***Helicobacter pylori*-Associated Gastritis Is Related to *babA2* Expression without Heterogeneity of the 3' Region of the *cagA* Genotype in Gastric Biopsy Specimens**

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Key Words

Antrum • BabA • *cagA* • Corpus • Gastritis • *Helicobacter pylori*

Abstract

Background: Cytotoxin-associated gene A (*cagA*) is the major virulence factor of *Helicobacter pylori* strains and affects the clinical outcome of patients. Blood group antigen binding adhesin (BabA) helps the strains adhere to the epithelial cell layer and is the most important adhesin of *H. pylori*. **Objectives:** We tried to study the association between the status of *babA2* and *cagA* in *H. pylori* strains and histological gastritis. **Methods:** Thirty-six patients were included. RNA was extracted from two frozen biopsy samples of the antrum and corpus, respectively, and *cagA/babA2* genotypes were analyzed with reverse transcription polymerase chain reaction and direct sequencing. Two gastric specimens of the antrum and corpus, respectively, were also stained with hematoxylin and eosin to analyze *H. pylori*-related gastritis. **Results:** In the antrum, 56% of the specimens were *babA2* positive and in the corpus 53%. The gastritis scores of activity and inflammation were associated with the presence of *babA2* in antrum specimens but not in corpus specimens. *cagA* gene encoding in the CagA EPIYA-D region was detected in all samples, and the sequence was completely identical

between those from the gastric corpus and antrum. **Conclusion:** *babA2* expression is heterogeneous and correlated with the extent of gastritis in the antrum, but not in the corpus, whereas *cagA* shows a monotonous genotype.

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Introduction

About 20–50% of adults in developed and 80–90% in developing countries are estimated to be infected by *Helicobacter pylori* [1]. The association between chronic *H. pylori* infection and the development of gastric carcinoma is generally accepted [2]. The International Agency for Research on Cancer has categorized *H. pylori* as a group 1 carcinogen [2]. Long-term infection with *H. py-*

Abbreviations used in this paper

<i>babA</i>	blood group antigen binding adhesin
<i>cagA</i>	cytotoxin-associated gene A
ESS	East Asian CagA-specific sequence
PG	pepsinogen
RT-PCR	reverse transcription polymerase chain reaction
WSS	Western CagA-specific sequence

lori results in glandular atrophy, and intestinal metaplasia is accepted to be an important risk factor in the development of gastric carcinoma [3–6]. As a marker of *H. pylori*, the cytotoxin-associated gene A (*cagA*) gene is the major virulence factor of *H. pylori* strains and is associated with severe disease outcome, most notably gastric carcinoma. Strains of *H. pylori* that express the CagA protein are associated with a threefold increased gastric cancer risk compared to *H. pylori* strains that do not express CagA protein [7–9]. The differential SHP-2-binding activities observed between the East Asian CagA-specific sequence (ESS) and the Western CagA-specific sequence (WSS) of CagA proteins are caused by the difference in a single amino acid at the pY+5 position, and such a difference between Western and East Asian CagA proteins may underlie the striking difference in gastric cancer incidence in these two geographic areas [10].

In Japan, most *H. pylori* express ESS, but the outcomes of patients who were infected by ESS-positive *H. pylori* differed; therefore, it is possible that the different structural subtypes of the *cagA* gene may be preferentially associated with specific *H. pylori*-related gastric diseases. The number of repetitions of the 5-amino acid sequence R1 (EPIYA) in the 3' region of the *cagA* gene was reported to vary, and the number of sequence R1 (EPIYA) may correlate with disease outcome [11, 12]. Relationships between the *cagA* genotype and variants in *H. pylori* strains and clinical disease were found in other countries [13].

The first step in the pathogenesis of *H. pylori* gastric mucosa colonization is the adhesion to the epithelial cell layer, and blood group antigen binding adhesin (BabA) is the most important adhesin of *H. pylori* [14, 15]. BabA of *H. pylori* is found to selectively adhere to the Lewis antigen of the host and thus may enhance the development of *H. pylori*-related diseases [16, 17]. The *babA* gene has two types: *babA1* and *babA2* (in which the BabA adhesin is expressed); *BabA1* is silent because of an incomplete signal peptide [16]. Recently, some studies showed that *babA2*- and *cagA*-positive *H. pylori* strain infection was associated with disease outcome, e.g. duodenal ulcer and gastric carcinoma [18, 19], but others showed that there were no correlations between the *babA2* or *cagA* status and clinical outcome [20].

In the present study, we aimed to determine whether or not there are subtypes of the *cagA* gene in the 3' region adjacent to the sequences that encode the EPIYA-D amino acids which are connected with histological gastritis. The association between the status of *babA2* and *cagA* in *H. pylori* strains and histological gastritis was also studied. Furthermore, to truly show natural *H. pylori* strain

Table 1. Clinical features of the 36 patients

Gender, males/females	20/16
Mean age (range), years	64.4 (41–90)
Gastric ulcer/duodenal ulcer/gastritis	4/5/27
<i>H. pylori</i> , positive/negative	36/0

colonization in the human stomach, RNA was directly extracted from frozen biopsy specimens in this study, in contrast to the usual methods that involved primary culture of the *H. pylori* isolates and then the extraction of the genomic DNA. Moreover, considering the difference in the physiological conditions between the antrum and the corpus, we studied the antrum and the corpus separately.

Patients and Methods

Patients

The initial study population consisted of 36 Japanese patients who underwent gastric endoscopy at the Department of Endoscopy, Hiroshima University, Hiroshima, Japan, between October 2001 and July 2002 (table 1). Patients who had a history of *H. pylori* eradication, a history of operation or had received treatment with proton pump inhibitors or antibiotics were excluded. The urea breath test (Ubit IR-200, Otsuka, Tokushima, Japan), and histological and serum antibody tests (E-plate, Eiken, Tokyo, Japan) were used to test for *H. pylori* infection; all patients had at least one positive result. Twenty male and 16 female patients (age range, 41–90 years; mean, 64.4 years) were included in the analysis of *cagA* and *babA2*. Of the 36 patients, 5 had duodenal ulcer, 4 had gastric ulcer and 27 had gastritis (15 patients had been treated with endoscopic mucosal resection for gastric tumor). Informed consent was obtained from all patients, and the protocol was approved by the hospital ethics committee.

Histological Evaluation

Gastric biopsy specimens (four samples, two from the greater curvature of the antrum and two from the greater curvature of the corpus) were obtained during gastric endoscopy. Two biopsy samples (one from the antrum and one from the corpus) were fixed with buffer formalin and embedded in a paraffin block. Sections were stained with hematoxylin and eosin to analyze *H. pylori*-related histology. The grade of gastritis was scored as follows: unaware of the endoscope results, (1) atrophy (0, absent; 1–3, from mild to marked); (2) activity, i.e. polymorphonuclear cell infiltrates (0, absent; 1–3, from mild to marked); (3) inflammation, i.e. mononuclear cell infiltrates (0, absent; 1–3, from mild to marked); (4) intestinal metaplasia (0, absent; 1–3, from mild to marked), and (5) *H. pylori* density (0, absent; 1–3, from mild to marked). All scores were based on the updated Sydney System [21].

Fig. 1. The sequence of PCR primers used and the DNA/amino acid sequence in the EPIYA-D region of the F32 strain.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Two biopsy specimens, each from the antrum and the corpus, were frozen by liquid nitrogen and stored at -80°C until used to extract RNA. RNA was extracted from the frozen biopsy specimen with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a first-strand cDNA kit (Amersham Biosciences UK Limited, Little Chalfont, UK). The reaction components were the same as those recommended by the manufacturer, and RT-PCR was performed using a thermal cycler (PTC-100TM Programmable Thermal Controller) under the following conditions: (1) 24°C for 10 min, (2) 37°C for 1 h, (3) 99°C for 5 min and then stored at -20°C until used. The resulting cDNA products were subjected to PCR for amplification of the *cagA*/*babA2* genes.

PCR and Primers for *babA2* Genotypes

Two microliters of DNA were added to 50- μl reaction mixtures containing: 5.0 μl of $10\times$ PCR buffer II, 0.25 μl of Taq DNA polymerase (5 U/ μl , Ampli Taq GoldTM; Applied Biosystems, Foster City, Calif., USA), 3.75 μl MgCl_2 (MgCl_2 solution 25 mM, manufactured by Rode/Applied Biosystems), 5.0 μl dNTP (2 mM each, Warrington, UK), 0.32 μmol of each primer and distilled water (sense primer, *bab7F*: CCA AAC GAA ACA AAA AGC GT, corresponding to bp 105–124 of AF033654, and the antisense primer, *bab7R*: GCT TGT GTA AAA GCC GTC GT, corresponding to bp 356–375 of AF033654). The primer was designed by Sheu et al. [22]. After denaturation of the samples (PCR at 95°C for 10 min), PCR was carried in the same thermal cycle as amplification of the *cagA* gene. Thirty-five amplification cycles, each comprising 95°C for 1 min, 58°C for 1 min and 72°C for 1 min followed by a final step at 72°C for 10 min, were performed.

The 271-bp PCR products were separated by electrophoresis on 1.5% agarose gel.

PCR and *cagA* Sequence Analysis

PCR primers were designed to analyze the DNA sequence in the vicinity of the EPIYA-D sequence (fig. 1). The sense (*cagA*2F) and antisense (*cagA*2R) primer were: 2F: CTC AAG TTG CTA

AAA AGG T, corresponding to bp 122–140 of THP432 (DU6) [23]; 2R: CAA TTC TTG TTC CCT TGA AAG C, corresponding to bp 366–387 of THP432 (DU6) [23]. One microliter of cDNA from the sample was used for *cagA* gene amplification in 24.5 μl of reaction mixture containing: 2.5 μl of $10\times$ PCR buffer II, 0.25 μl of Taq DNA polymerase (5 U/ μl , Ampli Taq Gold, Applied Biosystems), 1.5 μl MgCl_2 (MgCl_2 solution 25 mM, Rode/Applied Biosystems), 2.0 μl dNTP (2 mM each, Warrington), 0.25 μmol each of primer and distilled water. PCR were performed in the same thermal cycle as RT-PCR. PCR conditions were as follows: heating at 95°C for 10 min, followed by 50 cycles consisting of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min. The tubes were kept at 72°C for 10 min before storage at 4°C . The human GAPDH gene was amplified for PCR as a control. Primer F, ATC ATC CCT GCC TCT ACT GG (corresponding to bp 691–710 of human GAPDH), primer R, CCC TCC GAC GCC TGC TTC AC (corresponding to bp 859–878 of human GAPDH) [24], and the PCR components and conditions were exactly the same as for the amplification of the *cagA* gene.

A 266-bp PCR product (using primer *cagA*2F and *cagA*2R) and a 188-bp PCR product (using primer F and R) were obtained and separated by electrophoresis on 1.5% agarose gel. The 266-bp products were purified before use as the template DNA using the DNA and Gel Band Purification Kit (Amersham Biosciences) according to the manufacturer's instructions. The purified DNA fragments were subjected to sequence reaction using the BigDye Terminators v1.1 cycle sequencing kit (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instruction.

Serum Markers

Fasting blood samples used in the assay of serum pepsinogen (PG) and gastrin were obtained from all patients before endoscopy. Serum PG and gastrin levels were evaluated by a modified radioimmunoassay method [25] using Riabead Kits (Dainabot, Tokyo, Japan) and radioimmunoassay kit II (Dainabot) [26].

Statistics

The Mann-Whitney U test was used to assess differences between histological scores and the *babA2* genotype and differenc-

Fig. 2. **a** PCR analysis of the *babA2* gene of *H. pylori* extracted from frozen sample of a 75-year-old male patient (case 21). Lane C = 271-bp PCR product from the corpus; lane A = PCR product from the antrum; lane M = 200-bp marker; lane N = negative control; lane P = positive control. **b** PCR analysis of the *cagA* gene of *H. pylori* extracted from frozen sample of a 66-year-old female patient (case 19). Lane C = 266-bp PCR product from the corpus; lane A = 266-bp PCR product from the antrum; lane M = 200-bp marker; lane N = negative control; lane P = positive control. **c** 181-bp GAPDH from 2 patients after PCR. Lane M = 200-bp marker; A = antrum; C = corpus.

es between the *babA2* gene status and the serum PG levels. Fisher's exact test was employed to analyze categorical data. Data are presented as means $\pm$ SE, and $p < 0.05$ was considered to be statistically significant.

Results

babA2 Expression in the Antrum and Corpus

We extracted RNA directly from frozen biopsies, one each from the antrum and the corpus. Figure 2a shows 1.5% agarose gel electrophoresis of the *babA2* amplification products. Using Bab7F/Bab7R, the 271-bp product was obtained. The isolates were sequenced and were found to correspond with $>95\%$ homology to the *babA2* gene sequence (GeneBank accession No. AF001388). The *babA2*-positive rate was 56% in the antrum and 53% in the corpus. *babA2* expression differed between samples from the gastric corpus and the antrum (table 2).

Table 2. Comparison of *cagA/babA2* heterogeneity between the gastric antrum and the corpus

DNA status	<i>cagA</i> genotype (n = 31)		<i>babA2</i> expression (n = 36)	
	n	%	n	%
Corpus = antrum	31	100	27	75
Corpus $\neq$ antrum	0	0	9	25

Table 3. *babA2* genotype of *H. pylori* and gastritis score in the antrum and corpus

	<i>babA2</i> positive	<i>babA2</i> negative	p value
<i>Antrum</i>			
Atrophy	1.90 ± 0.19	1.75 ± 0.21	0.60
Activity	1.10 ± 0.15	0.50 ± 0.17	0.01
Metaplasia	0.40 ± 0.17	0.56 ± 0.20	0.54
Inflammation	2.10 ± 0.15	1.56 ± 0.17	0.02
<i>H. pylori</i> density	0.55 ± 0.60	0.68 ± 0.60	0.48
<i>Corpus</i>			
Atrophy	1.16 ± 0.22	1.11 ± 0.24	0.90
Activity	0.89 ± 0.12	0.59 ± 0.13	0.10
Metaplasia	0.26 ± 0.20	0.59 ± 0.21	0.27
Inflammation	1.73 ± 0.16	1.70 ± 0.17	0.90
<i>H. pylori</i> density	0.84 ± 0.76	0.64 ± 0.71	0.45

Means (SE) for 20 and 16 samples in the antrum and 19 and 17 samples in the corpus for the *babA2* genotype and the gastritis score, respectively.

Relationship between the babA Genotype and Histopathological Scores in the Antrum and Corpus

We examined the clinical features of the patients with or without *babA2* gene expression in the antrum and corpus. There were no significant differences regarding sex, age, disease or history of gastric cancer between the *babA2* gene-positive and -negative *H. pylori*-infected patients. Table 3 shows the *babA2* genotype of *H. pylori* and pathological histological features in the antrum and corpus. In the antrum, activity (polymorphonuclear cell infiltrates) and inflammation (mononuclear cell infiltrates) were significantly associated with the presence of the *babA2* gene (Mann-Whitney U test, $p < 0.05$), but there was no significant association in terms of atrophic change, intestinal metaplasia or *H. pylori* density. In the corpus, the grade of activity was higher in patients with *babA2*-positive *H. pylori* than in those without *babA2* expression, although the difference was not significant.

Fig. 3. Results of direct sequencing of *cagA* genes (**a**) and amino acid sequence of CagA (**a, b**). A standard type (**a**) and a minor mutated type (**b**; four different patterns, the underlined letters show differences in a single amino acid of CagA).

Relationship between the *babA* Genotype and Serum Markers of Gastritis

Next we tried to compare the serum levels of gastrin and PG between *babA2*-positive cases ($n = 22$; positive in the corpus or antrum) and *babA2*-negative cases ($n = 10$). The level of PG-II, a marker of gastric inflammation, was statistically higher in cases with *babA2*-positive than those without its expression (table 4).

cagA Gene Sequence in the Antrum and Corpus

Next, we examined the expression of the *cagA* gene. A 266-bp PCR product of the *cagA* gene was obtained by primer *cagA2F* and primer *cagA2R*. Amplification was shown using 1.5% agarose gel electrophoresis (fig. 2b). In all 36 cases examined, the *cagA* gene was expressed both in the antrum and the corpus.

cagA Gene: Subtype Sequences and Clinical Characteristics

Among 36 *H. pylori cagA* gene-positive patients, 31 sequences were noted: 30 patients were of the Asian type, and 1 patient (with duodenal ulcer) was of the western type. The *cagA* gene sequences that were extracted from gastric antrum biopsies were exactly the same as those extracted from the corpus (table 2). In 22 cases, the sequence was identical to the *H. pylori* standard strain F32 from the CagA residue containing EPIYA-D [10]. Another 8 had minor mutations upstream of EPIYA-D (fig. 3b).

Table 4. Comparison of levels of serum PG and gastrin between positive and negative cases of *babA2* heterogeneity (means $\pm$ SE)

DNA status	<i>babA2</i> positive ($n = 22$)	<i>babA2</i> negative ($n = 10$)	p value
Gastrin, pg/ml	190.9 $\pm$ 33.3	167.7 $\pm$ 41.9	0.67
PG-I, ng/ml	63.8 $\pm$ 6.6	48.0 $\pm$ 8.3	0.15
PG-II, ng/ml	27.9 $\pm$ 2.6	18.3 $\pm$ 3.3	0.03
PG-I/PG-II	2.39 $\pm$ 0.27	2.75 $\pm$ 0.34	0.41

There were no significant differences in sex, age, disease or the grade of histologic gastritis between cases with standard and mutated CagA.

Discussion

In our study, RNA and cDNA were extracted directly from frozen biopsy specimens, unlike the usual method where the *H. pylori* isolates are firstly cultured and then the genomic DNA is extracted. As expected, *H. pylori* must have remarkable capacity to adapt to changes in the local gastric environment because infections tend to persist for life once established. Some studies have also shown that most *H. pylori* can evolve from a benign to a more virulent lifestyle whenever this would be advantageous to

the bacterium [27]. The *babA2* expression pattern was different between the antrum and corpus, being higher in the antrum than in the corpus, in agreement with a report suggesting that the *bab* adhesin gene was metastable [27]. In addition, our results also showed that the *cagA* gene was positive both in the antrum and corpus, and the sequences of the *cagA* gene that were extracted from gastric antrum biopsies were completely identical with those extracted from the corpus, in harmony with our previously reported results [28]. We proposed that the main virulence genes of *H. pylori*, e.g. *cagA*, were not easily changed; other genes, e.g. the *babA* gene, was able to adapt to alterations in the host environment. Therefore, it is possible that *H. pylori* strains may undergo some changes depending on the culture conditions, and it should be controversial whether the biological characters of isolated colonies reflect the original character of the bacteria in vivo.

Recently, we have succeeded in examining the *cagA* status using paraffin-embedded tissue, but the size of the PCR product was limited to 100 bp in length and three times repeated PCR was needed for most cases [28]. However, the results were affected by the sample conditions, and paraffin-embedded biopsies may be obscured by DNA damage during fixation and embedding procedures, and some substances inhibited the extraction of RNA or the amplification of cDNA [29]. Frozen samples were used to examine the gene status since we assumed it could truly express the natural *H. pylori* colonization in the human stomach. Moreover, considering the different conditions in the different areas of the stomach and the topography of gastric inflammation [6], the status of the gastric corpus and antrum were studied separately in each patient.

The phosphorylated CagA protein specifically binds SHP-2 phosphatase, activates phosphatase activity and thereby induces the morphological transformation of the cell [30, 31]. The differential SHP-2-binding activities observed between WSS and ESS of CagA proteins are caused by the difference in a single amino acid at the pY+5 position [10]. In Japan, associations between the diversity of the 3' region of the *cagA* gene and clinical outcome were noted, e.g. the variety in the number of repetitions of the 5-amino acid sequence R1 (EPIYA) in the 3' region of the *cagA* gene was correlated with disease outcome [11, 12]. Our study suggested that there were some subtypes of the *cagA* gene in the 3' region near the sequence encoding the CagA protein EPIYA-D region, and the result showed that the sequence in the nearby 3' region encoding the CagA protein EPIYA-D region in Japan was not exactly

the same. However, there were no significant differences between the original (identical with F32 strain) and the mutated type with regard to changes in histological gastritis, e.g. atrophy, activity, inflammation and intestinal metaplasia both in the antrum and corpus. The results showed that there may be some variations in this region in Japanese patients; however, it could not result in CagA proteins with different immunogenicities, which then induce severe gastritis. The status of gastritis was not affected by the status of the CagA protein in Japanese patients, compatible with our previous report [28].

We demonstrated that the presence of another virulence gene, *babA2*, was associated with a higher grade of activity and inflammation in the antrum. In contrast, in the corpus, there was no statistically significant association between the presence of *babA2* and gastritis, and *p* values were relatively low for activity. In cases with *babA2* expression, an increased serum PG-II level suggests the presence of active gastritis in the stomach, in agreement with our histological data suggesting that although the *cagA* gene was the main virulence factor of *H. pylori*, the *H. pylori*-induced gastric damage did not depend on a single factor. Bab adhesin facilitates bacteria to adhere to the gastric epithelium and then to induce persistent inflammation. This is consistent with a recent report by Zambon et al. [19] showing that *cagA*-, *babA2*-positive *H. pylori* infection was related to a higher degree of activity and inflammation. They suggested that the *H. pylori*-induced gastric inflammation reaction does not depend on a single factor.

It is not clear why the significant association between the *babA2* expression and the grade of activity and inflammation was restricted to the antrum. However, to our knowledge, host factors also play an important role in the pathogenesis of the disease. In a study by Sheu et al. [22] on the host status for Lewis antigen expression in the stomach, the frequency of Lewis antigen expression was different between the gastric corpus and antrum. The intensity of Le^a or Le^x expression was higher in the antrum than in the corpus and the intensity of Le^b was higher in the corpus than in the antrum. They also showed that differences in Le antigens were correlated with special histological features. Lee et al. [32] found *babA2* gene to be significantly correlated with active and chronic inflammation, which is consistent with our result, and they also demonstrated that the host expression status of Le was significantly associated with clinicopathological features. Although we have not examined Le expression, not only the kind of the Lewis antigen but also the distribution of gastric Lewis antigen expression in the

human stomach may play a role in bacterial virulence and bacterial adherence [22, 32]. Moreover, other conditions, e.g. different acidic intensity in different areas of the stomach, maybe also be involved in the pathogenesis [33], possibly explaining the different results between the antrum and the corpus.

On the other hand, it is uncertain why *babA2* expression was not associated with the grade of atrophy or intestinal metaplasia. Zamboni et al. [19] disclosed that intestinal metaplasia was significantly associated with all the *H. pylori* virulence genes studied, suggesting that each had a potential pathogenic role in the onset of this precancerous lesion. However, we could not detect the relationship between the *babA2* pattern and the status of intestinal metaplasia in our specimens. *cdx-2* gene expression, which is a specific marker of intestinal differentiation of the gastric mucosa according to Hinoi et al. [34], was examined in the same samples, and no relation between *babA2* and *cdx-2* expressions was confirmed (data not shown). In atrophic/metaplastic conditions, the amount of bacteria or the amount of bacteria that adhered to the gastric epithelium was changed, partly explaining this discrepancy. However, the simple *H. pylori* density score did not correlate with the *babA2* phenotype, indicating the clearance of *H. pylori* in the metaplastic epithelium was not the main reason of this discrepancy.

We hypothesized that in atrophic gastritis, intragastric conditions, including low acid secretion, may contribute to the alterations in the *babA2* phenotype. In addition, the positive rate of *babA2* expression was lower than that in another report [20], and our patient cohort was relatively small, which may be considered a limitation of our study.

In conclusion, our study showed that (1) the *cagA* gene was uniformly expressed in all cases, and *cagA* gene variations adjacent to the EPIYA-D region were not associated with the grade of gastritis, and (2) *babA2*-positive expression was heterogeneous in the stomach and may play an important role in the induction of *H. pylori*-associated gastric inflammation.

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