceA, vacA, and cagA were helpful in predicting the clinical presentation of an H. pylori infection.

ACKNOWLEDGMENTS

This work was supported in part by the Department of Veterans Affairs and by NIH grant DK53659, as well as the generous support of Hilda Schwartz.

REFERENCES


