Restriction Fragment Length Polymorphism in *Helicobacter pylori* Associated with Gastric Cancer and Duodenal Ulcer

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More than 90% of 75 clinical isolates of *Helicobacter pylori* strains had the vacuolating cytotoxin gene *vacA* as demonstrated by polymerase chain reaction (PCR). A PCR restriction fragment-length polymorphism (RFLP) analysis revealed various gene subtypes, and some of the RFLPs correlated significantly with gastric cancer or duodenal ulcer. These findings suggest that specific types of *H. pylori* have factors that cause gastric cancer or duodenal ulcer.

Key words: *Helicobacter pylori*; RFLP; *vacA*; gastric cancer; duodenal ulcer

Helicobacter pylori infection might cause histological gastritis and contributes to peptic ulcer, gastric cancer and other gastrointestinal disorders (3). Patients infected with this bacterium can develop peptic ulcer or gastric cancer, but some people with *H. pylori* are asymptomatic. Although attempts to identify and characterize the pathogenic factors in this bacterium have been performed, no bacterial factors have yet been elucidated for these diseases (6–10). Attempts to classify *H. pylori* by their contribution to gastrointestinal disease have used genetic analysis of the bacterium, but the genetic diversity of *H. pylori* made such studies difficult except for duodenal ulcer (1, 8, 16).

Vacuolating cytotoxin (VacA) causes vacuolization in various mammalian cells, and it might cause tissue injury (7). The association of *vacA* subtypes with duodenal ulcers has been previously reported (1), but the association of *vacA* subtypes with other gastrointestinal diseases has not been examined. The purpose of this study was to clarify the association of specific *vacA* genotypes with specific gastrointestinal diseases caused by *H. pylori*.

The 75 *H. pylori* strains used in this study were obtained by biopsy from patients with gastrointestinal diseases (August 1991–September 1994). Of the 75 strains, 13 were from patients with duodenal ulcer (mean $\pm$ standard deviation, 54.3 $\pm$ 12.1 years), 22 with chronic gastritis (58.3 $\pm$ 18.5 years), 20 with gastric ulcer (49.7 $\pm$ 18.7 years) and 20 with gastric cancer (68.1 $\pm$ 15.7 years). No patient received nonsteroidal anti-inflammatory drugs or antibiotics.

The biopsy specimens were excised sequentially from

lesions or peripheral sites, followed by inoculation onto *H. pylori*-selective agar plates, which contained horse blood (10%) and Skirrow-supplement (SR069E; OXOID, Hampshire, UK). The cultured bacteria were identified as *H. pylori* by Gram staining and oxidase, catalase and urease tests.

Chromosomal DNA was isolated using a QuickPrep DNA purification kit (Pharmacia, Uppsala, Sweden). Two oligonucleotide primer sets that characterized the *vacA* gene were used for PCR: (1) first primer set, 5'-ATGGAAATACAACAACACA-3' (1 nt to 20 nt of *vacA*) and 5'-TTAGAACTATACCTCATTC-3' (3891 nt to 3872 nt of *vacA*); and (2) second primer set, 5'-TTTTACCTTTTTACACATTC-3' (–213 nt to –194 nt of *vacA*) and 5'-TGATGATAAGCTTGTGGTGTA-TGC-3' (3982 nt to 3959 nt of *vacA*) (5, 11, 12, 14). The *vacA* gene is about 4 kb (1 nt–3891 nt). *Taq* DNA polymerase (GIBCO BRL, New York, USA) was added after 2 min of preincubation at 94°C, which was followed by 35 cycles, each of which was at 94°C for 45 sec and 65°C for 4 min, with additional final incubation at 65°C for 10 min.

The amplified DNA was subjected to *Ban*I, *Xba*I or *Hpa*II digestion by adding 10 μ l of the PCR product to 10 μ l of 2 $\times$ concentrated reaction mixture, and the digested PCR product was analyzed by 2% agarose gel electrophoresis.

When the PCR for the first primer set was negative, the second primer set was used. By using the primer sets, 70 of 75 (93.3%) of the clinical isolates were determined by PCR. Before digestion with restriction endonucleases, the PCR products were visualized as a

Table 1. Profile of the PCR-RFLP of *vacA* of *H. pylori* from patients with gastric disorders

Disease	RFLP							Total
	A	B	C	D	E	Other	—*	
Duodenal ulcer	1	5	2	1	0	3	1	13
Chronic gastritis	2	2	3	2	0	12	1	22
Gastric ulcer	4	0	0	1	2	12	1	20
Gastric cancer	3	0	5	5	1	4	2	20
Total	10	7	10	9	3	31	5	75

* : Not amplified.

Fig. 1. Representative PCR-RFLP of *vacA*. A–E, PCR-RFLP; M, molecular weight marker.

single band.

Although PCR-RFLP analysis with *BanI* or *XbaI* provided no significant classification, 39 strains of the 70 PCR-amplified products were classified into 5 groups (types A–E) by *HpaII* digestion (Fig. 1, Table 1). The remaining 31 strains were not characterized by PCR-RFLP using *HpaII* digestion; therefore, they were not classified. The PCR-RFLP product of the *HpaII*-digested *vacA* sequences analyzed using the GenBank were not classified into these 5 groups (Fig. 2).

In type B, the ratio of isolates from patients with duodenal ulcer was significantly higher than that of isolates from patients with chronic gastritis ($p < 0.01$) (Fig. 3). Type B was not isolated from patients with gastric carcinoma or gastric ulcer (Fig. 3, Table 1); therefore, it was suggested that type B was associated with duodenal ulcer. On the other hand, the strains obtained from

Fig. 2. Schematic representation of PCR-RFLP. Lanes 1–5, type A–E; lane 6, GenBank accession number U056765; lane 7, U29401 (non toxic); lane 8, U0714511; lane 9, S7249414; lane 10, Z2688312. PCR-RFLPs of lanes 6 to 10 were from previously known nucleotide sequences of *vacA*. Fig. 3. Frequency distribution of the diseases that were positive for various *vacA* PCR-RFLPs. * : Not amplified.

patients with gastric cancer were predominantly of types C and D (Fig. 3, Table 1). The distribution of diseases that had the C strain was similar to that of diseases that had the D strain (Fig. 3). In addition, the RFLP of *Hpa*-

II digestion was similar for the strains (Fig. 1); therefore, the C and D strains were combined and re-classified as cancer-related. The C/D strain occurred in patients with gastric cancer at a significantly higher rate as compared to the other strains ($p < 0.01$, Table 1). Strains A and E were not characteristic (Fig. 3).

Previous studies revealed marked variation in the genome of *H. pylori* strains (4, 13), which suggests that *H. pylori* is heterogeneous. Our results suggest a correlation between *vacA* genotype and disease phenotype, which indicates that there are disease-specific strains. Some factors for the diseases may occur only in specific *H. pylori* strains.

In the case of duodenal ulcer, our results suggest that type B is the associated strain. It has been suggested that *vacA* genotypes are associated with inflammation and/or epithelial damage in the stomach (2). Weel et al. demonstrated a correlation between *H. pylori*-mediated duodenal ulcer and the cytotoxin-associated genes (*cagA*) and *vacA* (15). Atherton et al. characterized three families of the *vacA* signal sequence (s1a, s1b and s2) and two families of the *vacA* middle region allele (m1 and m2) (1, 2). Ito et al. reported that most of the clinical isolates of *H. pylori* that occur in Japan were s1a/m1, and that the middle region of *vacA* in strains that occur in Japan were markedly homologous, with more than a 96% identity, but that most divergence occurs in the middle region (9). In this study, the *vacA* RFLP of 39 clinical isolates were classified into 5 groups. Specific strains of *H. pylori* may have accumulated in patients in Japan.

Although epidemiological studies indicated that *H. pylori* causes gastric cancer, the carcinogenic factor of *H. pylori* remains unknown (3, 10). *H. pylori* infection causes most histological gastritis, and atrophic gastritis is a risk factor for adenocarcinoma of the stomach. In this study, PCR-RFLP analysis of *vacA* demonstrated that the specific types of *H. pylori*, which were different from those abundant in gastric and duodenal ulcers, were predominant in patients with gastric cancer. This result does not necessarily indicate that *vacA* of type C/D is responsible for gastric cancer since a gene really responsible for gastric cancer may exist near *vacA* and therefore link to specific types of *vacA*. However, the result suggests that strains of carcinogenic *H. pylori* are different from strains that cause duodenal or gastric ulcers. The selection of *H. pylori* strains should be considered for the characterization of the carcinogenic factors of *H. pylori*.

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