Evaluation of *Helicobacter pylori* vacA genotype in Japanese patients with gastric cancer

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Abstract

***Aim*—To examine the vacA genotypes of *Helicobacter pylori* strains in Japan and to define whether any specific genotype was associated with gastric cancer.**

***Methods*—The allelic variation of vacA gene was studied using a recently introduced polymerase chain reaction based vacA genotyping system.**

***Results*—80 *H pylori* strains were isolated from gastric biopsies of 40 patients with gastric cancer and 40 control subjects in a Japanese population. All strains were s1/m1 subtype and 79 of 80 strains were classified as s1a subtype.**

***Conclusions*—The recently proposed vacA genotyping system is applicable to Japanese *H pylori* strains and most strains have the s1a genotype, associated with increased virulence. While the high frequency of s1a/m1 vacA genotype might play a role in the increased incidence of atrophic gastritis and gastric cancer in Japanese subjects, it precludes its use as a predictor of clinical outcome of *H pylori* infection in Japan.**

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Since the discovery of *Helicobacter pylori*, many studies have implicated infection with this bacterium in the development of atrophic gastritis and gastric adenocarcinoma. However, few patients with *H pylori* infection develop gastric cancer, and there is increasing evidence that *H pylori* strains are highly diverse genomically. It is important to define whether strains with specific genotype are associated with the clinical outcome in patients with *H pylori* infection. This is particularly significant in Japan because the incidence of gastric cancer is much higher in Japanese than in western populations.[1] To date, possession of the cytotoxin associated gene A (cagA) is the most investigated non-conserved gene of *H pylori*.[2 3] Many studies have shown that infection of cagA positive strains increases the risk of both atrophic gastritis and gastric cancer in western populations.[4 5] However, the frequency of cagA is high in Japanese populations studied to date.[6 7] In Japan, therefore, cagA is not a useful marker for predicting clinical outcome, although recent serological analysis of responses to CagA suggests that seropositivity is associated with increased risk of gastric cancer.[8]

The vacuolating cytotoxin gene A (vacA) encodes the vacuolating cytotoxin which induces cytoplasmic vacuolation in eukaryotic cells.[9] All *H pylori* strains possess vacA, and diversity in this gene has been the subject of recent interest. Atherton *et al* proposed a polymerase chain reaction (PCR) based typing system to identify allelic variation in vacA.[10] There are two divergent regions, one in the second half of the signal sequence (s1a/s1b and s2) and the other in the mid-region of the gene (m1 and m2); s1a strains are associated with increased risk of peptic ulcer disease and enhanced gastric inflammation.[11] The initial PCR based typing system of vacA mid-region, however, has not been successful for categorising *H pylori* strains in Japanese populations, although a recent study using DNA sequencing showed that most strains isolated from patients with peptic ulcer and chronic gastritis were mid-region m1.[12] Recently Atherton *et al* have proposed a modified PCR based typing system which was applicable internationally and suitable for Japanese strains from patients with peptic ulcers.[13]

Our aims in this study were first to investigate whether the new PCR typing system was applicable for *H pylori* strains in Japanese patients with gastric cancer and normal subjects, and second to evaluate whether the heterogeneity in vacA was a useful marker to predict the clinical outcome in Japanese populations.

Methods

PATIENTS

Cancer patients and control subjects were selected from patients who were scheduled for upper gastrointestinal endoscopy for routine screening for gastric cancer at the Hirosaki University Hospital in northern Japan. We excluded patients who had received anti-ulcer agents or antibiotics two months before the examination or had previous histories of gastric tumours, gastric or duodenal ulcers, or gastric surgery. All patients provided informed consent before endoscopy. Cancer patients were enrolled into the study when their diagnosis was histologically confirmed. Control subjects were eligible if their endoscopic diagnosis was normal, or if atrophic gastritis was present without any evidence of ulceration, erosions, or neoplasia. The study was approved by the ethics committee of Hirosaki University.

In cancer patients and control subjects, biopsy specimens were taken for *H pylori* culture. Biopsy specimen was taken at least 2 cm away from tumours. Full histological diagnosis of the tumour type and stage was undertaken on resected stomach according to the Lauren system.

Table 1 Characteristics of the patients and the genotypes of H pylori isolates

	Cancer		
	Intestinal	*Diffuse*	*Control*
Number	29	11	40
Mean age (SD)	62.4 (8.4)	61.7 (12.6)	62.0 (10.3)
Range	43 to 79	43 to 83	43 to 83
Sex (M/F)	22/7	7/4	18/22
Signal sequence type			
s1	29	11	40
s1a	29	11	39
s1b	0	0	0
Unclassified	0	0	1
s2	0	0	0
Mid-region type			
m1	29	11	40
m2	0	0	0
cag A (+/−)	29/0	11/0	36/4

No significant difference was seen in patients' characteristics between groups.

H PYLORI CULTURE

Biopsy specimens were cultured for three to five days on Skirrow blood agar at 37°C. The bacteria were identified as *H pylori* by colony morphology, positive oxidase, catalase, and urease reactions. The clone picked strains were suspended in 1 ml phosphate buffered saline (PBS, pH 7.6) for DNA extraction.

DNA PREPARATION AND PCR ASSAY

One millilitre aliquots of bacteria in PBS were centrifuged at 10 000 *g* for five minutes. Bacterial DNA was extracted with phenol-chloroform-isoamylalcohol from bacterial pellet after digestion with 0.3 units of proteinase K at 55°C for two hours. The concentration and quality of each DNA sample was estimated by measuring A260/A280. The allelic variation of vacA signal sequence and mid-region was determined by PCR using primers previously reported.[11 13] Briefly, after five minutes denaturing at 95°C, 35 cycles of PCR including one minute denaturation at 95°C, one minute annealing at 52°C, and one minute polymerisation at 72°C were performed. The final cycle included an extension step for 10 minutes at 72°C. Each PCR amplification was performed using 0.1 μg of extracted DNA which was added to 50 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTPs, 100 pmol of each primer, and 0.5 units of Taq polymerase (TaKaRa Taq, Takara Biochemicals, Tokyo, Japan). PCR amplification was performed in duplicate for each DNA sample. PCR products were electrophoresed in 2% agarose gel; the vacA genotype was determined when the product, equivalent in size to the fragment described by Atherton *et al*,[11 13] was found.

STATISTICAL ANALYSIS

We used χ^2 analysis with Yate's correction or two tailed Fisher's exact test to compare the gender of the patients, and Student's *t* test to compare the age between groups. A probability (p) value of less than 0.05 was considered significant.

Results

H pylori strains isolated from 40 gastric cancer patients (mean age 62.1, 29 male, 11 female) and 40 control subjects (mean age 62.0, 22 male, 18 female) were tested for the vacA genotype. Histologically, 29 cancers were of the intestinal type and 11 cancers were of the diffuse type, and 35 were early gastric cancer. The vacA mid-region genotype was m1 in all *H pylori* strains from both cancer patients and control subjects. The signal sequence subtype was s1 in all strains. Classification of s1 was also performed. All strains isolated from cancer patients and 39 control subjects were of s1a subtype, and one strain from control subject was not classified into s1a or s1b. No strains showed s1b, s2, or m2 subtypes (table 1).

Discussion

This is the first study to investigate the allelic variation of *H pylori* vacA in gastric cancer patients and control subjects in a Japanese population. The results show that all strains are s1/m1 genotype and most are s1a, both in patients with gastric cancer and in control subjects; s1b, s2 and m2 subtypes, which are seen in western populations,[11] were not found.

Many studies have shown the associations between genomic diversity of *H pylori* strains and host variations in mucosal response and disease outcome.[3 11] In western populations, vacA genotype has been associated with both cytotoxin activity and enhanced gastric inflammation.[10 11] In vitro, the s1/m1 strains secrete larger amounts of toxin than s1/m2 and s2/m2 strains.[10] In vivo, m1 strains are associated with greater gastric epithelial damage than m2 strains[11]; s1a strains are also associated with greater mucosal neutrophil and lymphocyte infiltration than s1b or s2 strains.[11] These results suggest s1/m1 strains, especially s1a/m1, are more virulent than the other allelic variations. The initial vacA genotyping system proposed[10] was not applicable for categorising *H pylori* strains isolated from Japanese patients because of mutations within the primer annealing site in the mid-region.[12] The new modified PCR based vacA genotyping system, which was used successfully to genotype 13 strains from Japanese patients with peptic ulcer disease, showed all strains were s1a/m1 genotype.[13] In the present study, we show that the modified vacA genotyping system is applicable for 79 of 80 Japanese *H pylori* strains analysed and that, in a well defined population from the north east Japan, the dominant allele of vacA is s1a/m1 in both patients with gastric cancer and control subjects. Therefore it is not possible to associate any vacA genotype with gastric cancer in this population. Ito *et al* showed most strains isolated from patients with peptic ulcer disease and chronic atrophic gastritis were also of vacA s1a/m1 genotype in central Japan.[12] Thus studies to date show that most *H pylori* strains in Japan are vacA s1a/m1, even in patients with chronic gastritis.

In this study, all strains from cancer patients and 36 of 40 strains from control subjects were also cagA positive[7] as well as having the vacA s1a/m1 genotype. Our results are consistent with previous studies which showed a strong association between vacA signal sequence type s1 and cagA positivity.[11 12] The high prevalence of vacA s1a/m1 and cagA positive virulent *H*

pylori strains may be relevant to the high incidence of atrophic gastritis and gastric cancer in most Japanese populations.

In conclusion, the modified PCR based vacA genotyping system is applicable for Japanese *H pylori* strains.[13] However, in contrast to the USA, in Japan most strains are s1a/m1 and thus the vacA genotype is not useful in predicting the clinical outcome. It will be interesting to investigate vacA genotypes in other Asian and Pacific populations.

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