

cagA genotype and variants in Chinese *Helicobacter pylori* strains and relationship to gastroduodenal diseases

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Previous studies have implicated CagA [encoded by cytotoxin-associated gene A (*cagA*)] in *Helicobacter pylori*-associated gastroduodenal pathology and distinct subgenotypes of *cagA* may circulate in different pathological manifestations of *cagA*-positive *H. pylori* infection. To investigate *cagA* genotype and variants in Chinese *H. pylori* strains and explore their relationship with gastroduodenal diseases, the *cagA* status of 82 Chinese *H. pylori* strains was examined and variation in size of the 3' region of *cagA* in 71 of these strains was analysed by PCR. *cagA* was detected in 28 (100%) of 28 strains from peptic ulcer patients, two (100%) of two strains from gastric cancer patients, 32 (94.1%) of 34 strains from chronic gastritis patients and 17 (94.4%) of 18 strains from healthy volunteers. PCR products of the *cagA* 3' variable region were obtained from 71 (92.2%) of 77 Chinese *H. pylori* strains and could be classified into subgenotypes I, II and III, which gave PCR products of around 825, 900 and 950 bp, respectively. Subgenotype I *cagA* predominated in Chinese *H. pylori* strains (67/71), whereas subgenotype II *cagA* presented in two isolates from patients with chronic gastritis and subgenotype III presented in two isolates from healthy volunteers. Therefore, neither *cagA* nor its 3' region variants can be used as a sole marker for the presence of particular *H. pylori*-related gastroduodenal diseases in the Chinese population.

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INTRODUCTION

It is generally accepted that *Helicobacter pylori* strains that produce CagA, an antigenic outer-membrane protein of variable molecular mass from 128 to 140 kDa, are highly virulent. Several studies have implicated CagA in the development of duodenal ulcer and gastric adenocarcinoma in most western populations (Covacci *et al.*, 1993; Blaser *et al.*, 1995; Torres *et al.*, 1998; Bach *et al.*, 1999; Miehle *et al.*, 2000; Abasiyanik *et al.*, 2002; Nomura *et al.*, 2002a, b; Oliveira *et al.*, 2003; Wu *et al.*, 2003), whereas the majority of reports from east Asian countries (Miehle *et al.*, 1996; Pan *et al.*, 1997; Shimoyama *et al.*, 1997; Maeda *et al.*, 1998; Zheng *et al.*, 2000; Groves *et al.*, 2002; Lai *et al.*, 2002), as well as those from Estonia (Andreson *et al.*, 2002) and Argentina (Catalano *et al.*, 2001), showed a very high prevalence of *cagA*-positive *H. pylori*, irrespective of clinical manifestation. Furthermore, it was reported that a PCR primer set that amplified *cagA* from *H. pylori* isolated in one country failed

to detect *cagA* in isolates from another country (Miehle *et al.*, 1996; Pan *et al.*, 1997). These discrepancies have led to the hypotheses that there may be several distinct forms of CagA with an uneven geographical distribution, that differences in *cagA* subgenotypes may provide a marker for differences in virulence among *cagA*-positive *H. pylori* strains and that only some forms of CagA are associated with severe gastroduodenal diseases.

cagA is noted for its sequence diversity, both within and outside the variable 3' region of the molecule. Repeat sequences in the variable region are the major factor that contributes to the size variation and antigenic heterogeneity of CagA (Covacci *et al.*, 1993; Yamaoka *et al.*, 1998, 1999). It was also reported that strains carrying *cagA* with a short variable 3' region were distributed predominantly in Japanese, German and South African patients with duodenal ulcers, and that those with the longest *cagA* variable 3' region were found more frequently in patients with gastric cancer (Rudi *et al.*, 1998; Yamaoka *et al.*, 1998, 1999; Kidd *et al.*, 1999; Azuma *et al.*, 2002). However, studies conducted in Brazil (Rota *et al.*, 2001) revealed that variable 3' region

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Abbreviation: *cagA*, cytotoxin-associated gene A.

subgenotypes did not relate to clinical outcome, even though the presence of multiple subgenotypes of *cagA* was associated with gastric ulcer incidence.

To determine whether *cagA* genotype and subgenotype are correlated with *H. pylori*-associated gastroduodenal diseases in China, *cagA* status was investigated in 82 Chinese *H. pylori* isolates and *cagA* fragments (including the variable 3' region) of 77 different Chinese *H. pylori* isolates were amplified and amplicon size was compared.

METHODS

***H. pylori* clinical strains.** Experiments were performed with 82 well-characterized clinical *H. pylori* strains that were isolated from Chinese patients undergoing upper endoscopy, as well as the *cagA*-positive *H. pylori* reference strain Sydney Strain 1 (SS1). These strains were obtained from the culture collection of the Institute for Infections Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

***H. pylori* cultivation and DNA isolation.** *H. pylori* was cultured routinely on Columbia agar base (Oxoid) with 10% sheep blood and Oxoid antibiotic supplement for 3 days at 37 °C in a microaerophilic atmosphere (5% O₂, 10% CO₂ and 85% N₂). Bacteria were harvested and suspended in 0.1 M NaCl, 10 mM Tris/HCl and 1 mM EDTA (pH 8.0), SDS (1%, w/v) was added and the mixture was heated at 68 °C and then extracted by the phenol/chloroform/isoamyl alcohol method.

PCR for determining *cagA* status and size variation of the *cagA* 3' variable region. Primers *cagAF* (5'-GATAGGGATAACAGGCAAGC-3') and *cagAR* (5'-GGGGGTTGTATGATATTTTC-3'), which were designed to amplify a rather conservative 297 bp region (nt 151–446 of *cagA* of strain NCTC 11638) that was revealed by aligning 54 *cagA* gene fragments deposited in GenBank, were used to investigate the *cagA* status of *H. pylori* strains. Primers *cagAVF* (5'-CAAAAGATAACGGATAAAGT-3') and *cagAVR* (5'-CTGTTAGTAGCGTAATTGTC-3'), which corresponded to nt 2335–2354 and 3142–3161 of GenBank sequence AF202972, respectively, were used to amplify a fragment that included the *cagA* variable 3' region. According to *cagA* sequences deposited in GenBank, the expected molecular size of the PCR product of this primer set is 818–1052 bp, depending on the number of repeats and deletions present in the sequence of the gene. All PCR mixtures consisted of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, 25 pmol of each of the forward and reverse primers, 0.05–0.1 ng *H. pylori* genomic DNA and 2 U *Taq* polymerase (Sangon Biotechnological Co. Ltd, Shanghai, China) in a final volume of 50 μl. PCR was performed with a RoboCycler Gradient 40 PCR system (Stratagene) and comprised a preincubation of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C, and a final extension for 5 min at 72 °C. PCR products were electrophoresed in 2.0% agarose gels and visualized by staining with ethidium bromide under short UV light; molecular size of PCR products was estimated by comparison with a 100 bp DNA ladder (fragment sizes, 100–1000 bp in 100 bp increments), aided by scanning spectrophotometry using Smart-View 2001 software (version 5).

Statistical analysis. Data were analysed by using a χ^2 test. Probability levels (*P*) of < 0.05 were considered to be statistically significant.

RESULTS

Properties of the study subjects

H. pylori strains were isolated from 82 Chinese patients. Pathological manifestations of these patients were classified at the time of endoscopy as peptic ulcer (*n* = 28), gastric cancer (*n* = 2), chronic gastritis (*n* = 34) and normal (*n* = 18). For analysis of size variation of the *cagA* 3' region, four strains isolated from patients with chronic gastritis and one with peptic ulcer disease were exempted from study, due to loss of these strains during subculture.

cagA status of Chinese *H. pylori* strains as determined by PCR

By using primers *cagAF* and *cagAR* to amplify the conservative *cagA* fragment, the expected PCR product of 297 bp was obtained from 77 (93.9%) of 82 Chinese clinical isolates, as well as from the positive control strain, SS1. Two strains that produced no 297 bp amplicon were verified to be *cagA*-positive by amplifying the variable 3' region of the *cagA* gene (Table 1). In all, 79 (96.3%) of 82 Chinese *H. pylori* strains were *cagA*-positive; prevalence of *cagA* in patients with severe gastroduodenal diseases (30/30, 100%; two of two gastric cancer and 28 of 28 peptic ulcer patients) was not found to be significantly higher than in patients with gastritis (32 of 34, 94.1%; *P* = 0.177) or healthy volunteers (17 of 18, 94.4%; *P* = 0.192).

Size variation of the *cagA* 3' variable region of Chinese *H. pylori* strains

Amplification products of the fragment that included the variable *cagA* 3' region were obtained from 71 (92.2%) of 77 DNA samples. Six samples that yielded no amplification products were excluded from further analysis. Molecular size of PCR products varied from around 825 bp, in 67 (94.4%) Chinese *H. pylori* isolates and SS1, to around 900 bp in two (2.8%) isolates from patients with chronic gastritis and around 950 bp in two (2.8%) isolates from healthy volunteers, which were classified as subgenotypes I, II and III, respectively (Fig. 1, Table 2). These 3' variable region subgenotypes did not show a significant association with specific gastroduodenal disease (*P* = 0.125).

DISCUSSION

The first question posed in this study was whether *cagA* correlated with development of severe gastroduodenal diseases in China. Most of the Chinese *H. pylori* strains were *cagA*-positive (79/82, 96.3%), irrespective of gastroduodenal pathology; this provides further confirmation that *cagA* is not reliable for discriminating specific gastroduodenal disease-associated *H. pylori* strains in China (Pan *et al.*, 1997; Groves *et al.*, 2002; Lai *et al.*, 2002). The fact that two of five strains that were not detectable by *cagAF* and *cagAR* were verified to be *cagA*-positive by amplifying fragments that included the *cagA* 3' variable region showed that the

Table 1. *cagA* status of Chinese *H. pylori* strains, as determined by a PCR that detected a 297 bp consensus region (CR) and the 3' variable region (VR)

Clinical status	n	CR ⁺	CR ⁻ VR ⁺	Total	P value
Severe gastroduodenal disease*	30	30	0	30	–
Chronic gastritis	34	30	2	32	0.177
Healthy volunteer	18	17	0	17	0.192

*This group included 28 patients with peptic ulcer disease and two patients with gastric cancer.

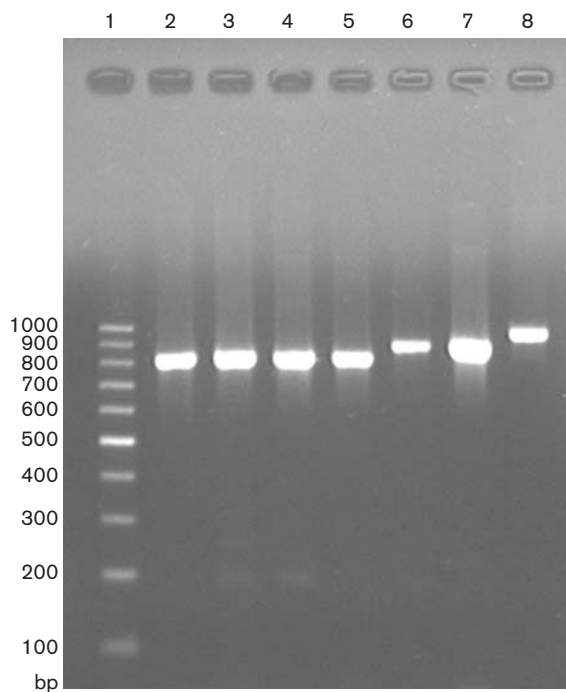


Fig. 1. *cagA* subgenotypes, as revealed by 2.0% agarose gel electrophoresis, of PCR products of the fragment that included the variable 3' region. PCR products from a representative group of strains are shown. Lane 1, 100 bp DNA ladder (100–1000 bp in 100 bp increments); lanes 2–5, subgenotype I *cagA* with a PCR product of around 825 bp; lanes 6–7, subgenotype II *cagA* with a PCR product of around 900 bp; lane 8, subgenotype III *cagA* with a PCR product of around 950 bp.

sensitivity of PCR methods for detecting *cagA* may be crucial for determining *cagA* status and its variants, especially when *H. pylori* strains are isolated from different populations or different geographical regions. This is in agreement with the results reported by Miehlke *et al.* (1996) and Pan *et al.* (1997).

When determining the size variation of fragments that included the *cagA* 3' variable region, primer *cagAVF* (located 168, 165 nt upstream from the forward primers) and *cagAVR* (located 11, 95 nt downstream from the reverse primers), which were reported by Yamaoka *et al.* (1998) and Rudi *et al.* (1998), respectively, were used to amplify this fragment, as

computer-assisted alignment of *cagA* sequences deposited in GenBank revealed a 39 nt deletion upstream of the former forward primers and several nucleotide substitutions in the former reverse primers in several Asian *cagA* sequences; therefore, the PCR products obtained with our primers may be 179 or 218 bp longer than those amplified with the primer set of Yamaoka *et al.* (1998) and 260 or 299 bp longer than those amplified with the set of Rudi *et al.* (1998), depending on the 39 nt deletion. *cagA* subgenotypes I (around 825 bp) and III (around 950 bp) in the present study correspond to types A (642–651 bp) and B/D (756 bp) in Japanese *H. pylori* strains (Yamaoka *et al.*, 1998) and to those with a PCR product of 552–558 or 654–660 bp in German *H. pylori* strains (Rudi *et al.*, 1998), respectively. No subgenotype corresponding to subgenotype II was found in previous reports and no subgenotype corresponding to that with a PCR product of 450 bp in Germany (Rudi *et al.*, 1998) was found in our strains or Japanese strains from a previous study (Yamaoka *et al.*, 1998), so there may be at least six variants of the *cagA* 3' region circulating in the world.

As shown by our results, *cagA* of subgenotype I (67/71, 94.3%) predominated in Chinese *H. pylori* strains, which is consistent with the distributions in Korea (124/128, 96.9%) and Japan (145/155, 93.5%) (Yamaoka *et al.*, 1998, 1999), implying that similar *cagA* subgenotypes may circulate in adjacent geographical areas. It is interesting that the correlation of *cagA* subgenotype C Japanese *H. pylori* strains with gastric cancer has not been found in our isolates or in Korean strains (Yamaoka *et al.*, 1999); as only two strains isolated from gastric cancer were studied and not all gastric cancer-associated *H. pylori* strains carry subgenotype C *cagA*, this subgenotype may have been excluded from our study, although it may be the case that there was no subgenotype C *cagA* in our, or Korean, *H. pylori* strains. Thus, our data did not show that the fragment length of the *cagA* 3' region correlates with disease in our population. We did not observe multiple *cagA* subgenotypes to be present in Chinese *H. pylori* strains; this finding is in agreement with that in Korean strains (Yamaoka *et al.*, 1999), whereas it is in contrast to the previous finding that multiple *cagA* subgenotypes were significantly more frequent in gastric cancer cases in the USA (Yamaoka *et al.*, 1999) and peptic ulcer cases in Brazil (Rota *et al.*, 2001). One possible reason may be that we have underestimated the occurrence of multiple *cagA* subgenotypes, as only a single biopsy was used in our study

Table 2. Relationship between *cagA* 3' variable region subgenotypes and *H. pylori*-related diseases

Subgenotype	Peptic ulcer (n = 26)	Gastric cancer (n = 2)	Chronic gastritis (n = 26)	Healthy volunteer (n = 17)	Total (n = 71)*
I	26	2	24	15	67
II	0	0	2	0	2
III	0	0	0	2	2

*Six of 77 strains, including one that was isolated from a patient with peptic ulcer disease, two from patients with chronic gastritis and one from a healthy volunteer, were excluded from this table, as these strains yielded no amplicon.

and a specific *cagA* subgenotype may become predominant during *H. pylori* subculture, or template competition may have occurred during the PCR amplification process. Therefore, to completely elucidate whether specific *cagA* subgenotypes correlate to *H. pylori*-associated diseases, it will be necessary to further test the *cagA* subgenotypes of *H. pylori* strains, especially those isolated from patients with gastric cancer or peptic ulcer disease in different geographical regions, as well as different isolates from one patient.

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