Published in final edited form as:
J Gastroenterol Hepatol. 2013 February ; 28(2): 291–296. doi:10.1111/jgh.12067.

Helicobacter pylori cagA 12-bp insertion can be a marker for duodenal ulcer in Okinawa, Japan

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Abstract

Backgrounds—*Helicobacter pylori cagA* can be classified into mainly two types (East-Asian-type and Western-type *cagA*) according to the repeat regions located in the 3′ region. Recent studies showed that the Western-type *cagA* in strains from Okinawa, Japan formed a different cluster (J-Western-type *cagA* subtype). We also reported that J-Western-type *cagA* possess a 12-bp insertion located in the 5′ region of *cagA* sequence.

Methods—The prevalence of 12-bp insertion in *cagA* in Okinawa and the United States (U.S.) was examined by DNA sequencing. We then designed the primer pair which can detect the 12-bp insertion only by polymerase chain reaction (PCR). The prevalence of strains with 12-bp insertion was examined in 336 strains isolated from Okinawa by PCR.

Results—In case of Western-type *cagA*/*vacA* s1m2 strains, the prevalence of 12-bp insertion was significantly higher in strains isolated from Okinawa than that from the U.S. ($P = 0.002$). Phylogenetic tree showed that strains with 12-bp insertion formed two individual clusters within J-Western-type *cagA* subtype; one is from Okinawa and another is from the U.S. Our designed primer set showed high sensitivity (100%) and specificity (90.8%) in Okinawa. The 12-bp insertion was found in 23.7%, 14.3%, 4.2%, and 4.0% of strains with duodenal ulcer (DU), gastritis, gastric cancer (GC), and gastric ulcer (GU), respectively ($P < 0.001$ for DU vs. GU) in Okinawa.

Conclusions—Although the mechanisms are unknown, the presence of 12-bp insertion was associated with the presence of DU and might have a suppressive action on GU and GC.

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Potential competing interests: The authors declare that they have no competing interests.

Keywords

Helicobacter pylori; 12-bp insertion; Okinawa; *cagA*

Introduction

Helicobacter pylori infection is now accepted as the major cause of chronic gastritis. Several epidemiological studies have shown that *H. pylori* infection is also linked to severe gastritis associated diseases, including peptic ulcer and gastric cancer (GC) [1]. In 1994, the International Agency for Research on Cancer categorized *H. pylori* as a group I carcinogen [2]. Although GC is one of the most common cancers, only a minority of individuals with *H. pylori* infection ever develop it. The prevalence of GC is approximately 3% in *H. pylori*-positive patients [3]. In addition to environmental factors (eg, diet) and host factors, virulence factors of *H. pylori*, such as *cagA* and *vacA*, have been demonstrated to be predictors of gastric atrophy, intestinal metaplasia, and severe clinical outcomes [4]. The most studied virulence factor of *H. pylori* is *cagA,* which is located at the end of an approximately 40-kb cluster of genes called *cag* pathogenicity island (PAI). *cag* PAI encodes a type-IV secretion system and transfers CagA protein into host cells [5]. CagA protein is believed to have oncogenic potential [6, 7], and *cagA*-positive strains are reported to be associated with severe clinical outcomes [4]. *cagA* can be classified into mainly two types (East-Asian-type *cagA* and Western-type *cagA*) according to the sequence located in the 3′ region of *cagA*[8-10].

Okinawa consists of small islands (2,276 km^2) in southwestern Japan. Though the prevalence of *H. pylori* in Okinawa is not significantly different from other parts of Japan [11,12], the incidence of GC (6.3 deaths/100,000 population) in Okinawa is the lowest in Japan (mean mortality rate of Japan; 11.8 deaths/100 000 population in 2009) (Center for Cancer Control and Information Services, National Cancer Center, Japan, [http://www.ncc.go.jp/]). Okinawa was under the rule of the United States (U.S.) after World War II (WWII) until 1972, and there are still many U.S. populations (the number of U.S. residents in Okinawa, military personnel, civilian employees, and their families, are estimated as 48,490 in 2009) (http://www.pref.okinawa.jp/annai/index.html). The different environmental factors and diets in Okinawa compared with mainland Japan are thought to be one reason for the lower incidence of GC [13]. Furthermore, we recently reported that different incidence of GC between Okinawa and mainland Japan might be explained by the high prevalence of Western-type *cagA* strains in Okinawa compared with other areas in Japan [14]. Intriguingly, recent studies using *cagA* full-sequenced data showed that the Western-type *cagA* detected in strains from Okinawa formed a different cluster compared to the original Western-type *cagA* and it was named the J-Western-type *cagA* subtype [15]. We also examined the *cagA* sequence data deposited in the GenBank and found that J-Western-type *cagA* strains possess a 12-bp insertion located in the 5′ region of *cagA* sequence compared to the original Western-type *cagA* strains [16]. However, the significance of 12-bp insertion, especially for clinical outcomes remains unclear. In this study, we designed the primer pair to be able to detect the 12-bp insertion by polymerase chain reaction (PCR) without DNA sequencing. In addition, we examined the prevalence of 12-bp insertion in Okinawa and the association between 12-bp insertion and clinical outcomes.

Methods

Patients and *H. pylori*

H. pylori strains were obtained from the gastric mucosa of *H. pylori*-infected patients who underwent endoscopy at University of the Ryukyus (Okinawa, Japan) between February

1993 and March 2005 and Michael E. DeBakey Veterans Affairs Medical Center (Houston, TX) between January 2001 and June 2006. Samples from Okinawa are same as our previous study [14]. Presentations included gastritis, duodenal ulcer (DU), gastric ulcer (GU), and GC. Gastric biopsy specimens were taken from the antrum. DU, GU, and GC were identified by endoscopy, and gastric cancer was further confirmed by histopathology [17]. Gastritis was defined as *H. pylori* gastritis in the absence of peptic ulcer or gastric malignancy. Patients with a history of partial gastric resection were excluded. Written informed consent was obtained from each participant, and the protocol was approved by the Ethics Committee of University of the Ryukyus (Japan) and Michael E. DeBakey Veterans Affairs Medical Center (U.S.).

Isolation and genotyping of *H. pylori*

Gastric biopsy specimens were taken from the antrum (pyloric gland area) and the corpus (fundic gland area). Antral biopsy specimens were used for the isolation of *H. pylori* using standard culture methods as previously described [18]. *H. pylori* DNA was extracted from confluent plate cultures using a commercially available kit (QIAGEN Inc., Santa Clarita, CA). The status of *cagA* based on the 3′ region (East-Asian-type or Western-type) and *vacA* (genotypes of s and m region; s1 or s2 and m1 or m2) was determined as described in our previous study [14]. To detect the 12-bp insertion, the new forward primer was designed on the basis of the sequence data we obtained. Forward primer was located the sequences including 12-bp insertion and was CAGYMF (+) (5′-AAT GGA GAG CCTACT GGA GAG CC-3′). The cagA M1(-) primer was used as reverse primer [19]. The PCR conditions were initial denaturation for 5 min at 95°C, 35 amplification steps (95°C for 30 s, 52°C for 30 s, and 72°C for 30 s), and a final extension cycle of 7 min at 72°C, using Blend Taq® DNA polymerase (TOYOBO, Japan). The expected lengths of PCR products amplified with the primers CAGYMF and cagA M1(-) was approximately 400 bp. The amplified fragment was detected by a 1.5% agarose gel electrophoresis using an ultraviolet transilluminator.

Nucleotide sequencing

The presence of 12-bp insertion was examined by sequencing using primer set cagA L2 (+) and cagA M1(-) as described previously [19]. PCR products (approximately 1,000 bp) were purified with QIAquick Purification Kit (QIAGEN) according to the manufacturer's instructions and DNA direct sequencing was performed using AB 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Phylogenetic analysis

For construction of phylogenetic tree based on the sequence of *cagA* including 12-bp insertion, *cagA* sequences of 14 strains with typical *cagA* genotypes based on the 3′ region (6 Western-type *cagA* strains [26695, NCTC11637, NCTC11638, F79, F80, and OK111], 2 J-Western-type *cagA* [J99 and OK180], 3 East-Asian-type *cagA* strains [F16, F37 and OK101], and 3 Amerindal-type *cagA* strains [Shi257, Shi329 and Shi417]) were obtained from Genbank that were deposited by other group. Among the strains, OK101, OK111 and OK180 were isolated from Okinawa. These sequences were compared with our data obtained from strains in Okinawa. Neighbor joining tree was constructed by MEGA 5.0 with 3,000 bootstrappings and using Kimura-2 parameters [20, 21].

Statistical analysis

The associations between the presence of 12-bp insertion and clinical outcome were analyzed with the chi-square test and Fisher's exact probability test. A multivariate logistic regression model was used to calculate the odds ratios (OR) of the clinical outcomes to adjust by age, sex, *cagA* type (Western-type or East-Asian-type), *vacA* m status (m1 or m2)

and the presence of 12-bp insertion. All determinants with P values of < 0.10 were entered together in the full model of logistic regression, and the model was reduced by excluding variables with P values of > 0.10. OR and 95% confidence interval (CI) were used to estimate the risk. A P value less than 0.05 was accepted as statistically significant. The SPSS statistical software package version 19.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

Results

The prevalence of 12-bp insertion in Okinawa and the U.S

We first examined the prevalence of 12-bp insertion in *cagA* in Okinawa and the U.S. We previously found that 12-bp insertion especially exist in J-Western-type *cagA* strains [16]. In our previous study, we did not distinguish J-Western-type *cagA* strains from typical Western-type *cagA* strains, and both types were classified as Western-type strains [14] . Therefore, we randomly selected 41 Western-type *cagA* strains isolated from Okinawa from the previous study [14] based on the 3′ region of *cagA* (including both J-Western-type and typical Western-type *cagA*). We also randomly selected 29 Western-type *cagA* strains isolated from the U.S. from our previous study [22] As control, we selected 28 East-Asian-type *cagA* strains based on the 3′ region of *cagA* (24 from Okinawa and 4 from the U.S.; these were isolated from Vietnamese who live in the U.S.) [14, 22]. Total of 98 samples (65 from Okinawa and 33 from the U.S.) were amplified by PCR and sequenced. Among 65 strains from Okinawa, 33 (50.7%) possessed 12-bp insertion (Table 1). On the other hand, the prevalence of 12-bp insertion was 18.1% (6/33) in strains from the U.S. The prevalence in strains from Okinawa was 83.9% (26/31) in Western-type *cagA*/*vacA* s1m2 strains, 50% (5/10) in Western-type *cagA*/*vacA* s1m1 strains, and 13.3% (2/15) in East-Asian-type *cagA*/*vacA* s1m2 strains. None of East-Asian-type *cagA*/*vacA* s1m1 strains possessed 12-bp insertion. In the U.S., the 12-bp insertion was only found in Western-type *cagA*/*vacA* s1m2 strains. Even in case of Western-type *cagA*/*vacA* s1m2 strains, the prevalence of 12-bp insertion was significantly higher in strains from Okinawa than that from the U.S. (83.9% vs. 37.5%, P = 0.002). This 12-bp insertion code the four amino acid PTGE or STGE in strains from Okinawa, whereas PNGE, PNGD and PIRE in strains from the U.S.

We constructed the phylogenetic tree using *cagA* sequence (approximately 1,000 bp) including 12-bp insertion (Figure 1). Phylogenetic tree showed that all strains with 12-bp insertion were in the J-Western-type *cagA* cluster, which was distinct from that of East-Asian-type *cagA* and typical Western-type *cagA* from Western countries. Interestingly, strains from Okinawa with 12-bp insertion formed one cluster different from U.S. strains with 12-bp insertion (Figure 1). In case of Okinawa, 2 East-Asian-type *cagA* strains with 12-bp insertion located in the same cluster with other strains with 12-bp insertion. This suggests that 2 strains have J-Western-type *cagA* structure in the 5′ region of *cagA*, whereas East-Asian-type *cagA* structure in the 3′ region of *cagA*. Three U.S. strains without 12-bp insertion also belonged to the same cluster with the U.S. strains with 12-bp insertion. Four East-Asian-type *cagA* strains from Vietnamese who live in the U.S. did not have 12-bp insertion, and were clustered with other East- Asian-type *cagA* strains.

Construction of primer set to detect the 12-bp insertion

To be able to detect the 12-bp insertion only by PCR, we designed new primer for specific 12-bp insertion on the basis of the sequence data we obtained. In Okinawa, all 34 samples with 12-bp insertion were positive by PCR. On the other hand, 3 out of 33 samples without 12-bp insertion showed the positive band in PCR, which means false-positive because these 3 strains did not have the similar sequence with forward primer. Altogether, sensitivity, specificity and accuracy were 100%, 90.8% and 95.3%, respectively in Okinawa. In the

U.S., all samples with 12-bp insertion were positive by PCR. On the other hand, none of samples without 12-bp insertion showed the positive band in PCR. Therefore, sensitivity, specificity and accuracy were all 100% in the U.S.

Association between the presence of 12-bp insertion and clinical outcomes

The prevalence of strains with 12-bp insertion was examined by PCR using our designed primer set. Total of 336 strains (98 from patients with gastritis, 101 with GU, 114 with DU and 24 with gastric cancer) in Okinawa were included in the final analysis (Table 2). The 12-bp insertion was the most found in 83.8% (31/37) in strains with Western-type *cagA*/*vacA* s1m2 genotypes, following 57.1 % (8/14) in those with Western-type *cagA*/*vacA* s1m1 genotypes. It was found in 2.3% (5/222) and 10.0% (2/20) of strains with East-Asian-type *cagA*/*vacA* s1m1 and *vacA* East-Asian-type *cagA*/s1m2, respectively. The prevalence of 12-bp insertion was significantly higher in strains with Western-type *cagA*/*vacA* s1m2 than other genotypes ($P < 0.001$).

Next, we examined the association between the presence of 12-bp insertion and clinical outcomes in Okinawa. The prevalence of *cagA* was significantly higher in strains from GU (89.0%), DU (90.4%) and GC (95.8%) than those from gastritis (77.6%) ($P = 0.03$, 0.01 and 0.04, respectively) (Table 3). The 12-bp insertion was found in 14.3%, 4.0%, 23.7%, and 4.2% of strains with gastritis, GU, DU, and GC, respectively. The presence of 12-bp insertion was significantly more prevalent in strains from DU than those from GU and GC (23.4 vs. 4.0, 4.2%) ($P < 0.001$ and $P = 0.03$, respectively). The presence of 12-bp insertion was significantly more prevalent in strains from gastritis (14.3%) than those from GU (4.0%) ($P = 0.01$). The presence of 12-bp insertion was tended to be higher in strains from DU than those from gastritis (23.7 vs. 14.3%), although the difference did not reach statistical significance ($P = 0.10$). In case of *cagA*-positive subjects, the presence of 12-bp insertion was significantly associated with DU compared with GU after adjustment by age, sex, *cagA* type, *vacA* m status in multivariate analysis (odds ratio [OR] = 4.72, 95% confidence interval [CI] = 1.46-15.23). Among 8 Western-type *cagA*/*vacA* s1m1strains with 12-bp insertion, 5 strains (62.5%) were isolated from DU. Furthermore, 4 (57.1%) out of 7 East-Asian-type *cagA* strains with 12-bp insertion were isolated from DU. In addition, the presence of 12-bp insertion was tended to be associated with DU compared with GC after adjustment by age, sex, *cagA* type, *vacA* m status in case of *cagA*-positive subjects in multivariate analysis (OR = 7.76, 95% CI = 0.97-61,66, $P = 0.05$).

Nucleotide sequence

Nucleotide sequence data reported are available under the DDBJ accession numbers AB725775-AB725874.

Discussions

cagA, which encodes a highly immunogenic protein (CagA), is the most extensively studied *H. pylori* virulence factor [23, 24]. *cagA* is a polymorphic gene and classified into mainly two types (East-Asian-type *cagA* and Western-type *cagA*) according to the repeat sequence located in the 3′ region of *cagA*[8-10]. Some reports showed that individuals infected with East-Asian-type *cagA* strains have an increased risk of peptic ulcer and/or gastric cancer compared with those infected with non East-Asian-type *cagA* strains [14, 25, 26].

Recent studies showed that Western-type *cagA* in Okinawa, Japan was different from that of the typical Western-type *cagA* found in Western countries according to the full-sequenced *cagA*, and it was names as J-Western-type *cagA*[15]. We recently examined the *H. pylori* in Okinawa with multi-locus sequence typing (MLST) using seven housekeeping genes [14]. Intriguingly, MLST analysis revealed that the majority of Western-type *cagA* strains in

Okinawa formed individual cluster and these did not belonged to cluster of strains isolated from ethnic Europeans, including people from countries colonized by Europeans. These findings supported that the origin of Western-type *cagA* strains in Okinawa is different from those of Western countries.

Recently, Duncan *et al.* as well as our group reported that J-Western-type *cagA* contain unique 12-bp insertion located in the 5′ region of *cagA*[16, 27]. In addition, they stated that J-Western-type *cagA* strains were found not only in Okinawa but also in other parts of the world [27]. Finally, they showed that J-Western-type *cagA* sequences were mainly found in strains possessing *vacA* m2. In this study, we examined the prevalence of 12-bp insertion in Okinawa and the U.S. As a result, the 12-bp insertion was more prevalent in strains in Okinawa than that from the U.S even among Western-type *cagA* strains. Intriguingly, the 12-bp insertion was found in 50.0% even in Western-type *cagA* /*vacA* s1m1 strains in Okinawa, which was higher than that of Western-type-*cagA* /*vacA* s1m2 strains in the U.S (37.5%). These findings suggest that 12-bp insertion was more specific in strains in Okinawa irrespective the status of *vacA* although it was more common in strains with *vacA* m2 genotype. In our previous study using MLST, Western-type *cagA* strains in Okinawa were classified into two groups [14]. One is individual group near hpAsia2 (genotype of strains mainly isolated in South Asia), and another was located in hspEAsia (genotype of strains isolated in East Asia). It is not clear whether there is any association between these groups by MLST and 12-bp insertion, which is processing in our subsequent study.

The presence of 12-bp insertion was inversely associated with the presence of GU. The prevalence of 12-bp insertion was the most prevalent in DU subjects, and followed by gastritis, GC and GU subjects. Furthermore, more than half of the strains with 12-bp insertion in Western-type *cagA*/*va cA* s1m1 and East-Asian-type *cagA* genotypes were isolated from DU subjects. This suggests that the presence of 12-bp insertion was associated with the presence of DU and might have a suppressive action on GU and GC. Duodenal ulcer promoting (*dupA*) gene is also a virulence factor for DU and protective factor for GC [28]. In our previous study, *in vitro* experiments showed that the presence of *dupA* was associated with increased susceptibility to low pH [28]. These findings assume the positive association between the presence of *dupA* and DU but not GC. It is possible that 12-bp insertion is also related with increased susceptibility to low pH and followed by the development of DU. Further studies will be necessary to investigate the mechanisms how 12-bp insertion of *cagA* is associated with DU. An additional *cagA* type, Amerindian *cagA*, was recently reported from populations of the Peruvian Amazon [29]. Amerindian *cagA* can be divided into 2 types, ie, AM-I and AM-II [30]. Interestingly, AM-II *cagA* has attenuated abilities to stimulate gastric epithelial proliferation and inflammation during infection compared to those of Western-type *cagA* or East-Asian-type *cagA*. The Amerindian CagA multimerization segment played an important role in those findings [30]. Our phylogenetic tree showed that the strains with 12-bp insertion formed individual cluster different from that of East-Asian-type *cagA* and typical Western-type *cagA* from Western countries. Furthermore, Western-type *cagA* with 12-bp insertion formed two clusters; one is from Okinawa and another is from the U.S. It remained unclear whether there are any differences of the biological activities between typical Western-type *cagA* and J- Western-type *cagA*, or two types (Okinawa and the U.S) of J- Western-type *cagA*. Several studies showed that the importance of N-terminus of CagA on host cell biology [31, 32]. However, these did not consider the presence or absence of 12-bp insertion. Low biological activities of *cagA* including 12-bp insertion may be inversely associated with GU. Further studies are needed to clarify the role of the J-Western-type *cagA* sequences or 12-bp insertion *in vitro* study.

In conclusion, we found that 12-bp insertion was more prevalent in strains from Okinawa than those from the U.S., especially in Western-type *cagA*/*vacA* s1m2 strains. In addition,

we designed the primer pair to be able to detect the 12-bp insertion only by PCR without DNA sequencing. The presence of 12-bp insertion was associated with DU and would be used as protective marker for GU and GC.

Acknowledgments

This report is based on work supported in part by grants from the National Institutes of Health (DK62813), grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (22390085 and 22659087), Special Coordination Funds for Promoting Science and Technology from MEXT of Japan, and Research Fund at the Discretion of the President, Oita University. The funders had no role in study design data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Ms. Kudo, Ms. Matsuda and Ms. Takahashi for their excellent technical assistance.

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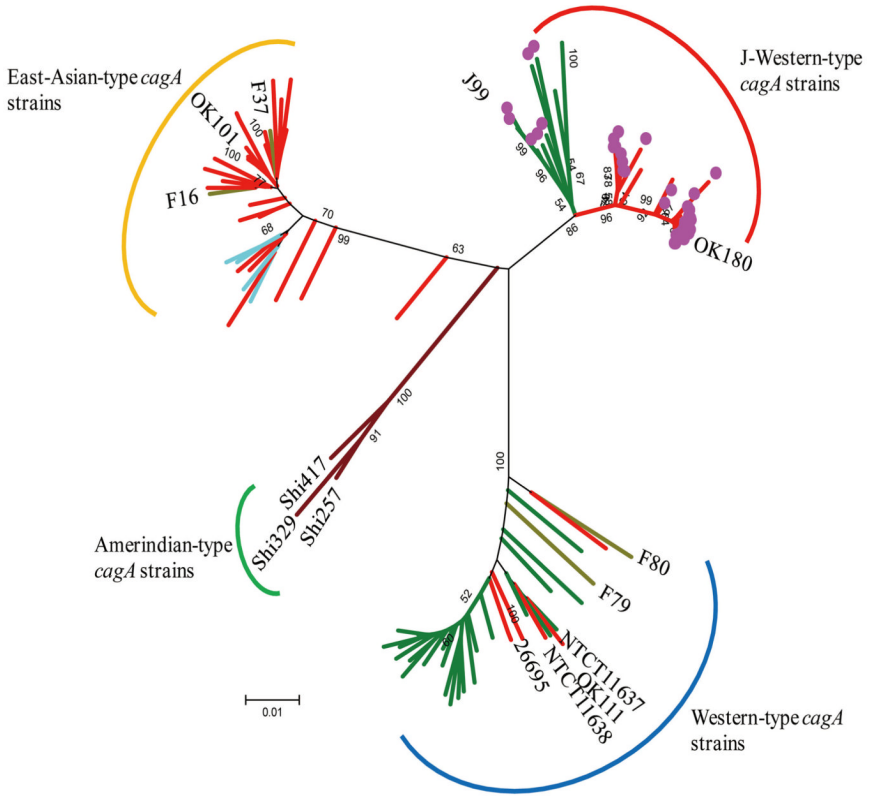

Fig.1. Phylogenetic tree constructed on the basis of the *cagA* sequence

Pylogenetic tree was constructed by using *cagA* sequence (about 1000 bp) including 12-bp insertion. *cagA* sequences of 14 strains (J99, 26695, NCTC11637, NCTC11638, F16, F37, F79, F80, OK101, OK111, OK180, Shi257, Shi329, and Shi417) were obtained from Genbank. Neighbor joining tree was constructed in MEGA 5.0 using bootstrapping at 3,000 bootstrap trials and through Kimura-2 parameters. Scale bars indicate the calculated distance. Red branch show the strains from Okinawa. Dark yellow branch show the strains from Fukui. Blue branch show the strains from Western countries. Light blue branch show the strains from Vietnamese who live in the U.S. Brown branch show the strains from Amerindian. Strains with 12-bp insertion were indicated as purple circle. Each *cagA* type was determined based on the 5′ region of *cagA* sequence (i.e., 2 East-Asian-type *cagA* strains based on the 3′ region of *cagA* sequence with 12-bp insertion located in the same cluster with other strains with 12-bp insertion, suggesting that 2 strains have J-Western-type *cagA* structure in the 5′ region of *cagA*, whereas East-Asian-type *cagA* structure in the 3′ region of *cagA).*

Table 1
The prevalence of 12bp insertion by DNA Sequencing

Okinawa, Japan	**Total**	**Positive**	
East-Asian-type *cagA*/*vacA* s1m1	9	0	(0.0%)
East-Asian-type *cagA*/*vacA* s1m2	15	2	(13.3%)
Western-type *cagA*/*vacA* s1m1	10	5	(50.0%)
Western-type *cagA*/*vacA* s1m2	31	26	(83.9%)
United State	Total	Positive	
East-Asian-type *cagA*/*vacA* s1m2	4	0	(0.0%)
Western-type *cagA*/*vacA* s1m1	12	0	(0.0%)
Western-type *cagA*/*vacA* s1m2	16	6	(37.5%)
Western-type *cagA*/*vacA* s2m2	1	0	(0.0%)

* *cagA* type was determined based on the 3′ region of *cagA* sequence

Table 2
The prevalence of 12bp insertion by polymerase chain reaction in Okinawa, Japan

	Total	Positive	
East-Asian-type *cagA*/*vacA* s1m1	222	5	(2.3%)
East-Asian-type *cagA*/*vacA* s1m2	20	2	(10.0%)
Western-type *cagA*/*vacA* s1m1	14	8	(57.1%)
Western-type *cagA*/*vacA* s1m2	37	31	(83.8%)
Western-type *cagA*/*vacA* s2m2	0	0	(0.0%)
cag A-negative/*vacA* s2m2	43	0	(0.0%)

* *cagA* type was determined based on the 3′ region of *cagA* sequence

Table 3

The association between the presence of 12-bp insertion and clinical outcomes

	Gastritis		Gastric ulcer		Duodenal ulcer		Gastric cancer	
		%		%		%		%
n	98		100		114		24	
mean age	54.4		55.6		54.3		64.8 *	
male	47	(48.0)	74	(74.0) *	69	(60.5)	17	(70.8) *
cag A	76	(77.6)	89	(89.0) *	103	(90.4) *	23	(95.8) *
12-bp insertion	14	(14.3)	4	(4.0) *	27	(23.7) **	1	(4.2) ***

*; $p < 0.05$ compared with gastritis

**; $p < 0.05$ compared with gastric ulcer

***; $p < 0.05$ compared with duodenal ulcer