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

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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; Miehlke 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 (Miehlke 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 (Miehlke 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...
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' -GATAGGGGATACACCCCGCCG-3') and cagAR (5' -GGGGTGTATGATAATTTTC-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' -AAAAGATAACCGATAAAAGT-3') and cagAVR (5' -CTGTATTAGCGGTAAATTTC-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 SmartView 2001 software (version 3).

**Statistical analysis.** Data were analysed by using a 2×2 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
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 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)

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>( n )</th>
<th>CR*</th>
<th>CR − VR*</th>
<th>Total</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe gastroduodenal disease*</td>
<td>30</td>
<td>30</td>
<td>0</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>Chronic gastritis</td>
<td>34</td>
<td>30</td>
<td>2</td>
<td>32</td>
<td>0.177</td>
</tr>
<tr>
<td>Healthy volunteer</td>
<td>18</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0.192</td>
</tr>
</tbody>
</table>

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


