Alanine-Threonine Polymorphism of Helicobacter pylori RpoB Is Correlated with Differential Induction of Interleukin-8 in MKN45 Cells

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Geographical differences in the genetic diversity of Helicobacter pylori isolates were examined by analyzing rpoB sequences. An extremely high level of allelic diversity among H. pylori strains was found. The rpoB sequences of Asian and non-Asian (North and South American, European, and South African) strains were found to differ. An amino acid polymorphism (alanine and threonine RpoB types) was found at the 497th residue by deduced amino acid analysis. RpoB with a threonine residue (RpoBThr) was uniquely present in East Asian countries, and two-thirds of the H. pylori isolate population in this region was RpoBThr; however, this type was rare or absent in Western countries, where RpoBAla predominated. RpoBThr strains induced a much larger amount of interleukin-8, a chemokine that plays an important role in chronic inflammation, than RpoBAla strains in cultured MKN45 cells.

Helicobacter pylori is disproportionately acquired during childhood and persists in its host for life. H. pylori infection typically leads to chronic inflammation of the gastric mucosa, and this is accompanied by mucosal damage, including the loss of acid-secreting parietal cells and the development of mucous cell metaplasia (30). H. pylori carriers have a higher risk of gastric diseases like gastric cancer, which is the second most common malignancy worldwide, and is particularly common in East Asian countries, such as Korea and Japan (8, 22). However, H. pylori factors involved in gastric carcinogenesis are not well understood.

The presence of the cytotoxin-associated gene A (cagA) of H. pylori has been proposed to be an important risk factor for the development of H. pylori-mediated gastric cancer (7). Recently, it was suggested that Src homology 2-containing tyrosine phosphatase (SHP-2) is an intracellular target of CagA protein (9) and that the prevalent CagA type in East Asian countries binds more strongly to SHP-2, and thus induces more cellular morphological changes, than the CagA type prevalent in Western countries (10). Moreover, it has been suggested that this difference may be correlated with the striking difference in the incidence of gastric cancer in these two geographical areas (10). However, even though nearly 100% of Korean and Japanese isolates possess cagA and express the East Asian type of CagA, relatively few infected individuals develop peptic ulcer or gastric cancer (6). The reason for this remains unresolved (28).

Phylogenetic analysis based on amino acid sequences often provides more significant information than analysis based on the nucleotide sequences of protein-coding genes (8, 20). However, such analyses have not been performed in previous population studies with cagA, oipA, or other housekeeping genes (5, 17, 29, 31). Thus, to test the hypothesis that certain H. pylori strains in Asia are uniquely prone to cause chronic inflammation and metaplastic changes in the gastric mucosa, we studied the population structure of H. pylori isolates from several countries by analyzing rpoB sequences. This allowed us to analyze the H. pylori population by both nucleotide and amino acid sequence analyses. rpoB encodes the β-subunit of RNA polymerase and is a highly conserved housekeeping gene. Comparisons of rpoB sequences have previously been used for phylogenetic analysis and for the differential identification of bacteria (14, 15, 19, 34).

MATERIALS AND METHODS

Bacterial strains. The DNAs of 535 clinical H. pylori isolates from 12 countries (Table 1) were analyzed; H. cinaedi was used as an outgroup. The strains, which were provided by D. Y. Graham, had been obtained from patients who had signed informed consent forms approved by institutional review boards in the United States.

Preparation of DNA. H. pylori DNAs were extracted from cultured bacteria and gastric biopsy specimens by the bead beater-phenol extraction method (14). A loopful of a culture of each isolate was suspended in 200 μl of TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl [pH 8.0]) and placed in a 2.0-ml
TABLE 1. Prevalence of H. pylori types on the basis of the rpoB amino acid, by country

<table>
<thead>
<tr>
<th>Geographical region and country</th>
<th>No. (%) of strains (n = 535)</th>
<th>rpoB&lt;sup&gt;64&lt;/sup&gt; type</th>
<th>rpoB&lt;sup&gt;66&lt;/sup&gt; type</th>
</tr>
</thead>
<tbody>
<tr>
<td>East and Southeast Asia</td>
<td></td>
<td>129 (32.4)</td>
<td>269 (67.6)</td>
</tr>
<tr>
<td>Korea</td>
<td>119</td>
<td>241</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Hong Kong</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tajikistan</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>77 (98.7)</td>
<td>1 (1.3)</td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>72</td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>South America</td>
<td>44 (93.6)</td>
<td>3 (6.4)</td>
<td></td>
</tr>
<tr>
<td>Colombia</td>
<td>42</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td>8 (100)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>4 (100)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>262</td>
<td>273</td>
<td></td>
</tr>
</tbody>
</table>

* The individual was born in Vietnam and immigrated to the United States.

Nucleotide sequence accession numbers. The rpoB sequences of H. pylori strains 26695 and J99 were retrieved from GenBank (accession nos. AE000625 and AE001540, respectively).

RESULTS

rpoB genotype. rpoB DNA (458 bp) containing a highly conserved region was amplified (16) and sequenced from 535 clinical H. pylori isolates obtained from 12 countries. Because many countries were represented by small numbers of isolates and, thus, may not represent the predominant strains of the particular geographical areas, we grouped strains by large geographical regions (Table 1). The rpoB sequences (363 bp) determined were aligned for the Homoplasy test, split decomposition analysis, and phylogenetic study. The Homoplasy index of H. pylori was found to be 0.519, which is higher than those reported for other bacteria (18, 23). This suggests frequent interstrain recombinations among the H. pylori population. Split decomposition analysis of rpoB showed a network topology and star phylogeny (data not shown), which are consistent with a recombinational population structure.

Another interesting finding was that although it was not robustly supported by bootstrap values, the H. pylori population could be separated into two major groups by nucleotide sequence analysis. In accord with previous reports on genotype analysis (1, 5, 8, 12, 13, 29, 32), the geographical distributions of these two groups were in agreement with their phylogenetic relationships. One group was termed the Asian group, and the other was termed the non-Asian group, which was mostly composed of Western H. pylori strains (North and South American, European, and South African strains) and included strains 26695 and J99 (Fig. 1A). Although marked genetic heterogeneity was observed, the clustering of H. pylori strains into...
FIG. 1. Phylogenetic relationships of 100 H. pylori isolates inferred from partial rpoB DNA sequences (A) and RpoB amino acid sequences (B). The H. pylori population was separated into an Asian group, to which most of the Asian strains belonged, and a non-Asian group, which was mainly composed of Western strains (North and South American, European, and South African strains), including strains H. pylori 26695 and H. pylori J99, by nucleotide sequence analysis (A). Two large groups (RpoB<sup>Ala</sup> and RpoB<sup>Thr</sup>) in the amino acid tree (B) were attributed to the identity of the 497th residue of each strain, which is either alanine or threonine. RpoB<sup>Thr</sup> strains have the suffix T. The tree was constructed by the neighbor-joining method in the PAUP package. The bootstrap values presented at the corresponding branches were evaluated from 1,000 replications, and values less than 50% are not indicated.
different groups by geographical regions by rpoB analysis was also compatible with the findings of other studies (5, 17, 29, 31).

Deduced amino acid. For protein-coding genes, phylogenetic relationships based on amino acid sequences are often more significant than those based on nucleotide sequences (8, 20). Population genetics data based on nucleotide sequences are often inadequate for the study of protein-coding genes because variations are usually found at the third bases of codons (the wobble position), and these variations do not af-
The amino acid sequence and thus result in synonymous substitutions (3, 8). Our analysis of the amino acid sequence of \( rpoB \) shows that the \( H. pylori \) strains could also be separated into another two large groups on the tree based on the amino acid sequences (Fig. 1B), and this was attributed to the identity of the 497th residue of each group, which was either alanine (GCT) or threonine (ACT). These groups were designated into another two large groups on the tree based on the amino acid sequences (Fig. 1B). Among the 398 strains from East Asian patients, 269 strains (67.6%) were of the RpoB Thr type, and 129 strains (32.4%) were of the RpoB Ala type. The result was obtained when the \( rpoB \) DNA from the RpoB Thr type was digested (lanes 4 and 5) [248 and 210 bp], while DNAs from the RpoB Ala type were not digested (lanes 1 to 3 [458 bp]). Lane M, size marker (6X174 replicative-form DNA digested with HaeIII). The numbers next to the gels are in base pairs.

**FIG. 2. Differentiation of RpoB Ala and RpoB Thr type \( H. pylori \) strains by PCR-restriction fragment length polymorphism analysis (with BsmFI) of \( rpoB \) DNA. Amplified \( rpoB \) DNAs (458 bp) of \( H. pylori \) were digested with BsmFI and electrophoresed in a 3% agarose gel. DNAs from the RpoB Thr types were digested (lanes 4 and 5 [248 and 210 bp] and lanes 6 and 7 [248, 116, and 94 bp]), while DNAs from the RpoB Ala types were not (lanes 1 to 3 [458 bp]). Lane M, size marker (6X174 replicative-form DNA digested with HaeIII).**

We also compared the clinical information with the RpoB Ala and RpoB Thr types of 398 strains from East Asian patients (Table 2). Because many strains were collected retrospectively and the clinical information for many patients was not available, it is not easy to conclude that the two RpoB types correlate with gastrointestinal disease groups. However, the distributions of the two RpoB types were not significantly different among four disease groups (gastric cancer, duodenal ulcer, gastritis, and others, excluding unknown) (\( P > 0.05 \) by chi-square test).

**FIG. 3. Secretion of IL-8 from MKN45 cells cocultured with the two different \( H. pylori \) types, RpoB Thr (\( n = 20 \)) and RpoB Ala (\( n = 20 \)). All the \( H. pylori \) strains were cagA positive (East Asian-type CagA). The amounts of IL-8 protein excreted by the cells was measured by enzyme-linked immunosorbent assay. The levels of IL-8 secreted by RpoB Thr-type infected cells were found to be significantly higher (\( P < 0.05 \) by the Mann-Whitney rank sum test) than the levels secreted by RpoB Ala-type infected cells.**

**Typing of \( H. pylori \) strains in biopsy specimens.** A similar result was obtained when the \( H. pylori \) strains in gastric biopsy specimens were typed by restriction fragment length polymorphism analysis. A restriction site, BsmFI, that distinguished between the RpoB Ala and RpoB Thr types was found on the basis of the \( rpoB \) sequences. Only the PCR product of RpoB Thr had one or two restriction sites for BsmFI and produced two (248- and 210-bp) or three (248-, 116-, and 94-bp) DNA fragments. DNAs of the RpoB Ala type, on the other hand, were not cleaved (458 bp) (Fig. 2). Of the 200 Korean biopsy specimens in which \( H. pylori \) was detected and identified, 136 samples (68%) contained the RpoB Thr type and the remainder (64 samples [32%]) contained the RpoB Ala type.

**TABLE 2. Distributions of the 394 East Asian \( H. pylori \) strains by PCR-restriction fragment length polymorphism analysis (with BsmFI) of \( rpoB \) DNA. Amplified \( rpoB \) DNAs (458 bp) of \( H. pylori \) were digested with BsmFI and electrophoresed in a 3% agarose gel. DNAs from the RpoB Thr types were digested (lanes 4 and 5 [248 and 210 bp] and lanes 6 and 7 [248, 116, and 94 bp]), while DNAs from the RpoB Ala types were not (lanes 1 to 3 [458 bp]). Lane M, size marker (6X174 replicative-form DNA digested with HaeIII).**

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>RpoB Thr type</th>
<th>RpoB Ala type</th>
<th>Subtotal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td>88 (62.2)</td>
<td>45 (33.8)</td>
<td>133</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>24 (75.0)</td>
<td>8 (25.0)</td>
<td>32</td>
</tr>
<tr>
<td>Gastritis</td>
<td>59 (62.3)</td>
<td>30 (33.7)</td>
<td>89</td>
</tr>
<tr>
<td>Benign gastric ulcer</td>
<td>17 (56.7)</td>
<td>13 (43.3)</td>
<td>30</td>
</tr>
<tr>
<td>Lymphoid hyperplasia</td>
<td>2 (100)</td>
<td>0 (0.0)</td>
<td>2</td>
</tr>
<tr>
<td>Within normal limit</td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
<td>4</td>
</tr>
<tr>
<td>Unknown</td>
<td>70 (67.3)</td>
<td>34 (32.7)</td>
<td>104</td>
</tr>
<tr>
<td>Subtotal</td>
<td>263 (66.8)</td>
<td>131 (33.2)</td>
<td>394</td>
</tr>
</tbody>
</table>

\( ^{a} \) Korea, Japan, Hong Kong, and Taiwan. 
\( ^{b} \) No pathological finding was observed.
DISCUSSION

*Helicobacter pylori* infection is a major cause of gastritis and is considered an important risk factor for stomach cancer. However, the presence of *H. pylori* alone does not sufficiently explain the striking difference in the geographical incidences of gastric cancer. While the association of the cag pathogenicity islands with an increased risk of gastric cancer was proved, it cannot explain the differences in disease presentations caused by the different CagA *H. pylori* types in different geographical areas, namely, Eastern and Western countries. Furthermore, in Korea and Japan essentially all isolates are positive for the cagA type that encodes the East Asian CagA type. Thus, to test the hypothesis that a more virulent *H. pylori* population exists in East Asia, we analyzed *H. pylori* isolates on the basis of the sequence of the protein-coding gene, rpoB, which encodes the β-subunit of DNA-dependent RNA polymerase.

DNA-dependent RNA polymerase is a principal enzyme in the transcriptional process and of many regulatory pathways that control gene expression in living organisms. It is evolutionarily conserved in sequence, structure, and function from bacteria to humans (19, 24, 34). A high level of genetic diversity among *H. pylori* strains has also been observed by 16S ribosomal DNA sequence analysis (27). However, as a protein-coding gene, rpoB provided several advantages over 16S ribosomal DNA for phylogenetic analysis, which offered only a moderate power to discriminate or distinguish between species and strains (14, 15, 19). With rpoB analysis the can be performed at both the nucleotide and the amino acid sequence levels and the *H. pylori* strains can be grouped according to both the amino acid and the nucleotide sequences. The amino acid sequence-based grouping of *H. pylori* led to the discovery of a novel RpoB polymorphism (RpoBAla–RpoBThr) at residue 497. The prevalence of the RpoBThr type was notable only in *H. pylori* isolates from East and Southeast Asia. However, because the *H. pylori* strains used for rpoB analysis were collected retrospectively and the clinical information for many patients was unknown, we are very cautious not to conclude that a correlation between an RpoB type with a certain gastrointestinal disease group exists.

Gastric cancer is generally thought to arise through a series of mucosal changes leading to atrophic gastritis caused by chronic *H. pylori* infection. Chronic *H. pylori* infection causes abnormal changes in the gastric mucosa, such as severe infiltration of the lamina propria by polymorphonuclear and mononuclear cells, and increases in epithelial cell proliferation, resulting in atrophic gastritis and focal intestinal metaplasia in an animal model (11). The proinflammatory chemokine IL-8 plays an important role in *H. pylori*-related inflammation by recruiting neutrophils and lymphocytes into the gastric mucosa (2, 4). We measured the IL-8 levels in cultured MKN45 cells after *H. pylori* infection. Of interest, strains polymorphic at the 497th residue induced different amounts of IL-8 secretion, with strains with the RpoBThr type inducing more IL-8 secretion than those with the RpoBAla type. This difference in levels of IL-8 secretion did not correlate with the recA group (group I and group II), as defined in a previous report (17; data not shown).

These data suggest that additional factors are responsible for enhanced virulence among *H. pylori* strains and may provide important clues to the question of why the incidence of *H. pylori*-induced clinical disease differs so markedly between East Asia and the West. While it is clear that the presence of the East Asian-type CagA cannot be solely credited with this difference in IL-8 induction, the specifics of the RpoB polymorphism in IL-8 induction have yet to be explored. The RpoB polymorphism may affect the function of RNA polymerase. Although the presence of the RpoB polymorphisms correlated with the geographical locations of the isolates and with the levels of IL-8 induction in vitro, it did not correlate with clinical presentation. The question remains whether RpoB polymorphisms are directly involved in clinical outcomes or whether they are actually a marker linked to another factor responsible for increased virulence, independent of the polymorphism. Studies are planned to test the effects of the Thr→Ala substitution on IL-8 secretion in vitro to investigate whether the polymorphism is directly related to the induction of enhanced IL-8 secretion.

In conclusion, this *H. pylori* population study based on rpoB nucleotide sequences, analysis of their deduced amino acid sequences, and the IL-8 assay provides evidence that a polymorphism in RpoB may be related to the pathogenesis of *H. pylori*-associated gastric diseases. Because of different host-parasite interactions, we suggest that the classification of *H. pylori* strains according to the RpoB polymorphism should be an integral part of the study of *H. pylori*-mediated pathogenesis.

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REFERENCES


