Genetic variation in Helicobacter pylori
Pan, Z.

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Chapter 5

Relation between vacA subtypes, cytotoxin activity, and disease in Helicobacter pylori-infected patients from the Netherlands

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Relation Between vacA Subtypes, Cytotoxin Activity, and Disease in Helicobacter pylori-Infected Patients From The Netherlands

Zhi-Jun Pan, M.D., René W. M. van der Hulst, M.D., Ph.D., Guido N. J. Tytgat, M.D., Ph.D., Jacob Dankert, M.D., Ph.D., and Arie van der Ende, Ph.D.

Departments of Medical Microbiology and Gastroenterology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

OBJECTIVE: The vacuolating cytotoxin of Helicobacter pylori (H. pylori) is encoded by vacA, of which allelic variation has been described. In the U.S., H. pylori strains with the signal sequence allele s1a are associated with enhanced gastric inflammation and with peptic ulcer disease (PUD). The ml middle region allele is linked with more severe gastric epithelial damage. However, the distribution of H. pylori genotypes and the association with disease may be different in other geographical areas. The aim of this study was to establish whether vacA types among H. pylori isolates from Dutch patients are associated with disease.

METHODS: The cytotoxin activity of the H. pylori isolates from 34 PUD patients and 46 patients with functional dyspepsia (FD) was assessed by an in vitro assay using HeLa cells as indicator cells. The vacA types and cagA status of the isolates were assessed by polymerase chain reaction (PCR).

RESULTS: vacA s1-type H. pylori displayed cytotoxin activity more frequently than s2 vacA-type H. pylori (p = 0.003). This difference was not significant when only cagA+ H. pylori were considered. H. pylori isolates with the ml vacA type exhibited a higher cytotoxin activity, independent of cagA (p = 0.006). Ninety-four percent (32/34) of the PUD patients and 74% (34/46) of the FD patients were infected with s1 vacA-type H. pylori (p = 0.04). When only cagA+ H. pylori were considered, s1 vacA type was not associated with disease. In addition, neither the s1a nor s1b subtypes correlated with disease.

CONCLUSIONS: An association between vacA subtypes and disease could not be established in this patient population, due to the strong linkage between vacA s1 type and cagA. (Am J Gastroenterol 1999;94:1517-1521. © 1999 by Am. Coll. of Gastroenterology)

INTRODUCTION

Helicobacter pylori (H. pylori) is the major cause of chronic superficial gastritis in humans, an important etiological factor in the pathogenesis of peptic ulcer disease (PUD) and some forms of gastric cancer (1, 2). Various virulence factors of H. pylori involved in the onset of gastric infection have been described, but so far, two loci associated with clinical manifestations, the cytotoxin-associated gene A (cagA) and vacA encoding the vacuolating cytotoxin (VacA), have been identified (for a review see Reference 3).

The cagA gene encodes a large immunodominant protein (CagA) of unknown function and is part of the 40-Kb cag pathogenicity island (PAI) (4, 5). This region, with a different GC content than the rest of the chromosome, comprises at least 22 genes and inactivation of many of these abrogates the inflammatory response induced in cultured epithelial cells by cag-positive strains. Strains that do not produce the CagA protein generally lack the entire cag PAI.

In Western countries, only 60–70% of the patients with functional dyspepsia (FD) and virtually all patients with PUD are colonized with cagA+ H. pylori (6). In addition, the cag PAI is predominantly found in H. pylori isolated from cases associated with more severe active gastritis and gastric cancer (7, 8).

The cytotoxin VacA induces vacuoles in cultured mammalian cells (9, 10). Although approximately 30–60% of H. pylori isolates produce measurable vacuolating cytotoxin activity in vitro, all possess vacA (10). In addition, the purified toxin was found to cause gastric epithelial damage and mucosal ulceration in a mouse model (11). Sixty to seventy percent of the H. pylori isolates from Western patients with PUD display cytotoxin activity, whereas 30–50% of isolates from FD patients are toxigenic (6, 12). Analysis of vacA in H. pylori isolates from patients in the U.S. showed that two types of the signal sequence, s1 (which can be subtyped into s1a and s1b) and s2, and two types of the middle region, ml and m2, of vacA can be distinguished (13). In the U.S., H. pylori strains with the s1a vacA allele are associated with higher levels of vacuolating cytotoxin activity than the s1b or s2 allele and are associated with enhanced gastric inflammation and PUD. The ml middle region allele is associated with higher levels of toxin activity and more severe gastric epithelial damage than the m2 allele (13, 14).
Recently, we found that vacuolating activity of \textit{H. pylori} from Chinese patients was not associated with a particular disease status, and the distribution of \textit{vacA} alleles was distinct from that seen in \textit{H. pylori} isolates from patients in the U.S. (15). In addition, none of the \textit{vacA} alleles was associated with clinical manifestations in \textit{H. pylori}-infected patients, due to the homogeneous distribution of the \textit{vacA} alleles. In this report we studied the relation between \textit{vacA} types of \textit{H. pylori} from 80 \textit{H. pylori}-infected Dutch patients with clinical manifestations.

**MATERIALS AND METHODS**

**Patient Population**

Eighty consecutive \textit{H. pylori}-infected dyspeptic adults underwent gastroendoscopy at the Department of Gastroenterology in the Academic Medical Center, Amsterdam. Thirty-four of them had PUD, as they had an active ulcer or scars from ulcers were seen at endoscopy. Twenty-one had duodenal ulcers, 16 had an ulcer in the ventriculi, and three had ulcers in the duodenum and ventriculi. The remaining 46 patients suffered from FD, i.e., no ulcers or scars from ulcers were observed at endoscopy.

**Endoscopy, Histopathology, and \textit{H. pylori} Cultures**

During each endoscopic procedure, three antral and three corpus mucosal biopsy specimens were obtained by use of biopsy forceps, which were cleansed with a detergent, disinfected with 70% ethanol, and autoclaved after each examination. One antrum and one corpus biopsy specimen were placed in 2 ml of phosphate-buffered saline at 4°C and used for bacteriological cultivating. The other four specimens were fixed in 10% formalin for histopathological examination. Bacteriological and histological assessments of the mucosal biopsy specimens were carried out as previously described (16, 6). Cultures were prepared by smearing biopsy specimens on the surface of horse blood agar plates (75% defibrinated horse blood Columbia agar base, Oxoid CM 331, Unipath, Basingstoke, England) and horse blood agar plates containing Skirrow supplement (Unipath). \textit{H. pylori} organisms were identified on the basis of typical colony morphology, characteristic appearance on Gram staining, and positive urease, oxidase, and catalase test. \textit{H. pylori} grown on the primary culture plates of each antrum or corpus biopsy specimens were collected separately using swabs. Thus, \textit{H. pylori} populations from either the antrum or corpus biopsy specimens from the 80 patients were assessed for their \textit{cagA} status.

**Vacuolating Cytotoxin Assay**

\textit{H. pylori} isolates were assessed for cytotoxin activity by a modification of the procedure described by Leunk et al. (9) and Tee et al. (17), recently described by Fan et al. (15). Briefly, an aliquot was scraped from the frozen \textit{H. pylori} culture suspensions, thawed, cultured for 4 days on 7% horse blood agar plates and then subcultured for another 3 days at 37°C under microaerobic conditions. A small sample was inoculated in 6 ml of Brucella Broth (Difco Laboratories, Surrey, England) supplemented with 5% v/v fetal bovine serum (GIBCOBRL, Life Technology, Paisley, Scotland). The initial optical density at 540 nm (A540) was adjusted to a value between 0.1 and 0.3. The cultures were incubated at 37°C with an atmosphere of 10% CO₂, 5% O₂, and 85% N₂ on a gyratory shaker at 140 rpm for 48 h to an optical density of A540 between 0.4 and 1.3. The liquid \textit{H. pylori} cultures were centrifuged at 3000 \times g for 20 min, and the supernatants were used to inoculate \textit{HeLa} cells. Confluent \textit{HeLa} cell cultured in Dulbecco's modified Eagle medium (DMEM, GIBCOBRL, Life Technology) with 10% of fetal calf serum (FCS) (GIBCOBRL, Life Technology) were trypsinized and adjusted to a density of 2 \times 10^⁶ cells per ml. One milliliter of this cell suspension was seeded into each well of a 24-well microtiter plate. After 48 h incubation at 37°C in 5% O₂ and 95% CO₂, culture medium was changed for 0.8 ml of culture test medium (DMEM with 25 mmol/L HEPES, pH 7.4) and 0.8 ml of \textit{H. pylori} culture supernatant was added. Uninoculated Brucella Broth medium was used as a negative control, and a culture supernatant of the cytotoxin-positive Dutch \textit{H. pylori} strain (isolate no. 157) was used as a positive control. After further incubation at 37°C in 5% O₂ and 95% CO₂ for 24 and 48 h, cells were examined microscopically for the presence of intracellular vacuoles. The supernatants of all \textit{H. pylori} isolates were tested in duplicate and scored as positive if > 50% of the cells/well contained vacuoles. Culture supernatants without cytotoxin activity after 30-fold concentration were considered to be cytotoxin negative.

**Preparation of Genomic DNA for Polymerase Chain Reaction (PCR)**

After thawing of an aliquot of the frozen primary cultures, bacterial suspensions were inoculated on horse blood agar plates and cultured at 37°C for 3 days in a microaerobic environment. Bacteria were harvested and genomic DNA were extracted using Insta Gene Matrix (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions.

**\textit{vacA} Genotyping**

PCR-based typing of \textit{vacA} was assessed according to Atherton et al. (13). For \textit{vacA} signal typing, each strain was typed as s1a, s1b, or s2 by performing three separate PCRs, each using one primer specific to the signal sequence type, and one conserved primer (13). The \textit{vacA} midregion was typed as m1 or m2 by two separate PCRs, each using one pair of
primer specific to the m1 or m2 midregion. Thermal cycling for each set of primers (0.5 μmol/L each) was at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, for a total of 35 cycles.

PCR Amplification of cagA

The cagA status of the H. pylori isolates was assessed by PCR as described (6). The cagA status of the H. pylori isolates was confirmed by Southern blotting as described (6).

Statistical Analysis

The χ² test with Yates’ continuity correction (or Fischer exact test, when applicable) was used for analysis of categorical data. Two-sided significances are reported. Exact 95% confidence intervals of the difference in proportion were calculated using Stat Exact, version 3.0.

RESULTS

Prevalence of cagA+ and vacA Type of H. pylori Among Dutch H. pylori-Infected Patients With PUD or FD

In total, 26 (33%) of the H. pylori isolates induced vacuoles in HeLa cells in vitro. Nineteen (56%) of the 34 patients with PUD and seven (15%) of the 46 patients with FD were colonized with H. pylori capable of inducing vacuoles in HeLa cells. Thus, patients with PUD were more often infected with cytotoxic-positive H. pylori (p = 0.0003; 95% confidence limits of the difference in proportion: 60–21%).

Sixty (75%) of the 80 patients, 28 (94%) of 34 patients with PUD and 26 (56%) of 46 patients with FD, were colonized with cagA-positive H. pylori (p = 0.002; difference: 33%; 95% confidence limits: 12–54%).

Relation Between Cytotoxin Activity, cagA Status, and vacA Genotype of H. pylori Isolates

The relation between vacA subtypes, cagA status, and in vitro cytotoxic activity is displayed in Table 1. Twenty-four (92%) of 26 Tox+ H. pylori isolates and 36 of 54 (67%) of the Tox− H. pylori isolates were cagA positive (p = 0.03; difference: 25%; 95% confidence limits: 3–48%).

Fifty percent (13/26) of the Tox+ H. pylori and 15% (8/54) of the Tox− H. pylori were of the s1a/m1 vacA subtype (p = 0.002; difference: 35%; 95% confidence limits: 12–60%). The single subtype m1, but not s1a, showed a correlation with the cytotoxic activity of H. pylori. Twenty-seven (77%) of 26 Tox+ H. pylori isolates and only 13 (24%) of 54 of the Tox− H. pylori are of m1 type (p = 3 × 10⁻⁵; difference: 53%; confidence limits: 30–75%). Among the 60 cagA+ H. pylori, 19 (32%) of the 24 Tox+ H. pylori and 11 (31%) of the 36 Tox− H. pylori were of the m1 vacA subtype (p = 0.0006; difference: 48%; 95% confidence limits: 23–72%). Hence the association between m1 vacA type and Tox+ H. pylori is not dependent on the cagA status of the H. pylori isolate.

All 26 of the Tox+ H. pylori and 74% (40/54) of the Tox− H. pylori were of the s1 vacA type (p = 0.003; difference: 26%; 95% confidence limits: 6–46%). All 24 cagA+ H. pylori isolates with measurable cytotoxin activity had the s1 vacA type and 34 of 36 (94%) cagA+ H. pylori without cytotoxin activity had the s1 vacA type.

None of the 26 Tox+ H. pylori and 26% (14/54) of the Tox− H. pylori were of the s2m2 vacA subtype (p = 0.003; difference: 26%; 95% confidence limits: 6–46%). Most (12/14) of the s2m2 H. pylori isolates were cagA negative. Thus, s2 vacA types are predominantly found with cagA-negative H. pylori.

Relation Between cagA Status and vacA Type of H. pylori Isolates and Disease in H. pylori-Infected Patients

The relationship between cagA status, vacA subtypes, and patient’s disease is shown in Table 2. The H. pylori isolates of 71% (24/34) of the PUD patients and of 54% (25/46) of the FD patients were cagA positive (p = 0.03; difference: 25%; 95% confidence limits: 12–54%).

Table 1. Relation Between Cytotoxin Activity, cagA Status, and vacA Type of H. pylori Isolates From 80 H. pylori-Infected Patients From The Netherlands

<table>
<thead>
<tr>
<th>H. pylori Phenotype</th>
<th>vacA</th>
<th>cagA+</th>
<th>cagA−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tox+ (n = 26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1a/m1</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>s1a/m2</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>s1a/m(−)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>s1b/m1</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>s1b/m2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>s2/m</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tox− (n = 54)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1a/m1</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>s1a/m2</td>
<td>18</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>s1a/m(−)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>s1b/m1</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>s1b/m2</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>s2/m</td>
<td>2</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Relation Between cagA Status and vacA Type of H. pylori Isolates and Clinical Manifestations in 80 H. pylori-Infected Patients From The Netherlands

<table>
<thead>
<tr>
<th>H. pylori Genotype</th>
<th>Diagnosis at Endoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vacA</td>
</tr>
<tr>
<td>PUD (n = 34)</td>
<td></td>
</tr>
<tr>
<td>s1a/m1</td>
<td>10</td>
</tr>
<tr>
<td>s1a/m2</td>
<td>11</td>
</tr>
<tr>
<td>s1a/m(−)</td>
<td>2</td>
</tr>
<tr>
<td>s1b/m1</td>
<td>7</td>
</tr>
<tr>
<td>s1b/m2</td>
<td>1</td>
</tr>
<tr>
<td>s2/m</td>
<td>2</td>
</tr>
<tr>
<td>FD (n = 46)</td>
<td></td>
</tr>
<tr>
<td>s1a/m1</td>
<td>8</td>
</tr>
<tr>
<td>s1a/m2</td>
<td>11</td>
</tr>
<tr>
<td>s1a/m(−)</td>
<td>0</td>
</tr>
<tr>
<td>s1b/m1</td>
<td>5</td>
</tr>
<tr>
<td>s1b/m2</td>
<td>3</td>
</tr>
<tr>
<td>s2/m</td>
<td>1</td>
</tr>
</tbody>
</table>

PUD = peptic ulcer disease; FD = functional dyspepsia.
the FD patients were of the s1a vacA type (n.s.; difference: 16%; 95% confidence limits: –6–40%). In addition, s1b, the m1, or the m2 vacA type did not show correlation with disease. Together, 94% (32/34) of the PUD patients and 74% (34/46) of the FD patients were infected with s1 vacA-type H. pylori (p = 0.04; difference: 20%; 95% confidence limits: 0–40%). However, the s2 vacA type is predominantly found with cagA – H. pylori. Twelve of the 14 s2m2-type H. pylori isolates were cagA negative. Hence, the correlation of s1 vacA type with PUD was not observed when only the 60 cagA + H. pylori were considered.

DISCUSSION

This paper describes the correlation between cytotoxin activity of the H. pylori isolate, the cagA status of the H. pylori isolate, and disease in a large population (n = 80) of H. pylori-infected patients from the Netherlands. The vacA type m1 appeared to be associated with in vitro cytotoxin activity, whereas the vacA m2 type is associated with H. pylori with low or no in vitro cytotoxin activity. The s2m2 vacA subtype is strongly linked to the cagA – status of the H. pylori. Thus vacA s1 type seems to be correlated with PUD and s2 type with FD. However, the vacA s2 type is almost exclusively found in cagA – H. pylori, and consequently a correlation between the s1 type and disease could not be established.

H. pylori of the s1a/m1 vacA subtype was more often found with cytotoxin activity, most likely due to the correlation between the sole m1 vacA subtype and cytotoxin activity. These results are in agreement with those of Atherton et al. (14). However, in contrast to the results of Atherton et al., only the m1 vacA type, but not the s1a vacA type, was associated with cytotoxin activity of H. pylori isolates from Dutch patients. The m2 vacA subtype was more often found with H. pylori that produced only low levels of or no cytotoxin activity.

In this study the s2m2 vacA type was strongly linked with the cagA – status of H. pylori, i.e., only 3% (2/60) of the cagA + H. pylori and 60% (12/20) of the cagA – H. pylori were of the s2m2 vacA type. This might indicate selection against this vacA allele when the cag pathogenicity island is present. The s1a vacA subtype did not show a significant association with disease. Correlation between vacA s1 type and PUD was observed, but this association is difficult to interpret because s1 is strongly linked with cagA + status, which is strongly associated with PUD. In addition, neither of the vacA types showed correlation with disease in patients. The lack of correlations between vacA type and clinical manifestations presented here is in contrast to those described by Atherton et al. (14). They observed a correlation between the s1a subtype of vacA and PUD, as well as a higher grade of gastritis. The reason for this difference is unclear. It is known that H. pylori from patients from different geographical regions can have different distributions of vacA alleles. In particular, H. pylori from Asian patients displays a different vacA allele pattern (15, 18). However, the study of Atherton et al. and this study were both performed in Western countries. In addition, in our study, the proportion of patients infected with a cagA + H. pylori and the proportions of the different vacA alleles are similar to what is observed for other patient populations from Western countries, indicating that the H. pylori population in this area is similar to that of the rest of the Western world. Nevertheless, this does not exclude a difference in distribution of vacA alleles between H. pylori among Dutch patients and among patients from the U.S.

The occurrence of the s1 vacA type largely overlapped with the occurrence of cagA. Only two of 60 cagA + H. pylori isolates (one from a PUD patient and one from a FD patient) had the s2 vacA type. Therefore, both parameters are equal in discriminating ulcerogenic H. pylori strains. The fact that cagA – and s2-type H. pylori are rarely isolated from patients with PUD might indicate that FD patients infected with cagA + s1 H. pylori are at risk for developing an ulcer. The course of infection in these patients might be just too short to have an ulcer develop. Alternatively, host factors may contribute to the development of ulcers in H. pylori-infected patients.

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Reprint requests and correspondence: Arie van der Ende, Ph.D., Academic Medical Center, Department of Medical Microbiology, University of Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands.

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