Vacuolating Toxin Production in Clinical Isolates of *Helicobacter pylori* with Different *vacA* Genotypes

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A vacuolating cytotoxin encoded by *vacA* in *Helicobacter pylori* is known as a potential virulent determinant. The relationship between different *vacA* alleles, vaculating ability, and *H. pylori*–related diseases was investigated. Genetic analysis of 119 isolates from Taiwanese patients revealed that 104 strains were s1a/m2, 13 strains were characterized as the s1a/m1T type, which was more homologous to the s1a/m1 strains, and 2 were characterized as the s1a/m1Tm2 chimeric type. Production of high-grade cytotoxin among 11 strains with s1a/m1T was higher (72.7%) than among 66 strains with s1a/m2 (21.2%) (*P* < .01). Peptic ulcer occurred in 76.9% of 13 patients with s1a/m1T strains compared with 46.2% of 104 patients with s1a/m2 strains (*P* < .05). These results suggest that s1a/m1T strains are associated with increased cytotoxic activity and higher ulcer prevalence than are s1a/m2 strains.

*Helicobacter pylori*, the most common pathogen of the human gastrointestinal tract, is associated with chronic active gastritis, peptic ulcer disease, and gastric cancer [1–5]. Although the molecular basis for development of gastrointestinal pathology remains largely unknown, several studies have shown that some strains are more pathogenic, possibly due to the production of an active vacuolating cytotoxin (VacA) and a cytotoxin-associated antigen (CagA) [6–9]. The *cagA* gene, though with an unknown function [10, 11] and not a virulence factor, is located at one end of a pathogenicity island that contains genes required for interleukin-8 induction by gastric epithelial cells [12]. VacA can induce the formation of acidic vacuoles in cultured cells and in primary human mucosal epithelial cells [13, 14].

In a mouse model of *H. pylori* infection [15], cytotoxic but not nontoxic strains promote gastric injuries resembling the pathologic conditions observed in humans. About 50% of *H. pylori* strains have vaculating cytotoxic activity in vitro, and the presence of cytotoxin-producing ability is epidemiologically associated with gastric damage and peptic ulceration [9, 16]. On the basis of the expression of CagA and VacA, clinical isolates of *H. pylori* are grouped into at least two major types [17]: Type I bacteria produce VacA toxin and CagA; type II bacteria do not express VacA or CagA. Type I bacteria are more virulent and are closely associated with peptic ulceration [8, 15].

Two regions of the *vacA* gene [18–21] are highly divergent between cytotoxic and nontoxic strains: the amino terminal signal sequence region and a 700-bp central region [16]. Strains are typed as s1 (which can be subtyped into s1a and s1b) or s2 in the signal sequence region and as m1 or m2 in the middle region [16]. *vacA* alleles of all combinations (except *vacA* s2/m1) have been observed. Type s1 *vacA* alleles are associated with increased virulence and peptic ulceration [16, 22]. It has been suggested that *vacA* s1a genotype and *cagA* positive status, two closely associated phenotypes, are valuable predictors of the duodenal ulcer risk for an *H. pylori*–infected person [8, 16, 22, 23].

We previously showed that a much higher proportion of Taiwanese patients (98%) are infected with *cagA*+ strains [24] than in most Western countries (~60%) [7, 8, 16, 17, 23]. Our data agree well with recent studies of *H. pylori* isolates from patients in other Asian countries [25–27], indicating that there may be specific populations of *H. pylori* strains in these regions. The object of this study was to characterize *vacA* genotypes of 119 *H. pylori* isolates from Taiwanese patients with various gastrointestinal diseases. We also determined in vitro vaculating activity among different *H. pylori* strains. We sought to test the hypothesis that s1a strains are associated with increased virulence and to correlate the relationship between *vacA* genotypes, cytotoxin-producing ability, and *H. pylori*–related diseases.

**Materials and Methods**

*Bacterial strains.* One hundred nineteen clinical isolates that were previously characterized for *cagA* and *vacA* [24] were used to assess the *vacA* genotypes: 34 isolates were from persons with duodenal ulcers, 26 from persons with gastric ulcers, 44 from subjects with nonulcer dyspepsia, and 15 from persons with gastric cancer. All isolates described possess *vacA*, and all but 3 (97, 137, 1137) have *cagA*. Two well-characterized strains, *H. pylori* 60190 (ATCC 49503; CagA+/VacA+, cytotoxic) [28] and Tx30a (ATCC...
H. pylori strains were recovered from frozen stocks on Wilkins-Chalgren agar with 10% sheep blood, 1% IsoVitalex, 6 μg/mL vancomycin, and 2 μg/mL amphotericin B in a microaerophilic atmosphere for 2–4 days at 35°C. After passages onto fresh plates, the bacteria were cultured in 25 mL of Brucella broth supplemented with 2% fetal bovine serum and 0.2% β-cyclodextrin in a microaerophilic atmosphere for 2 days at 35°C. H. pylori growth was determined by measuring the optical density of the broth culture medium at 600 nm.

Genotyping and sequencing of vacA. Chromosomal DNA was prepared from H. pylori strains as described previously [24]. The vacA signal sequence type of each isolate was determined by polymerase chain reaction (PCR) amplification of extracted DNA as described [16]. In brief, each strain was typed as s1a, s1b, or s2 by three separate PCR amplifications using oligonucleotide primers as follows: 5’-GTCACATCAACCGCGAAC-3’ (s1a-F) and 5’-GTGTTGAGTGGCGCCAAAC-3’ (s1b-F) and VA1-R for amplification of a 0.19-kb fragment; 5’-AGGCCATACCGCAAG-3’ (s1b-F) and VA1-R for amplification of a 0.19-kb fragment; and 5’-GCTAACACCGCAATGACC-3’ (s2-F) and VA1-R for amplification of a 1.99-bp fragment. The vacA midregion was typed as m1, m1T, m1Tm2, or m2 by separate PCR amplifications using different sets of primers as follows: 5’-GGCTAAAATGCGGGTC-3’ (m1-F) and 5’-CCATTGTTACCTGTAGAAAC-3’ (m1-R) for amplification of a 0.29-kb fragment from type m1 strains; m1T-F (5’-GGCCACATGCTTTGATTGCCG-3’) and m1T-R (5’-CTCTTAGGGCTAAAAGAAC-3’), which contained nucleotides of strain 184 that corresponded to 2741–2759 and 3010–3030 bp, respectively, of GenBank sequence U05676, for amplification of a 0.29-kb product from type m1 strains; m1T-F and m2-R for amplification of a 0.3-kb product from type m1Tm2 strains; and 5’-GGAGGCCCAGCGAACATGG-3’ (m2-F) and 5’-CATAACTAGCCGCTTGC-3’ (m2-R) to amplify a 0.35-kb fragment from type m2 strains.

For sequence analysis of vacA signal sequence region, 5’-GGTGAAGATCGCATATT-3’ (SA1) and VA1-R (which contained nucleotides 681–700 and 1037–1055, respectively, of GenBank sequence U05676) were used for amplification of a 0.37-kb fragment encoding the vacA signal sequences. For sequence analysis of the vacA middle region, MD-F (5’-GTCATTTAGGGCCCT-TTTG-3’) and MR-R (5’-TTAAGGTGTCGGCTGTAAGCG-3’) (which contained nucleotides 1925–1943 and 3374–3399, respectively, of U05676) were used to amplify a 1.48-kb fragment. Sequencing reactions were done on both DNA strains by using the 1.48-kb product as a template and by using each of the primers as follows: MD-F, MD-R (5’-GCTCAAAATAT-TCAAAGG-3’, corresponding to 2621–2639 bp of U05676), ME-F (5’-CAATACAAACAGGCTATTACG-3’, corresponding to 2380–2401 bp of U05676), ME-R (5’-GAGCGGTGTGAAA-TCC-3’, corresponding to 2912–2929 bp of U05676), MF-F (5’-CCATGGAATTATTTGACG-3’, corresponding to 2621–2639 bp of U05676), MF-R (5’-CACCATTGTTGACC-3’, corresponding to 3154–3172 bp of U05676), and m1T-F or m2-F. Nucleotide sequences of PCR-amplified vacA fragments were determined on both strands by the dideoxy chain termination procedure.

For sequence analysis, we used the University of Wisconsin Genetics Computer Group (Madison, WI) package and the BLAST network service of the National Center for Biotechnology Information (Bethesda, MD). The sequences compared in this study were derived from GenBank (accession numbers of 12 m1 strains: U05676 for 60190, Z26683 for 185–44, U07145 for NCTC 11638, S72494 for CCUG 17874, AF001358 for ATCC 43526, AE000598 for 26695, U91575 for F84, U91576 for F71, U91577 for F94, U91578 for F37, U91579 for F79, and U91580 for F80; accession numbers of 2 m2 strains are U29401 for Tx30a and U05677 for 87–203).

Vacuolating cytotoxin assay. Broth culture supernatants were precipitated by 50% ammonium sulfate. The precipitates were recovered in PBS, dialyzed, and concentrated to 50-fold using the same buffer. Alternatively, broth culture supernatants were concentrated 50-fold by use of a 100-kDa ultrafiltration membrane [28, 29]. The concentrated dialysates were used for cell culture vacuolating assay. HeLa cells were cultured in Dulbecco’s MEM (DMEM) containing 10% fetal bovine serum in 96-well plates. Toxin preparations were serially diluted (1:5–1:160) in DMEM supplemented with 2% fetal bovine serum, 10 mM ammonium chloride, 100 μM penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin. We incubated 10 μL of toxin preparations with HeLa cells in 96-well plates for 24 h at 37°C. Cell vacuolation was examined by microscopy. The maximum dilution of the sample that produced >50% HeLa cell vacuolation was defined as the score of cytotoxic activity in a sample (0, 5, 10, 20, 40, 80). Uninoculated broth was used as a negative control. High-grade cytotoxin activity was defined as a score ≥40, low-grade cytotoxin activity was scored as 5–20, and a nontoxic activity was scored as <5. We found strain 60190 to be a high-grade cytotoxin producer and Tx30a to be a tox⁻ strain [28] by use of broth culture concentrates from ammonium sulfate treatment or by ultrafiltration.

Statistical methods. The associations between H. pylori genotypes and peptic ulceration or between genotypes and cytotoxin-producing ability were analyzed by χ² analysis with Yates’s correction or by Fisher’s exact test.

Nucleotide sequence accession numbers. The nucleotide sequences were submitted to GenBank under accession numbers AF030399, AF030400, AF030401, and AF030402.

Results

Genotyping and sequence analysis of vacA. By use of a previously described method [16], all 119 H. pylori strains from Taiwanese patients with various gastrointestinal diseases were found to s1a signal sequence type. Sequence analysis of the 33-aa vacA signal sequences for 8 Taiwanese strains also indicated that they were much more homologous to the published s1a strains (91% mean amino acid identity) than to the s2 strains (56% mean amino acid identity) in this aligned region (figure 1).

For the middle region of vacA, 104 strains were characterized as m2 type, but the remaining 15 strains yielded negative results for both m1 and m2 typing by Atherton’s method. Sequence analysis of a 1.48-kb fragment spanning the middle region was
Figure 1. Comparison of aligned nucleotide sequences encoding putative vacA signal sequences (A) and deduced amino acid sequences for 8 Taiwanese strains (49, 52, 61, 62, 122, 1604, 1711) and for published strains 60190 (s1a) and Tx30a (s2). Nucleotide sequences of strains 52, 122, and 1604 are identical to those of strain 61 and are represented by signal sequences of strain 61 (A). Deduced amino acid sequences of signal region of all 8 Taiwanese strains were identical and are represented by those of strain 61 (B). Dots indicate nucleotide or amino acid identity compared with sequence listed above.

then done for 2 unassigned Taiwanese strains (61 and 184). By aligning and comparing the 0.73-kb region (corresponding to bp 2321–3052 of U05676) of these strains, there was 96.6% nucleotide identity and 95.5% amino acid identity (table 1). Further analysis of the aligned nucleotide sequences revealed 91.4% mean nucleotide identity and 89.7% mean amino acid identity between these 2 strains and 12 published m1 strains compared with 71.2% mean nucleotide identity and 56.4% mean amino acid identity between these 2 isolates and 2 published m2 strains. Several mutations were found in m1 primer regions that resulted in negative PCR amplification. These results indicate that the 2 Taiwanese strains are much more homologous to m1 strains than to m2 strains. Above all, strains 61 and 184 were nearly identical (98% mean nucleotide identity) to 3 Japanese m1 strains (F84, F71, and F94) [30].

Table 1. Divergence analysis of a 0.73-kb middle region of the H. pylori vacA gene.

<table>
<thead>
<tr>
<th>Strains</th>
<th>vacA m1 type</th>
<th>vacA m1T type</th>
<th>vacA m2 type</th>
</tr>
</thead>
<tbody>
<tr>
<td>F94</td>
<td>88.4</td>
<td>98.1</td>
<td>71.3</td>
</tr>
<tr>
<td>F37</td>
<td>84.8</td>
<td>87.7</td>
<td>71.3</td>
</tr>
<tr>
<td>F80</td>
<td>91.4</td>
<td>92.2</td>
<td>71.5</td>
</tr>
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<td>60190</td>
<td>85.7</td>
<td>88.3</td>
<td>70.9</td>
</tr>
<tr>
<td>61</td>
<td>98.8</td>
<td>88.3</td>
<td>71.5</td>
</tr>
<tr>
<td>184</td>
<td>95.9</td>
<td>95.5</td>
<td>71.3</td>
</tr>
<tr>
<td>55</td>
<td>57.0</td>
<td>56.6</td>
<td>93.9</td>
</tr>
<tr>
<td>62</td>
<td>55.7</td>
<td>55.7</td>
<td>95.5</td>
</tr>
<tr>
<td>Tx30a</td>
<td>57.0</td>
<td>57.0</td>
<td>96.3</td>
</tr>
</tbody>
</table>

NOTE. Nucleotide and deduced amino acid sequences of a 0.73-kb region of vacA (corresponding to 2321–3052 bp of strain 60190) were aligned and analyzed. Sequences in this table are derived from GenBank: U05676 for 60190, U91577 for F94, U91578 for F37, U91580 for F80, and U29401 for Tx30a. Sequences of strains 61, 184, 55, and 62 were obtained for this study and have been submitted to GenBank. Bold type indicates amino acid identity; nonbold numbers show nucleotide identity.
signed (non-m1T and non-m2) strains, we determined the nu-
cleotide sequence of a 0.55-kb fragment spanning the PCR
middle typing region. By sequence analysis of the aligned mid-
dle typing region (corresponding to bp 2301–2726 of GenBank
sequence U29401), these 2 strains were highly homologous to
the m1T strains (184 and 61) in the region spanning the m1T-
F primer sequence corresponding to 2701–2810 bp of U05676
(92.5% mean nucleotide identity) [18]. Nevertheless, they were
much more homologous to the m2 strains (strains 55, 62, and
Tx30a) in the region spanning the m2-R primer region corre-
sponding to 2540–2640 bp of Tx30a (91.6% mean nucleotide
identity) [16, 18], thus showing an m1T/m2 chimeric type.
Taken together, this suggests three families of vacA alleles
(m1T, m2, and m1Tm2) in the midregion locus and only one
type of vacA signal sequence (s1a) for Taiwanese isolates. In
total, the vacA genotypes of H. pylori isolates were as follows:
13 s1a/m1T, 104 s1a/m2, and 2 s1a/m1Tm2.

Vacuolation of HeLa cells induced by H. pylori culture su-
pernatants with different vacA genotypes. The intracellular
vacuoles produced by strain 60190 and cytotoxic strains were
similar to those described by Leunk et al. [13]. To examine
whether a specific genotype was associated with cytotoxic activ-
ity, 11 s1a/m1T strains, 66 s1a/m2 strains, and 2 s1a/m1Tm2
strains were randomly selected for in vitro vacuolating assay.
After incubation of toxin preparations for 24 h, 57 (72%) of
the 79 isolates produced detectable vacuolating activity com-
pared with ~50% of H. pylori strains in previous studies [9,
13, 16, 29]. Since additional culture supernatants scored as
toxic genotypic have been reported when assays were run for 48 h
[9], a much higher frequency of toxigenic strains than found
in Western populations might be anticipated. One possible rea-
son for the higher proportion of cytotoxic strains found in our
study might be attributed to the fact that all Taiwanese strains
are s1a but not s2. Of 2 strains with the s1a/m1Tm2 type, 1
was a high-grade cytotoxin producer and 1 was noncytotoxic.
Of 11 strains with the s1a/m1 vacA genotype, 8 (72.7%) were
high-grade cytotoxin producers, 2 (18.2%) produced a low-
grade cytotoxin, and 1 (9.1%) was noncytotoxic. Of 66 strains
with s1a/m2 vacA genotype, 14 (21.2%) were high-grade cyto-
toxin producers, 32 (48.5%) were low-grade cytotoxin produc-
ters, and 20 (30.3%) were tox−. As shown in figure 2, produc-
tion of high-grade cytotoxin among strains with s1a/m1T was sig-
ificantly higher (72.7%) than among s1a/m2 strains (21.2%).
Statistical analysis indicated that vacA s1a/m1T strains pro-
duced higher levels of vacuolating activity than vacA s1a/m2
strains (P < .05, Fisher’s exact test). The median cytotoxin
activity scores of s1a/m2 and s1a/m1T strains were 16 and 45,
respectively. These results suggest that s1a/m1T strains are
associated with higher levels of cytotoxin-producing activity
than are s1a/m2 strains.

Relationship between vacA genotypes and gastrointestinal
diseases. Infection with the s1a/m1T vacA type was found in
10 (16.7%) of 60 subjects with peptic ulceration but only in 1
(2.3%) of 44 subjects with nonulcer dyspepsia (table 2, P <
.05), indicating that persons infected with s1a/m1T strains are
more likely to have peptic ulcer disease than are persons in-
fected with s1a/m2 strains. Although 2 (13.3%) of 15 persons
infected with type s1a/m1T had gastric cancer compared with
1 (2.3%) of 44 nonulcer dyspepsia patients infected with s1a/
m1T strains, there was no statistically significant association.
When the vacA genotype was compared with cagA status, the
only 3 cagA− strains among 173 Taiwanese isolates [24] were
typed as s1a/m2, and all were associated with nonulcer dyspe-
sia. Of 116 cagA+ isolates, 13 were s1a/m1T and 103 were
s1a/m2. Despite the association of all 3 cagA− strains with
the s1a/m2 type, we could not conclude that there was any
significance between cagA status and specific vacA genotype
since there are many fewer cagA− than cagA+ strains in Taiwan
[24].

Discussion

In this study, 3 different H. pylori genotypes (s1a/m1T, s1a/
m2, and s1a/m1Tm2) were found in Taiwan. Five genotypes
(s1a/m1, s1b/m1, s1a/m2, s1b/m2, and s2/m2) have been found
in the United States [16]. A high proportion (87%) of the
Taiwanese H. pylori clinical isolates were typed as s1a/m2,
suggesting there is less mosaicism in vacA alleles of H. pylori
isolates in Taiwan. These results are similar to those of a recent
study in Japan [30]. In Japan, only s1a/m1 and s1b/m1 types
were found and an extremely high proportion of clinical isolates
(97%) were s1a/m1 vacA type. However, unlike the Japanese
strains, which were highly homologous to each other (all typed
as the m1 allele type in the middle region) [30], both m1T
(m1-like) and m2 types were found in Taiwan. This suggests
that the degree of allelic heterogeneity and recombination in the vacA gene might vary by geographic area.

We showed that all 116 cagA+ isolates were signal sequence type s1a and that the only 3 cagA− strains among 173 Taiwanese isolates [24] were also s1a. Thus, although cagA+ status and vacA s1a type (two closely associated phenotypes) are proposed as markers of ulcerogenic potential [16, 22], they cannot be useful for prediction of peptic ulcer risk in Taiwan. Instead, the finding that s1a/m1T strains were more closely associated with higher levels of vaculating activity and that vacA s1a/m1T strains (72.7%) occurred more frequently in peptic ulceration than did vacA s1a/m2 strains (21.2%), suggested that the s1a/m1T type might be a potential ulcerogenic marker for peptic ulceration in Taiwan. However, since only a small proportion of patients were infected with the s1a/m1T type, such an assessment would have a limited effect in practice.

Despite the strong association between the s1a/m1T vacA genotype and high cytotoxin-producing activity, 27% of s1a/m1T strains produced low-grade or nondetectable cytotoxin. Similarly, for s1a/m2 strains, 14 (21%) of 66 s1a/m2 isolates produced high-grade cytotoxin, although a relatively high proportion of strains were associated with low or no cytotoxin-producing activity. A mature VacA polypeptide consists of 2 domains of relative molecular masses of 34 and 58 kDa [20]. Thus, it is possible that other regions in the vacA gene in addition to the middle region may also play a role in cytotoxic activity, since the middle region of vacA encodes only part of the 58-kDa domain [16]. Alternatively, there are unidentified elements that might influence the vaculating activity. Further investigation will be required to understand the molecular basis between tox+ and tox− strains as well as the roles of these 2 distinct subunits in cytotoxin action.

Acknowledgments

Hung-Jung Wang and Chun-Hsien Kuo contributed equally to this work. We thank Dr. J.-C. Yang for technical support.

Table 2. Relationship between a vacA genotype and its association with gastrointestinal disease for 119 H. pylori isolates.

<table>
<thead>
<tr>
<th>vacA type (n)</th>
<th>Peptic ulcer*</th>
<th>Nonulcer dyspepsia*</th>
<th>Gastric cancer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1a/m1T (13)</td>
<td>10 (76.9)</td>
<td>1 (7.7)</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td>s1a/m2 (104)</td>
<td>48 (46.2)</td>
<td>43 (41.3)</td>
<td>13 (12.5)</td>
</tr>
<tr>
<td>s1a/m1Tm2 (2)</td>
<td>2 (100)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* vacA s1a/m1T strains were more frequently associated with peptic ulceration than were vacA s1a/m2 strains (P < .05, χ² test with Yates’s correction). 

References