Role of Deletion Located between the Intermediate and Middle Regions of the *Helicobacter pylori vacA* Gene in Cases of Gastroduodenal Diseases[▽]

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Received 5 May 2009/Returned for modification 19 June 2009/Accepted 22 August 2009

The vacuolating cytotoxin gene of *Helicobacter pylori*, *vacA*, induces cytoplasmic vacuolation in gastric epithelial cells. Recently, the *vacA* intermediate (i) region, which is located between the signal (s) and middle (m) regions, was identified as a third polymorphic determinant of *vacA* activity. In *vacA*, there are approximately 81-bp deletions between the *vacA* i and m regions (denoted the d region). The aim was to clarify the roles of the *vacA* d region in relation to *H. pylori*-related diseases and histopathological gastric mucosal changes. We assessed the *vacA* signal s-, m-, i-, and d-region genotypes and *cagA* status in *H. pylori* isolates recovered from Western countries ($n = 266$) and East Asian countries ($n = 244$) by PCR. In East Asian countries, there were no relationships between the *vacA* genotypes and the clinical outcomes and histopathological changes. In Western countries, strains with the *vacA* s1, m1, i1, or d1 (no deletion) genotype significantly increased the risk for the development of gastric cancer compared with the risk from strains with the s2, m2, i2, or d2 genotype (adjusted odd ratios, 3.17 [95% confidence interval {CI}, 1.07 to 9.45] for s1, 10.65 [95% CI, 3.36 to 31.35] for m1, 8.57 [95% CI, 2.85 to 25.81] for i1, and 8.04 [95% CI, 2.67 to 24.16] for d1). The highly virulent *vacA* genotypes significantly enhanced neutrophil infiltration and gastric atrophy in univariate analysis, whereas only the *vacA* d-region genotype was significantly associated with neutrophil infiltration and gastric atrophy in both the antrum and the corpus by multiple linear regression analysis. The presence of the *vacA* d1 genotype in *H. pylori* strains could be an improved predictor of histological inflammation and the potential for atrophy compared with the presence of the *vacA* s-, m-, and i-region genotypes in Western countries.

Gastric cancer arises through steps related to the presence of a chronic *Helicobacter pylori* infection, which leads to the precursor lesion, atrophic gastritis. The progression of gastric mucosal atrophy varies within and between different populations; and the differences relate to differences in *H. pylori* virulence, host genetic factors, and/or environmental factors. The important *H. pylori* virulence factors and host genetic factors are related to an increased inflammatory response and include the presence of the *cag* pathogenicity island, polymorphisms of host inflammation-related cytokines (5, 6), drug metabolism-related enzymes (19, 22), and growth factors (5, 6, 12, 19, 22, 30).

The vacuolating cytotoxin (VacA) was one of the first putative virulence factors discovered in *H. pylori* (4). Virtually all *H. pylori* strains possess a *vacA* gene; however, the in vitro vacuolating activities for cell lines vary considerably among strains; and the differences in the vacuolating activities are related to differences in the *vacA* structures at the signal (s) region (s1 and s2) and the middle (m) region (m1 and m2) (1). The s region encodes part of the signal peptide and the N terminus of the mature protein, whereas the m region encodes part of

the 55,000-Da (55K) C-terminal subunit. The amount of toxin produced also varies according to the *vacA* m-region genotypes: *vacA* s1-m1 strains induce greater vacuolating activity than *vacA* s1-m2 strains (1, 11).

Recently, a third polymorphic determinant of vacuolating activity was described as being located between the s and m regions and was termed the intermediate (i) region (16). Two i-region subtypes were described: the i1 and i2 subtypes. Among Western strains, *vacA* s1-m2 strains were noted to vary in their i-region genotypes; *vacA* s1-m1 and s2-m2 strains were exclusively i1 and i2, respectively. The *vacA* s1-i1-m2 strains induced vacuolation in rabbit kidney RK13 cells, whereas s1-i2-m2 strains did not. Clinically, the prevalence of the *vacA* i1 genotype in patients with gastric cancer was 80%, which was significantly higher than that in patients in an Iranian population with nonulcer dyspepsia (37%) (16). Subsequent studies showed that infection with *vacA* i1 strains was associated with gastric cancer in patients in Iranian and Italian populations and gastric ulcer in patients in Iraqi and Italian populations (2, 8, 16). This allowed the conclusion that the *vacA* i-region genotype might be a better predictor of the carcinogenic potential of *H. pylori* than the previously used *vacA* s- and m-region genotypes. However, our recent study shows that determination of the *vacA* genotypes for the combination of three regions (the s, m, and i regions) did not provide any advantage as a disease determinant marker over determination of s- and

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[▽] Published ahead of print on 2 September 2009.

FIG. 1. Nucleotide sequences of *vacA* d1 or d2 region from eight strains. Strains Tx30a and OK180 have the *vacA* d2 genotype; and strains 26695, NATC11637, F-71, 249a3, ATCC 49503, and J99 have the *vacA* d1 genotype. The *vacA* d2 genotype had a 69- to 81-bp deletion between the *vacA* i and m regions.

m-region genotypes in East Asian and Southeast Asian countries (15).

Examination of the information for 49 complete, nonpartial *vacA* gene sequences covering the *vacA* s to m regions deposited in the GenBank database showed that 37 strains were of the s1-i1-m1 genotype, 10 were of the s1-i1-m2 genotype, 1 was of the s1-i2-m2 genotype, and 1 was of the s2-i2-m2 genotype. Interestingly, there was a deletion of 81 bp between the *vacA* i region and the *vacA* m region in two strains (Fig. 1), both of which were of the i2-m2 genotype. In contrast, the remaining 47 strains had either no deletion (31 strains) or a short deletion ranging from 9 bp (5 strains) to 23 bp (1 strain); and there was no correlation between the presence of a short deletion and the *vacA* s-, m-, and i-region type. The frequency of the 81-bp deletion and the short deletion in clinical strains is unclear, and

it is also unknown whether the presence of the 81-bp deletion and the short deletion in clinical strains is associated with other *vacA* genotypes (e.g., the i2 and/or m2 genotype) and with the clinical outcome and/or the degree of gastric histopathological mucosal injury. Of the various deletions, we focused on the 81-bp deletion in this study because the 81-bp deletion was expected to have correlations with the *vacA* genotype. We also examined whether the *vacA* deletion status and/or a combination of the genotypes of the *vacA* s, m, and i regions and the deletion is of advantage as a marker of disease that may be used in addition to currently used *vacA* genotyping systems.

MATERIALS AND METHODS

Patients. *H. pylori* strains were obtained from infected patients from the United States, Colombia, South Korea, and Japan (Table 1). The study popula-

TABLE 1. Demographic characteristics of Western and East Asian patients enrolled in this study

Characteristic	Result for patients from:					P value ^a
	United States	Colombia	South Korea	Japan	Total	
No. of patients	170	96	124	120	510	
No. of males/no. of females	142/28	55/41	94/30	75/45	366/144	<0.01
Mean age (yr) $\pm$ SD	49.8 $\pm$ 1.1	57.1 $\pm$ 1.5	47.5 $\pm$ 1.2	56.9 $\pm$ 1.3	52.3 $\pm$ 0.6	<0.01
No. (%) of patients with the following disease ^b :						
Gastritis	91 (53.5)	32 (33.3)	33 (26.6)	27 (22.5)	183 (35.9)	<0.01
GU	30 (17.6)	0 (0.0)	27 (21.8)	33 (27.5)	90 (17.6)	
DU	40 (23.5)	25 (26.0)	24 (19.4)	28 (23.3)	117 (22.9)	
GC	9 (5.3)	39 (40.6)	40 (32.3)	32 (26.7)	120 (23.5)	

^a P values were used to analyze the differences in the demographic data among patients from the United States, Colombia, South Korea, and Japan.

^b Abbreviations: GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer.

tion consisted of patients with gastric ulcers, duodenal ulcers, gastric cancer, or gastritis alone. Gastritis was defined as *H. pylori* gastritis without peptic ulcers or gastric malignancy. None of the patients had received nonsteroidal anti-inflammatory drugs or steroids at least 3 months prior to endoscopy. None had previously received treatment for *H. pylori* infection. Informed consent was obtained from all patients under protocols approved by the local hospital's ethics committee.

Genotyping of *vacA* s, m, and i regions and *cagA* status by PCR methods. Chromosomal DNA was isolated from confluent plate cultures expanded from a single colony by using the QIAamp tissue kit (Qiagen Inc., Santa Clarita, CA), according to the manufacturer's instructions. Genotyping of the *vacA* s, m, and i regions and *cagA* status were determined by PCR methods, as described previously (1, 10, 16, 28).

Detection of *vacA* d region between i and m regions. We designated the region covering the 81-bp deletion between the i and m regions as the deletion (d) region and designed the following PCR primers to cover that region: 5'-ACTA ATATTGGCACACTGGATTG-3' (forward primer VAS-5F) and 5'-CTCGC TTGATTGGACAGATTG-3' (reverse primer VAGF-R). The PCR conditions were 95°C for 90 s; then 35 cycles of 95°C for 30 s, 53°C for 60 s, and 72°C for 30 s; and finally, 72°C for 5 min. The fragments classified two types: type d1 without the 81-bp deletion (approximately 367 to 379 bp due to the short deletion) and type d2 with the 81-bp deletion (approximately 298 bp). In this study, a short deletion of 9 to 23 bp was included in the d1 genotype.

Histology. Gastric biopsy specimens were taken from the antrum (pyloric gland area) and the corpus (fundic gland area). The biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and cut into sequential 4- μ m sections. The *H. pylori* density, gastritis activity (neutrophil infiltration), and atrophy were graded from 0 (absent/normal) to 5 (maximal), as described previously (7). The score is presented as the mean scores for biopsy samples from the corpus and antral areas.

It is well known that atrophic gastritis poses a high risk for the development of gastric ulcer and gastric cancer, and antral dominant gastritis is associated with the development of duodenal ulcer; therefore, the gastritis group contained a mixture of patients, some of whom would be destined to develop severe clinical outcomes. Use of the Operative Link on Gastritis Assessment (OLGA) staging system was recently advocated. By use of that system, gastritis staging combined with *H. pylori* infection provided clinically relevant information on the overall status of the gastric mucosa and the implications of that status for prognosis, therapy, and management (17, 18). We therefore analyzed the risk of certain clinical outcomes by comparing not only all gastritis patients but also gastritis patients with patients with OLGA stages 0 and I as a control group.

Data analysis. Statistical differences in demographic characteristics, *vacA* genotypes, and *cagA* status among the different geographic groups were determined by one-way analysis of variance or the chi-square test. Differences in the histological scores among the different *vacA* genotype groups were analyzed by using the Mann-Whitney U test. A multiple linear regression analysis was performed to determine which factor(s) was related to the severity of the histology, with age, sex, bacterial factors, and clinical outcome being explanatory variables. The selection of variables was by backward stepwise deletion in the logistic regression and by the F value out and F value in stepwise method in the linear regression, where both F values were 2.0. A P value of less than 0.05 was accepted as statistically significant. Calculations were carried out by using StatView (version 5.0) statistical software (SAS institute Inc., Cary, NC) or HALBAU statistical software (Gendai-sugaku-sha, Kyoto, Japan).

RESULTS

The *vacA* genotypes and *cagA* status were determined for 510 *H. pylori* isolates, including 266 strains from Western countries (170 strains from the United States and 96 strains from Colombia) and 244 strains from East Asian countries (124 strains from South Korea and 120 strains from Japan) (Table 1). The mean age of the subjects, the ratio of the patients by sex, and the population of individuals with *H. pylori*-related diseases were significantly different ($P < 0.01$) among the different countries (Table 1). Therefore, the odds ratio (OR) for the development of peptic ulcer and gastric cancer was adjusted by age and sex in subsequent analyses.

The prevalence of *vacA* genotypes and *cagA* status. We combined the data from the different East Asian and Western countries into the East Asian and the Western groups, respectively. East Asian strains had a higher prevalence of *vacA* s1, m1, i1, and d1 genotypes than Western strains (Table 2). The prevalence of *vacA* s-, i-, and d-region genotypes and *cagA* status were similar between U.S. and Colombian strains and between Japanese and South Korean strains. In contrast, the prevalence of *vacA* m genotypes differed significantly among East Asian strains but not among strains from Western countries ($P < 0.01$) (Table 2).

In agreement with the findings of previous studies (15, 16), all *vacA* s1-m1 strains from East Asian countries were of the i1 genotype and, with the exception of one strain of the i2 genotype, all *vacA* s1-m1 strains from Western countries were exclusively of the i1 genotype (99.8%) (Table 2). In addition, with the exception of one strain of the i1 genotype, all s2-m2 strains from Western countries were exclusively of the i2 genotype (99.8%). The prevalence of the *vacA* i1 and i2 genotypes was similar among s1-m2 strains from Western countries (45.5% and 55.5%, respectively), which was in agreement with the findings of previous studies (15, 16); however, only 6 of 18 s1-m2 strains from East Asian countries (33.3%) showed the i2 genotype. When the d-region genotypes were taken into account, i1 and i2 strains exclusively showed the d1 and d2 genotypes, respectively (98.9% and 93.7%, respectively), with the exceptions being one Western strain of the i1-d2 genotype, seven Western strains of the i2-d1 genotype, and one East Asian (South Korean) strain of the i2-d1 genotype (Table 2). Overall, the *vacA* s-, m-, i-, and d-region genotypes as well as the *cagA* status were significantly correlated with one another

TABLE 2. Prevalence of virulence factors and different ethnic groups

Virulence factor	Genotype	No. (%) of patients							<i>P</i> value ^a
		Western countries			East Asian countries			Total	
		United States	Colombia	Total	Korea	Japan	Total		
<i>vacA</i>									
s region	s1	141 (83.9)	74 (77.1)	215 (80.8)	124 (100)	120 (100)	244 (100)	459 (90.0)	<0.01
m region	m1	106 (62.4)	65 (67.7)	171 (64.3)	109 (87.9)	117 (97.5)	226 (92.6)	397 (77.8)	<0.01
i region	i1	122 (71.8)	69 (71.9)	191 (71.8)	120 (96.8)	118 (98.3)	238 (97.5)	429 (84.1)	<0.01
d region	d1	126 (74.1)	71 (74.0)	197 (74.1)	121 (97.5)	118 (98.3)	239 (98.0)	436 (85.5)	<0.01
s-m-i combination	s1-m1-i1	105 (61.8)	65 (67.7)	170 (63.9)	109 (87.9)	117 (97.5)	226 (92.6)	396 (77.6)	<0.01
	s1-m1-i2	1 (0.6)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.2)	
	s1-m2-i1	16 (9.4)	4 (4.2)	20 (7.5)	11 (8.9)	1 (0.8)	12 (4.9)	32 (6.3)	
	s1-m2-i2	19 (11.2)	5 (5.2)	24 (9.0)	4 (3.2)	2 (1.7)	6 (2.5)	30 (5.9)	
	s2-m2-i1	1 (0.0)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.2)	
	s2-m2-i2	28 (17.1)	22 (22.9)	50 (18.8)	0 (0.0)	0 (0.0)	0 (0.0)	50 (9.8)	
s-m-i-d combination	s1-m1-i1-d1	105 (61.8)	65 (67.7)	170 (64.0)	109 (87.9)	117 (97.5)	226 (92.6)	396 (77.6)	<0.01
	s1-m2-i1-d1	16 (9.4)	4 (4.2)	20 (7.5)	11 (8.9)	1 (0.8)	12 (4.9)	32 (6.3)	
	s1-m2-i2-d2	15 (8.8)	5 (5.2)	20 (7.5)	3 (3.2)	2 (1.7)	5 (2.0)	25 (4.9)	
	s2-m2-i2-d2	28 (16.5)	20 (20.8)	48 (18.0)	0 (0.0)	0 (0.0)	0 (0.0)	48 (9.4)	
	Other ^b	6 (3.5)	2 (2.1)	8 (3.0)	1 (0.8)	0 (0.0)	1 (0.4)	9 (1.8)	
<i>cagA</i>	Pos./neg. ^c	143 (84.1)	78 (81.3)	221 (83.1)	121 (97.6)	120 (100)	241 (98.8)	462 (90.6)	<0.01

^a P values were used to analyze differences in the demographic data between patients from Western countries and those from East Asian countries.

^b The other types of the *vacA* s-m-i-d combination included s2-m2-i2-d1 (*n* = 2), s1-m2-i2-d1 (*n* = 5), s1-m1-i2-d1 (*n* = 1), and s2-m2-i1-d2 (*n* = 1).

^c Pos./neg., positive/negative.

among the Western strains (Table 3). However, nearly all East Asian strains had identical genotypes (*vacA* s1, m1, i1, and d1 and *cagA* positive).

Influence of *vacA* genotype and *cagA* status on clinical outcome. In East Asia, there was no significant association between the clinical outcome and the *vacA* genotype or *cagA* status (Fig. 2). In contrast, the *vacA* d1 genotype was significantly associated with gastric cancer in Western countries (for all patients versus patients with gastritis, the adjusted OR is 8.04 [95% confidence interval {CI}, 2.67 to 24.16]; for all patients versus patients with OLGA stages 0 and I, the OR is 33.78 [95% CI, 11.26 to 101.39]) (Table 4). Similarly, the *vacA* s1, m1, and i1 genotypes were significantly associated with gastric cancer (Fig. 2 and Table 4). When all patients were compared with patients with OLGA stages 0 and I, the *vacA* s1, m1, i1 and d1 genotypes were significantly associated not only with gastric cancer but also with gastric ulcer and duodenal ulcer in Western countries (Table 4). In the multiple linear regression analysis by use of a factor(s) related to the clinical outcome, no single factor was found to be associated with the risk of gastroduodenal disease.

TABLE 3. Relationship among different *H. pylori* virulence factor genotypes for patients from Western countries

<i>vacA</i> genotype	R value			Presence of <i>cagA</i>
	<i>vacA</i> m1	<i>vacA</i> i1	<i>vacA</i> d1	
s1	0.410 ^a	0.462 ^a	0.506 ^a	0.535 ^a
m1		0.617 ^a	0.591 ^a	0.385 ^a
i1			0.678 ^a	0.432 ^a
d1				0.475 ^a

^a *P* < 0.01.

Histology. The gastric mucosa in patients with gastric cancer and gastric ulcer is generally atrophic, and the gastric mucosa in patients with duodenal ulcer generally has enhanced inflammation; thus, the inclusion of those patients in histological analyses might result in a potential bias. We therefore limited the evaluation of the histological analyses by using only patients with gastritis (for the Western population, *n* = 123; for the East Asian population, *n* = 60).

For the patients from East Asian countries, there was no

A. East-Asia

B. Western

FIG. 2. Prevalence of strains with *vacA* s1, m1, i1, and d1 genotypes and *cagA* status for strains from all patients of East Asian (A) and Western (B) origin. For strains from Western countries, the *vacA* m1, i1, and d1 genotypes significantly increased the risk of gastric cancer compared with the risk of gastritis alone. *, *P* < 0.05 versus gastritis alone; #, *P* < 0.05 versus mild gastritis alone.

TABLE 4. Age and sex-adjusted risk for peptic ulcer and gastric cancer in relation to *H. pylori* virulence factors in Western countries

Virulence factor	Gastric ulcer (<i>n</i> = 30)			Duodenal ulcer (<i>n</i> = 65)			Gastric cancer (<i>n</i> = 48)		
	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value
All patients vs patients with gastritis (<i>n</i> = 123)									
<i>vacA</i> s1 vs s2 region	1.62	0.52–5.00	0.40	2.15	0.92–5.01	0.08	3.17^a	1.07–9.45	0.04
<i>vacA</i> m1 vs m2 region	1.77	0.71–4.40	0.22	1.31	0.69–2.49	0.42	10.65	3.36–31.35	<0.01
<i>vacA</i> i1 vs i2 region	2.65	0.95–7.41	0.06	1.55	0.78–3.01	0.21	8.57	2.85–25.81	<0.01
<i>vacA</i> d1 vs d2 region	2.54	0.91–7.11	0.08	1.87	0.91–3.87	0.09	8.04	2.67–24.16	<0.01
<i>cagA</i> presence vs absence	3.25	0.85–12.41	0.09	3.52	1.16–10.64	0.03	3.52	1.16–10.64	0.03
All patients vs patients with OLGA stages 0 and I (<i>n</i> = 93)									
<i>vacA</i> s1 vs s2 region	8.36	2.78–25.16	<0.01	5.25	2.21–12.37	<0.01	16.55	5.28–51.83	<0.01
<i>vacA</i> m1 vs m2 region	7.25	3.30–15.68	<0.01	2.61	1.41–4.82	<0.01	16.04	7.11–36.24	<0.01
<i>vacA</i> i1 vs i2 region	12.54	4.80–31.80	<0.01	3.30	1.70–6.41	<0.01	42.73	14.27–127.99	<0.01
<i>vacA</i> d1 vs d2 region	9.99	3.88–25.74	<0.01	3.43	1.71–6.89	<0.01	33.78	11.26–101.39	<0.01
<i>cagA</i> presence vs absence	14.91	4.02–55.33	<0.01	6.20	2.54–15.16	<0.01	13.81	4.62–41.32	<0.01

^a Boldface data indicate statistically significant results.

significant association between histology and *H. pylori* factors (data not shown). For the patients from Western countries, the *vacA* s1, m1, i1, and d1 genotypes were significantly associated with increased scores for gastric mucosal atrophy and neutrophil infiltration in both the antrum and the corpus compared with the scores for patients with the *vacA* s2, m2, i2, and d2 genotype (Fig. 3).

Multiple linear regression analysis was performed to determine which factor(s) was related to the severity of the histology. The *vacA* d1 genotype was significantly associated with severe neutrophil infiltration (partial regression coefficients, 1.12 ± 0.17 in the antrum and 0.94 ± 0.17 in the corpus; $P < 0.001$ for both comparisons) and mucosal atrophy (partial regression coefficient, 0.60 ± 0.30 in the antrum and 0.63 ± 0.35 in the corpus; $P = 0.04$ for both comparisons) in Western countries (Table 5). The *vacA* m1 genotype also remained in the final model for antral and corpus mucosal atrophy, although the differences did not reach statistical significance (Table 5). Interestingly, no other variables (i.e., the *vacA* s1 or i1 genotype or *cagA* status) remained in the final model.

When patients with gastroduodenal diseases were added, the *vacA* d1 genotype was also observed to be significantly associated with severe neutrophil infiltration and mucosal atrophy, which is the same result obtained when patients with gastroduodenal diseases were not included (data not shown).

***vacA* genotypes in other Asian countries.** Because most of the strains in East Asia were of the *vacA* d1 and i1 genotypes and there was no significant association between clinical outcomes and the *vacA* d1 and i1 genotypes, we evaluated the prevalence of the *vacA* s, m, i, and d genotypes and *cagA* status among strains from other Asian countries in a preliminary analysis (Table 6). In China, Vietnam, and Thailand, all strains had the *vacA* d1 and i1 genotypes and were *cagA* positive; these were the same *vacA* genotypes and *cagA* status as strains from East Asian countries (Table 6).

DISCUSSION

The role of VacA in the pathogenesis of *H. pylori* disease remains unclear (13). VacA is expressed as a 140K prototoxin

FIG. 3. Gastric mucosal atrophy score (A and B) and inflammation activity (neutrophil infiltration) (C and D) in gastric antrum and corpus among the different *vacA* genotype groups in Western countries. Patients harboring strains with the *vacA* s1, m1, i1, and d1 genotypes have a significantly increased atrophy score and significantly increased inflammation activity in both the gastric antrum and the corpus than patients harboring strains with the *vacA* s2, m2, i2, and d2 genotypes. The bottoms and tops of the columns indicate the 25th and 75th percentiles of the data, respectively; the horizontal lines in the columns indicate the 50th percentile (median); and the bottoms and tops of the bars of the columns indicate the 10th and 90th percentiles, respectively; circles represent data outside the 10th and 90th percentiles. *, $P < 0.01$; **, $P < 0.05$.

TABLE 5. Final model of *H. pylori* factors associated with histology obtained by multiple linear regression analysis

Specimen	Pathology	Factor	Partial regression coefficient ^a	P value
Antrum	<i>H. pylori</i> density	None		
	PMN ^b infiltration	<i>vacA</i> d1 vs d2	1.12 ± 0.17	<0.01
	Atrophy	<i>vacA</i> d1 vs d2	0.60 ± 0.30	0.04
	Atrophy	<i>vacA</i> m1 vs m2	0.48 ± 0.27	0.08
Corpus	<i>H. pylori</i> density	None		
	PMN infiltration	<i>vacA</i> d1 vs d2	0.94 ± 0.17	<0.01
	Atrophy	<i>vacA</i> d1 vs d2	0.63 ± 0.35	0.04
	Atrophy	<i>vacA</i> m1 vs m2	0.52 ± 0.27	0.06

^a The partial regression coefficients are presented as the means ± standard errors.

^b PMN, polymorphonuclear leukocyte.

that undergoes amino- and carboxyl-terminal processing, yielding a mature 88K secreted product called the VacA toxin (3). Partial proteolytic digestion of the mature 88K toxin yields two fragments, one of 33K (p33) and one of p55K (p55). Amino acid sequences located within a hydrophobic region near the amino terminus of the p33 domain are required for the formation of anion-selective membrane channels (9), and the p55 domain is responsible for VacA binding to host cells (25). The expression of the p33 domain, as well as the expression of about 111 amino acids from the N terminus of the p55 domain, is required for vacuolating activity (24, 25). Both recombinant p33 and p55 domains have been shown to associate with lipid bilayers (14) and to bind to HeLa cells (23); and an interaction between them is required for binding, internalization, and vacuolating activity (23). Although Wang and Wang (26) reported that the deletion of a region of about 100 amino acids from the C-terminal portion of the p55 domain significantly decreased the binding activity of the toxin to cells, the role of the N-terminal portion of the p55 domain remained unclear. The d regions of the *vacA* gene are located in the N-terminal side of the p55 domain, and the d regions might be considered to be related to VacA binding to host gastric cells and vacuolating activity. In our preliminary experiments, we tried to investigate the biological role of the *vacA* d region by immunoblot analysis for VacA production and enzyme-linked immunosorbent assay methods for cytokine (i.e., interleukin-8) production. However, since there were few *H. pylori* strains with *vacA* d1 and d2 mosaic patterns and these strains had no effect on the *vacA* s, m, and i regions (e.g., s1-i1-d2-m1), we could not demonstrate a significant difference in the biological roles of the *vacA* d1 and d2 regions (unpublished data).

The gastritis group contained a mixture of patients, some of whom would be destined to develop severe clinical outcomes. It is well known that atrophic gastritis poses a high risk for the development of gastric ulcer and gastric cancer and that antrum-dominant gastritis is associated with the development of duodenal ulcer. In fact, in the present study, the grade of gastric atrophy among gastric cancer patients and gastric ulcer patients was significantly higher than that among patients with gastritis alone (data not shown). We therefore used patients with OLGA staging system stages 0 and I as the control group. When we used all gastritis patients as the control group, only

TABLE 6. Demographic characteristics of patients from other Asian countries

Characteristic	Result for patients from:		
	China	Vietnam	Thailand
No. of patients	48	7	31
No. of males/no. of females	36/12	5/2	19/12
Mean age (yr) ± SD	59.7 ± 1.2	36.7 ± 1.9	52.7 ± 1.7
No. (%) of patients with the following disease ^a :			
Gastritis	29 (60.4)	7 (100)	16 (51.6)
GU	0 (0)	0 (0)	5 (16.1)
DU	0 (0)	0 (0)	10 (32.3)
GC	19 (39.6)	0 (0)	0 (0)
No. (%) of patients harboring isolates with the following <i>vacA</i> genotypes:			
s region, s1	48 (100)	7 (100)	31 (100)
m region, m1	31 (64.6)	6 (85.7)	17 (54.8)
i region, i1	48 (100)	7 (100)	31 (100)
d region, d1	48 (100)	7 (100)	31 (100)
No. (%) of patients with the following s-m-i combination:			
s1-m1-i1	32 (66.7)	6 (85.7)	17 (54.8)
s1-m1-i2	0 (0.0)	0 (0.0)	0 (0.0)
s1-m2-i1	16 (33.3)	1 (14.3)	14 (45.2)
s1-m2-i2	0 (0.0)	0 (0.0)	0 (0.0)
s2-m2-i1	0 (0.0)	0 (0.0)	0 (0.0)
s2-m2-i2	0 (0.0)	0 (0.0)	0 (0.0)
No. (%) of patients with the following s-m-i-d combination:			
s1-m1-i1-d1	32 (66.7)	6 (85.7)	17 (54.8)
s1-m2-i1-d1	16 (33.3)	1 (14.3)	14 (45.2)
s1-m2-i2-d2	0 (0.0)	0 (0.0)	0 (0.0)
s2-m2-i2-d2	0 (0.0)	0 (0.0)	0 (0.0)
Others	0 (0.0)	0 (0.0)	0 (0.0)
No. (%) of patients positive/negative for <i>cagA</i>	48 (100)	7 (100)	31 (100)

^a Abbreviations: GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer.

the *vacA* m1, i1, and d1 genotypes were significantly related to gastric cancer. In contrast, these genotypes were significantly related not only to gastric cancer but also to gastric and duodenal ulcers when we used patients with mild gastritis as the control group. Most previous studies did not take the heterogeneity of gastritis into account, and this might be one reason why some previous studies, even those performed in Western countries, could not find a relationship between the *vacA* genotype and the clinical outcome. However, these relationships could not be detected in Asian countries, where most strains exclusively possessed the *vacA* s1, m1, i1, and d1 genotypes and were *cagA* positive. In Asian countries, other virulence factors (e.g., the presence of the duodenal ulcer-promoting [*dupA*] gene [12]) and/or host genetic factors (e.g., inflammation-related cytokines [20]) should have influences on gastric injury. Moreover, the evaluation of populations from Africa and Southeast Asia, where, enigmatically, the frequency of infection is high but the incidence of

gastroduodenal disease is low, might help us obtain more evidence on the influence of the *vacA* d region.

We previously reported that although the prevalence of the *vacA* s- and m-region genotypes differed significantly among strains from different countries, there were no relationships between the *vacA* genotype and the risks of disease, probably due to the small sample size (27, 29). We therefore randomly selected strains from large stocks in our university strain bank for which histology information for the patients was available to avoid sample bias. In univariate analyses, not only the *vacA* d-region genotype but also the s-, m-, and i-region genotypes were associated with the histological grade of atrophy and inflammatory cell infiltration; importantly, however, multivariate analysis revealed that only the *vacA* d1 genotype was significantly associated with atrophy and inflammatory cell infiltration. Although there was a significant association between the i-region genotype and the clinical outcome in Western countries, as reported in previous studies (8, 16), the increased risk of disease associated with the i-region genotype was the same as that associated with the *vacA* s- and m-region genotypes. In addition, in combination analysis of the *vacA* s-, m- and i-region genotypes, carriage of the *vacA* i1 genotype had no significant additive effect on the risk for the development of peptic ulcer or gastric cancer in either geographically defined group. Moreover, multivariate analysis revealed no significantly increased risk of *H. pylori*-related disease in association with carriage of a strain of the *vacA* i1 genotype and is thus inconsistent with the hypothesis of Rhead et al. (16), who suggested that the *vacA* i-region genotype might be a better predictor of gastric cancer than the s- and m-region genotype in an Iranian population. We were also unable to confirm their observation that only strains with the naturally occurring *vacA* s1-m2 genotype had variations in the *vacA* i region, that strains of the s1-i1-m2 genotype are more virulent, and that the s1-i1-m2 genotype is more likely associated with gastric cancer than the s1-i2-m2 genotype (16). In contrast, the current study showed that there was also no significant additive effect of infection with a strain of the *vacA* s1-i1-m2 subtype compared with infection with a strain of the s1-i2-m2 subtype on the risk of development of peptic ulcer and gastric cancer in both Western and Asian countries.

In conclusion, we consider the genotype of the *vacA* d region to be a histologically more sensitive marker of severe inflammation and gastric mucosal atrophy than the genotype of the *vacA* s or m region and the genotype of the *vacA* i region, as recently reported, and therefore, the d-region genotype might be one of risk factors for gastric cancer and peptic ulcer. However, the biological role of the d region is still unclear; and combination analysis of the genotype of this region and the host genetic genotype, which plays a significant role in the pathogenesis of *H. pylori*, may also be required. Therefore, future studies with isogenic strains with mutations in the d region will be necessary. This study might be limited due to the insufficient sample size. This limitation might cause a discrepancy in the differences in histology and disease risk determined on the basis of the *vacA* genotype. Future studies with a sufficient sample size will thus also be required.

ACKNOWLEDGMENTS

The material presented here is based upon work supported in part by the Office of Research and Development Medical Research Service of the U.S. Department of Veterans Affairs and by Public Health Service grant DK56338, which funds the Texas Medical Center Digestive Diseases Center. Y. Yamaoka is supported in part by NIH grant DK62813.

We thank Oscar Gutierrez (Universidad Nacional de Colombia, Bogota, Colombia) for providing clinical samples from Colombia.

We have no conflicts of interest to declare.

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