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Original Article

The Genetic Diversity of *Helicobacter pylori* Virulence Genes Is Not Associated with Gastric Atrophy Progression

Masahide Kita[a], Kenji Yokota[b]*, Hiroyuki Okada[c], Susumu Take[a], Ryuta Takenaka[a], Yoshiro Kawahara[c], Keiji Oguma[d], Osamu Matsushita[d], and Kazuhide Yamamoto[a]

Departments of [a]Gastroenterology and Hepatology, and [d]Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, [b]Graduate School of Health Sciences, Okayama University, [c]Department of Endoscopy, Okayama University Hospital, Okayama 700–8558, Japan

Atrophy of the gastric mucosa is a precursor of intestinal-type gastric cancer, and *Helicobacter pylori* infection causes atrophic gastritis. The aim of this study was to determine whether the genetic diversity of *H. pylori* virulence genes is associated with the development and progression of gastric atrophy in humans. We isolated and cultured *H. pylori* strains from patients with gastric ulcer and duodenal ulcer accompanied by atrophic gastritis in background mucosa. *H. pylori* strains were stored at −80℃ prior to the experiments being carried out. We analyzed iceA, babA, vacA, cagA, and cagE genes by PCR. The cagA gene was analyzed through sequencing of the C-terminal region containing the EPIYA motif, which is related to tyrosine phosphorylation. Severe atrophy was observed in patients with gastric ulcer. The major phenotype of the vacA gene was s1c/m1 (93%). The cagA gene was detected in all strains. The cagE gene was not detected in 2 and 5 strains from the mild cases and severe cases, respectively. The major cagA EPIYA motif, which is amino acids repeat in the C terminus, was the A-B-D type (44 of 58 strains). The virulence genes were not statistically associated with the severity of atrophy in the background gastric mucosa in humans. Not only identification of bacterial virulence factors but also studies of the host response will be necessary to investigate the progression of gastric atrophy and subsequent cancer development in humans.

Key words: *Helicobacter pylori*, virulence genes, chronic atrophic gastritis

Atrophy in the stomach is conventionally defined as loss of glandular tissue from repeated or continuing mucosal injury and is a common denominator in all pathological processes causing progressive mucosal damage, including long-standing *Helicobacter pylori* infection. Chronic atrophic gastritis (CAG) is an established precursor of intestinal-type gastric cancer, which is the second most common cause of cancer-related deaths worldwide [1, 2]. *H. pylori* infection has been identified as a major risk factor for both gastric inflammation and carcinogenesis [3]. Only a few patients develop symptomatic diseases such as ulcers with clinical symptoms, MALT lymphoma, or gastric cancer despite one-half of the population in Japan being infected with *H. pylori*. This clinical diversity is assumed to be caused by the interplay of environmental factors, host susceptibility, and diversity in the pathogenicity of different *H. pylori* strains.

Bacterial virulence genes such as vacuolating toxin (*vacA*) and cytotoxin-associated gene A (*cagA*), which

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*Corresponding author. Phone:+81-86-235-6846; Fax:+81-86-235-6846
E-mail:yokochan@md.okayama-u.ac.jp (K. Yokota)

have long been established as important virulence factors of *H. pylori*, play important roles in CAG [4–11]. The vacuolating cytotoxin (*vacA*), which is encoded by the *vacA* gene, is a virulence factor of *H. pylori* that has been associated with epithelial cell damage. This cytotoxin is present in all *H. pylori* strains and comprises 2 variable parts in the *vacA* gene: the s-region (signal) and the m-region (middle). While *vacA* s1/m1 strains produce higher levels of *vacA* activity *in vitro* than *vacA* s1/m2 strains do, *vacA* s2/m2 strains do not produce any detectable activity. CagA undergoes tyrosine phosphorylation [12]. Phosphorylation of CagA occurs within the C-terminus of the protein and is mediated by members of the Src family of tyrosine kinases [13]. The major phosphorylation motif is a cluster of Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence repeats that share homology to c-Src consensus phosphorylation sites. The number of these EPIYA motifs varies from 1–5 repeat units depending on the individual CagA protein species [13]. According to the C-terminal sequence of the EPIYA motifs of *cagA*, there are 2 types of *H. pylori* strains: the East Asian *cagA* (A-B-D) and Western *cagA* (A-B-C) types. The degree of gastric inflammation, activity of gastritis, and atrophy are significantly higher in patients infected with the East Asian *cagA*-type strains than in patients infected with the *cagA*-negative or the Western-*cagA*-type strains due to the high capability of phosphorylated CagA to bind to tyrosine phosphatase SHP-2. In addition, the adhesion molecules of IceA and BabA are also associated with some diseases in Western countries. There is greater prevalence of iceA2-positive strains reported among patients with non-ulcer dyspepsia, while *H. pylori* strains possessing the iceA1 allele are more prevalent in peptic ulcer disease. Bacterial attachment to the human gastric epithelial lining mediated by the fucosylated Lewis b (Leb) histo-blood group antigen and the Leb-binding adhesion BabA of some *H. pylori* strains have been shown to contribute to the bacterium's pathogenicity [14]. However, an association between these genes and progression of atrophy has not been reported.

We previously demonstrated that the grade of gastric atrophy is closely related to the development of gastric cancer after receiving *H. pylori* eradication therapy [15]. In this study, we isolated *H. pylori* strains from age-matched patients with severe and mild CAG. Then, we examined whether the presence of individual virulence genes was associated with the progression of gastric atrophy in these patients.

Materials and Methods

Subjects. Patients (n = 58 cases; average: 51.9 years; range, 47–63 years) on whom an upper endoscopy had been carried out were recruited for this study. They were diagnosed at Okayama University Hospital and Nippon Kokan Fukuyama Hospital as having a digestive ulcer with atrophic gastritis, and then followed up for 10 years [15]. Gastric mucosal atrophy was evaluated according to the endoscopic atrophic border scale described by Kimura and Takemoto [16, 17], which correlates with the results of histologic evaluation [18, 19]. Atrophy was classified as mild (C-1 and C-2 patterns), moderate (C-3 and O-1 patterns), or severe (O-2 and O-3 patterns).

The patients were divided into 2 groups: patients with mild atrophy (n = 35; C-1 and C2 patterns) and patients with severe atrophy (n = 23; O-2 and O3 patterns), including 4 cases with severe atrophy and gastric cancer (Table 1). The mean ages ± SD of the mild atrophy group and severe atrophy groups were 51.6 ± 4.1 and 52.5 ± 4.2 years, respectively. The mean ages were not significantly different between the 2 groups.

Ethical approval was granted by the Ethics Committee of the Okayama University and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Bacterial strains. Antral biopsy specimens were homogenized under sterile conditions in 100 μL of sterile saline using a homogenizer. The homogenate was plated onto selective Brain Heart Infusion agar (BHI) with 7% horse blood containing vancomycin (20 mg/L), bacitracin (200 mg/L), and amphotericin B (2 mg/L). Agar plates were incubated under microaerobic conditions at 37℃ for 7 days until small

Table 1 Number of *H. pylori* isolates from patients with atrophy

Disease	Mild atrophy	Severe atrophy
Gastric ulcer	9	16
Duodenal ulcer	26	3
Gastric cancer	0	4
Total	35	23

grey translucent colonies appeared. Gram stains and assays for oxidase and urease were performed. Colony morphology was consistent with the characteristic shape of *H. pylori* colonies. Single colonies were used for DNA extraction.

DNA extraction. DNA was extracted from bacterial pellets using QuickGene-810 (FUJIFILM) according to the manufacturer's instructions. Quantification of DNA from all strains was performed spectrophotometrically.

PCR and sequencing. We used the primers for *vacA*, *cagE*, *iceA*, and *babA* previously reported by Chomvarin *et al.* [20]. PCR for *cagA* and the sequence of its C-terminal region were analyzed according to the report by Higashi *et al.* [21]. DNA direct sequencing was performed using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems) according to the manufacturer's recommendations. The full-length amino acid sequences of each gene were constructed and translocated from the nucleotide sequence and aligned and analyzed with GENETYX-MAC version 11.2.3 (Software Development, Tokyo, Japan).

Statistical analysis. Fisher's exact test or the Chi-square test was used for analysis of categorical data. A p-value of < 0.05 was considered statistically significant.

Results

Severity of atrophy. Patients with gastric ulcer showed severe atrophy (64%) in the background gastric mucosa, but severe atrophy was observed in only 10% of patients with duodenal ulcer (Table 1). Gastric atrophy was significantly (Chi square test p value = 0.000011) progressed in patients with gastric ulcer.

Polymorphism of vacA. Fifty-four of the 58 strains were identified as the vacA s1/m1 type. Only 2 s1/m2 strains were detected in patients with mild atrophy, and 1 s2/m1 strain was detected. VacA s/m genes were not significantly (Chi square test p value = 0.18) associated with atrophy in the Japanese patients (Table 2).

Presence of the cagA, cagE, iceA, and babA2 genes. The numbers of strains testing positive for *cagA*, *cagE*, *iceA*, and *babA2* genes are shown in Table 3. The cagA and babA2 genes were detected in all strains. The *cagE* gene was detected in 51 (87.9%) strains. The *iceA* gene was detected in 47 (81.0%) strains. The *iceA1* gene was detected in 27 (77.1%) of 35 strains from patients with mild atrophy and in 13 (56.5%) of 23 strains from patients with severe atrophy. The percentages of strains testing positive for these genes among the patients with mild or severe atrophy are shown in Fig. 1. However, of these genes were not significantly (Chi square test p value = 0.89) associated with grade of atrophy.

Polymorphism of cagA. Almost all strains were the East Asian *cagA* type. Mutation of the B domain and repeat of the A or B domains were detected in some strains. Only 2 strains of the the Western cagA type were detected in the mild atrophy group (Table 4). EPIYA motifs were not significantly (Chi square test p value = 0.68) associated with grade of atrophy.

Table 2 Alleles of the vacA gene in *H. pylori* isolates from patients with gastric atrophy

Allele	Mild atrophy (n = 35)	Severe atrophy (n = 23)		Total (n = 58)
		Without GC	With GC (n = 4)	
vacA s1/m1	33	18	3	54
vacA s1/m2	2	0	1	3
s1a	8	4	2	14
s1b	0	0	0	0
s1c	34	16	3	53
vacA s2/m2	0	1	0	1

GC: gastric cancer

Discussion

Atrophic gastritis is considered a precursor lesion of intestinal-type gastric cancer. It is unknown whether the genetic diversity of *H. pylori* virulence genes is associated with the development and progression of atrophic gastritis and cancer in Japanese patients. A study of the European dyspeptic population with chronic gastritis indicated that the risk of presenting with atrophic gastritis was enhanced by the simultaneous presence of anti-cagA and anti-vacA antibodies [22]. Another report indicated that CagA, VacA, HcpC, and GroEL were independent predictors of atrophic gastritis and, in combination, were strongly associated with chronic atrophic gastritis [23]. These 2 reports were serological investigations, and thus what the association between individual vacA or cagA genotypes and atrophy were not well defined. This indicated that immune response might play a important roles in atrophy in Europeans. In contrast, there have been no reports regarding the association between bacterial genotype and atrophy in North-East Asians, including the Japanese. We have reported that genetic diversity in the *cagA*, *vacA*, *babA*, and *iceA* genes from different age-matched atrophy groups was not detected in this study.

Several pathogenic genes associated with diseases have been reported. However, associations between

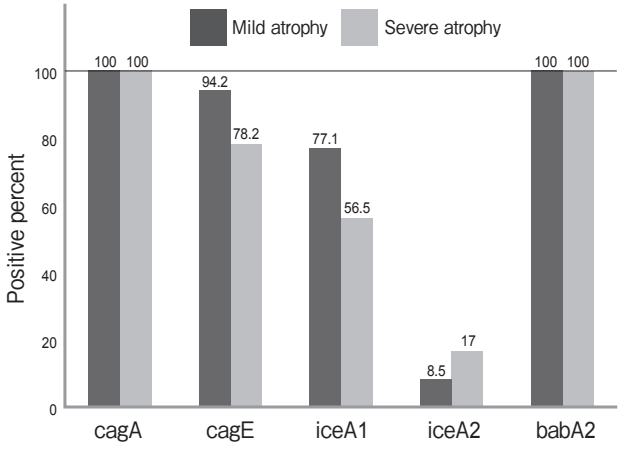

Fig. 1 Percentage of positive for *cagA*, *cagE*, *iceA*, *babA* and *babA2* The presence of *cagA*, *cagE*, *iceA*, and *babA* was detected by the PCR method. Percentage of positive samples was calculated as the number of positive samples divided by the total number of samples (mild: 35 samples; severe 23 samples).

Table 3 Presence of the cagA, cagE, iceA, babA1 and babA2 genes in *H. pylori* isolates from patients with gastric atrophy

Genes	Mild atrophy (n = 35)	Severe atrophy (n = 23)		Total (n = 58)
		Without GC	With GC (n = 4)	
cagA	35	19	4	58
cagE	33	14	4	51
iceA1	27	10	3	40
iceA2	3	4	0	7
babA2	35	19	4	58

GC: gastric cancer

Table 4 Gene structure of EPIYA motifs of *cagA* gene in *H. pylori* isolates from patients with gastric atrophy

EPIYA motifs	Mild atrophy (n = 35)	Severe atrophy (n = 23)		Total (n = 58)
		Without GC	With GC (n = 4)	
A-B-D	25	15	4	44
A-(B)-D	6	2	0	8
A-B-B-D	0	1	0	1
A-B-A-B-D	0	1	0	1
A-A-B-D-D	1	0	0	1
A-C	2	0	0	2

GC: gastric cancer

pathogenic genes and severity of diseases have not been identified in East Asian countries. The diversity of pathogenic genes is related to geographic and ethnic distribution. A number of investigators have reported geographical "enigmas" (*i.e.*, African, Asian, Indian, and Costa Rican enigmas) based on their perceptions that the outcomes they expected were not achieved in a particular population or region [24–28]. Clinical outcomes induced by *H. pylori* infection in humans may be not be associated with genetic types of virulence.

In the present study, we investigated 58 age-matched cases randomly selected from among 1342 previously reported patients [13]. Because of the small size, the generalizability of our results to broader definition of genotypes and diseases for Japanese patients is limited. On the other hand, some study recently reported that the EPIYA motif was important for gastric cancer. Ferreira *et al.* analyzed 53 strains from European patients with atrophic gastritis and carcinoma and found that the number of *H. pylori* EPIYA C motifs was important in better defining gastric carcinoma risk [28]. A study of 70 South American patients indicated that the EPIYA C motif was not associated with gastric cancer, and two SNPs in positions 1039 and 1041 of cagE showed a highly significant association with cancer [29]. In our Japanese data, the EPIYA C motif was detected in only 2 strains, and EPIYA A-B-D variation was also not associated with grade of atrophy. The results of this study may provide preliminary information not to progress atrophy to high-risk individuals, if the effects of these variants are confirmed in further investigations.

Most of infected subjects develop some degree of atrophic chronic gastritis during their lifetime, but despite the fact that CAG is a premalignant condition, very few patients develop gastric cancer. We could not determine whether virulence genes are associated with the development of gastric mucosa atrophy in this study. We are of the opinion that the development of atrophy in the human stomach may also be associated with severe inflammation and a host immune response during lifelong infection with *H. pylori*.

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References

1. Dixon MF: Pathology of gastritis and peptic ulceration. *Helicobacter pylori* Mobley HL, Mendz GL, and Hazell SL eds, ASM press washington DC (2001) 459–470.
2. Forman D and Burley VJ: Gastric cancer: global pattern of the disease and an overview of environmental risk factors. Best Pract Res Clin Gastroenterol (2006) 20: 633–649.
3. Peek RM Jr and Blaser MJ: *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer (2002) 2: 28–37.
4. Podzorski RP, Podzorski DS, Wuerth A and Tolia V: Analysis of the vacA, cagA, cagE, iceA, and babA2 genes in *Helicobacter pylori* from sixty-one pediatric patients from the Midwestern United States Diagn Microbiol Infect Dis (2003) 46: 83–88.
5. Huang JQ, Zheng GF, Sumanac K, Irvine EJ and Hunt RH: Meta-analysis of the relationship between CagA seropositivity and gastric cancer. Gastroenterology (2003) 125: 1636–1644.
6. Kuipers EJ, Perez-Perez GI, Meuwissen SG and Blaser MJ: *Helicobacter pylori* and atrophic gastritis: importance of the cagA status. J Natl Cancer Inst (1995) 87: 1777–1780.
7. Dhar SK, Soni RK, Das BK and Mukhopadhyay G: Molecular mechanism of action of major *Helicobacter pylori* virulence factors. Mol Cell Biochem (2003) 253: 207–215.
8. van Doorn LJ, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, de Boer W and Quint W: Clinical relevance of the cagA, vacA, and iceA status of *Helicobacter pylori*. Gastroenterology (1998) 115: 58–66.
9. Peek Jr RM, Thompson SA, Donahue JP, Tham KT, Atherton JC, Blaser MJ and Miller GG: Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori* gene, iceA, that is associated with clinical outcome. Proc Assoc Am Physicians (1998) 110: 531–544.
10. Yamaoka Y, Kodama T, Gutierrez O, Kim JG, Kashima K and Graham DY: Relationship between Helicobacter pylori iceA, cagA, and vacA status and clinical outcome: studies in four different countries. J Clin Microbiol (1999) 37: 2274–2279.
11. Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Covacci A, Engstrand L and Borén T: *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science (1998) 279: 373–377.
12. Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M and Meyer TF: Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell Microbiol (2000) 2: 155–164.
13. Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ and Covacci A: c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol (2002) 43: 971–980.
14. Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, Miehlke S, Classen M and Prinz C: Clinical relevance of the *Helicobacter pylori* gene for bloodgroup antigen-binding adhesin. Proc Natl Acad Sci U S A (1999) 96: 12778–12783.
15. Take S, Mizuno M, Ishiki K, Nagahara Y, Yoshida T, Yokota K, Oguma K, Okada H and Shiratori Y: The effect of eradicating *Helicobacter pylori* on the development of gastric cancer in patients with peptic ulcer disease. Am J Gastroenterol (2005) 100: 1037–1042.
16. Kimura K and Takemoto T: An endoscopic recognition of the atrophic border and prevent gastric cancer and its significance in chronic gastritis. Endoscopy (1969) 3: 87–97.
17. Sakaki N, Arakawa T, Katou H, Momma K, Egawa N, Kamisawa T,

Yamada Y, Tu Y, Ishikawa C and Ishiwata J: Relationship between progression of gastric mucosal atrophy and *Helicobacter pylori* infection: Retrospective long-term ebdscopic follow-up study. J. Gastroenterol (1997) 32: 19–23.
18. Ito S, Azuma T, Murakita H, Hirai M, Miyaji H, Ito Y, Ohtaki Y, Yamazaki Y, Kuriyama M, Keida Y and Kohli Y: Profile of *Helicobacter pylori* cytotoxin derived from two areas of Japan with different prevalence of atrophic gastritis. Gut (1996) 39: 8000–8006.
19. Sato K, Kimura K, Taniguchi Y, Yoshida Y, Kihira K, Takimoto T, Kawata H Saifuku K, Ido K, Takemoto T, Ota Y, Tada M, Karita M, Sakaki N and Hoshihara Y: Distribution of inflammation and atrophy in the stomach of *Helicobacter*-positive and -negative patients with chronic gastritis. Am J Gastroenterol (1996) 91: 963–969.
20. Chomvarin C, Namwat W, Chaicumpar K, Mairiang P, Sangchan A, Sripa B, Tor-Udom S and Vilaichone RK: Prevalence of *Helicobacter pylori* vacA, cagA, cagE, iceA and babA2 genotypes in Thai dyspeptic patients. Int J Infect Dis (2008) 12: 30–36.
21. Higashi H, Yokoyama K, Fujii Y, Ren S, Yuasa H, Saadat I, Murata-Kamiya N, Azuma T and Hatakeyama M: EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. J Biol Chem (2005) 280: 23130–2317.
22. The Eurohepygast Study Group: Risk factors for atrophic chronic gastritis in a European population: results of the Eurohepygast study. Gut (2002) 50: 779–785.
23. Lei Gao, Weck MN, Michel A, Pawlita M and Brenner H: Association between chronic atrophic gastritis and serum antibodies to 15 *Helicobacter pylori* proteins measured by multiplex serology. Cancer Res (2009) 69: 2973–2980.
24. Holcombe C, Omotara BA, Eldridge J and Jones DM: *H. pylori*, the most common bacterial infection in Africa: a random serological study. Am J Gastroenterol (1992) 87: 28–30.
25. Miwa H, Go MF and Sato N: *H. pylori* and gastric cancer: the Asian enigma. Am J Gastroenterol (2002) 97: 1106–1112.
26. Singh K and Ghoshal UC: Causal role of *Helicobacter pylori* infection in gastric cancer: an Asian enigma. World J Gastroenterol (2006) 12: 1346–1351.
27. Tsuji S: The 'Costa Rican enigma' of *Helicobacter pylori* CagA and gastric cancer. J Gastroenterol (2006) 41: 716–717.
28. Goh KL, Cheah PL, Md N, Quek KF and Parasakthi N: Ethnicity and *H. pylori* as risk factors for gastric cancer in Malaysia: a prospective case control study. Am J Gastroenterol (2007) 102: 1–5.
29. Ferreira RM, Machado JC, Leite M, Carneiro F and Figueiredo C: The number of *Helicobacter pylori* CagA EPIYA C tyrosine phosphorylation motifs influences the pattern of gastritis and the development of gastric carcinoma. Histopathology (2012) 60: 992–998.
30. Rizzato C, Torres J, Plummer M, Muñoz N, Franceschi S, Camorlinga-Ponce M, Fuentes-Pananá EM, Canzian F and Kato I: Variations in *Helicobacter pylori* cytotoxin-associated genes and their influence in progression to gastric cancer: implications for prevention. PLoS One (2012) 7: e29605.