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A new subtype of 3′ region of *cagA* gene in *Helicobacter pylori* strains isolated from Zhejiang Province in China

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Abstract

AIM: To isolate the subtypes of 3′ region of *cagA* gene in *Helicobacter pylori* (*H pylori*) strains from Zhejiang Province in China and to investigate their relations to *H pylori*-associated gastroduodenal diseases.

METHODS: One hundred and thirty-seven *H pylori* clinical strains were isolated from the gastric mucosa specimens of 74 patients with chronic gastritis, 61 with peptic ulceration, and 2 with gastric cancer. Bacterial genomic DNA was extracted and 3′ region of *cagA* gene was amplified by polymerase chain reaction (PCR). Subtypes of 3′ region of *cagA* gene were determined by the size of PCR amplified segments. The sequences of the subtypes were analyzed by PCR-based sequencing.

RESULTS: Of the 137 *H pylori* isolates from Zhejiang Province, 132 (96.4%) yielded PCR products that could be classified into three groups of subtypes, named as subtypes I, II, and III according to their sizes. The sizes of subtypes I, II, and III were 648-650 bp, 705-707 bp, and 815 bp, respectively. Among the 132 *cagA*-positive *H pylori* strains, 123 (93.2%) belonged to the group of subtype I, 6 (4.5%) presented subtype II, 1 (0.8%) was subtype III, and 2 (1.5%) presented subtypes I and III both. The primary structure of subtype I was composed of 3 repeats of R1, 1 repeat of R2 and 1 repeat of R3. Subtype II possessing 4 repeats of R1, 2 repeats of R2 and 1 repeat of R3 was a newly found type of 3′ region of *cagA* gene which had not been reported before. The primary structure of subtype III consisted of 4 repeats of R1, 1 repeat of R2 and 2 repeats of R3. Comparison of the sequences of subtype I strains with the corresponding sequences deposited in GenBank, showed a similarity of 95.0% (94.0-96.1%) for nucleotide sequences and 95.9% (94.9-97.4%) for deduced amino acid sequences. Comparison of the sequences of subtype III strains with the corresponding sequences deposited in GenBank, showed a similarity of 93.9% (90.8-96.9%) for nucleotide sequences and 93.2% (90.2-96.2%) for deduced amino acid sequences. Among subtype II strains, the nucleotide and deduced amino acid sequences showed a similarity of 95.2% (94.1-96.5%) and 96.4% (93.8-97.9%), respectively. There were no statistical differences in the distribution of subtypes of 3′ region of *cagA* gene among different *H pylori*-associated gastroduodenal diseases ($\chi^2 = 11.544$, $P > 0.05$).

CONCLUSION: There are three subtypes (I, II, and III) of 3′ region of *cagA* gene in *H pylori* strains isolated from Zhejiang Province, and subtypeIis predominant. Subtype II is a newly found subtype of 3′ region of *cagA* gene. The result of this study does not support the view that the subtypes of 3′ region of *cagA* gene in *H pylori* isolated from Zhejiang Province are correlated with the clinical outcomes of *H pylori* infection.

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INTRODUCTION

Although *Helicobacter pylori* (*H pylori*) is present in stomachs of at least half of the world's population[1], only a small proportion of the carriers develop symptomatic diseases[2]. The clinical spectrum of *H pylori* infection ranges from asymptomatic gastritis to peptic ulcer and gastric cancer[3]. The causes of different outcomes of *H pylori* infection may include the virulence of infectious strains, the susceptibility of hosts, and environmental cofactors[4-8]. The cytotoxin-associated gene A (*cagA*) is located at one end of a 40-kilobase DNA segment called *cag* pathogenicity island (*cag* PAI), which contains open reading frames encoding for a putative *H pylori* secretion system that may be associated with export of virulence factors to the extracellular compartment[9,10]. The presence of *cagA* gene can be considered as a marker for *cag* PAI and is associated with more virulent *H pylori* strains[11]. The structure of *cagA* gene contains a 5' highly conserved region and a variable 3' region, in which the presence of a variable number of repeat sequences results in a protein (CagA) with a molecular mass of 120 to 140 ku[12]. Yamaoka *et al*.[13] from Japan found that the structural organization of 3' region of *cagA* gene in Japanese *H pylori* isolates could be divided into four types (types A to D), and type C was associated with gastric atrophy and carcinoma. However, the genetic structure of 3' region of *cagA* gene in Chinese *H pylori* strains has been little exploited. In this study, we attempted to investigate the subtypes of 3' region of *cagA* gene in *H pylori* strains isolated from Zhejiang Province in China and their relations to *H pylori*-associated gastroduodenal diseases.

MATERIALS AND METHODS

H pylori isolates

A total of 137 *H pylori* isolates were obtained from *H pylori*-infected patients at the Second Affiliated Hospital of Zhejiang University and the Renmin Hospital of Daishan County in Zhejiang Province. The patients, consisting of 95 men and 42

women with a mean age of 42.6 years (ranging from 16 to 71 years), were classified into 3 groups including chronic gastritis ($n = 74$), peptic ulcer ($n = 61$), and gastric cancer ($n = 2$), according to the results of endoscopic and histological examination.

H pylori culture

Bacteria isolated from biopsy specimens were cultured on ECY selective solid medium[14] at 37 ℃ for 5 d, under 100% humidity and microaerophilic conditions (50 mL/L O_2, 100 mL/L CO_2, and 850 mL/L N_2). *H pylori* strains were identified by the following criteria: Gram staining, colony morphology, rapid urease test, and catalase test. The cultured bacteria were defined to be *H pylori* if they formed typical colonies on the medium, were negative Gram stain with curved or spiral shape, and positive for urease and catalase production[15].

Preparation of H pylori genomic DNA

The bacteria were harvested from the agar plates, then genomic DNA was extracted and purified from each *H pylori* isolate using cetyltrimethyl ammonium bromide (CTAB), phenol-chloroform-isoamyl alcohol, and ethanol precipitation[16].

Amplification of 3' region of cagA gene by PCR

The primers 5'-ACCCTAGTCGGTAATGGGTTA-3' (CAG1) and 5'-GTAATTGTCTAGTTTCGC -3' (CAG2) described by Yamaoka *et al*.[13] were used to amplify 3' region of *cagA* gene in this study. PCR was performed in a volume of 25 μL containing 2.5 μL of 10×PCR buffer, 2 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2 mmol/L dNTPs, 0.5 μL of 20 μmol/L primer sets, 0.2 μL of Taq DNA polymerase, 1 μL of bacterial genomic DNA, and 15.8 μL of H_2O. PCR amplification was performed as following: an initial denaturation at 95 ℃ for 3 min, followed by 30 cycles, each consisting of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, and extension at 72 ℃ for 45 s. The final cycle included a further extension at 72 ℃ for 7 min to ensure the full extension of PCR products. PCR products were analyzed by 20 g/L agarose gel electrophoresis with ethidium bromide staining.

Sequences analysis

PCR products were purified with the DNA purification kit (Shanghai Shenyou Biotechnology Co., Ltd.) according to the manufacturer's instructions. Purified PCR products were consigned to Shanghai BioAsia Biotechnology Co., Ltd. for sequencing. Biological software DNAssist (version 1.0) was used to analyze the sequences of 3' region of *cagA* gene and to compare them with the corresponding sequences deposited in GenBank.

Statistical analysis

The categorical data were analyzed using Chi-square (χ^2) test, and $P<0.05$ was considered statistically significance.

RESULTS

PCR products of 3' region of cagA gene

PCR products of 3' region of *cagA* gene were electrophoresed on 20 g/L agarose gel (containing 0.5 μg/mL ethidium bromide). Of the 137 *H pylori* strains isolated from Zhejiang Province, 132 (96.4%) strains yielded PCR products of three different sizes. The three different-size PCR-amplified segments were respectively named as subtype I (648-650 bp), subtype II (705-707 bp), and subtype III (815 bp) (Figure 1). Among the 132 *cagA*-positive strains, 123 (93.2%) presented subtype I, 6 (4.5%) presented subtype II, 1 (0.8%) presented subtype III, and 2 (1.5%) presented both subtypes I and III.

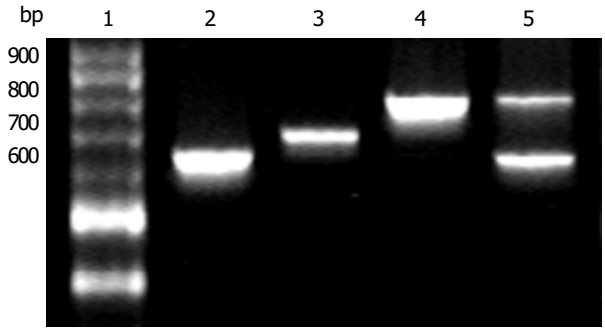

Figure 1 Amplified products of 3' region of *cagA* gene by PCR (20 g / L agarose gel electrophoresis) Lane 1: 100 bp DNA ladder; Lane 2: subtype I (648 bp); Lane 3: subtype II (705 bp); Lane 4: subtype III (815 bp); Lane 5: from a patient with both subtypes I and III.

Sequence analysis of subtypes of 3' region of cagA gene

The primary structure of 3' region of *cagA* gene was composed of a variable number of repeat regions, including R1 (15 bp), R2 (42 bp) and R3 (147 bp). The primary structures of the subtypes of 3' region of *cagA* gene in this study are illustrated in Figure 2. The primary structure of subtype I was composed of 3 repeats of R1, 1 repeat of R2 and 1 repeat of R3, so subtype I was equal to type A in Japanese *H pylori* strains reported by Yamaoka *et al*.[13]. The primary structure of subtype III consisting of 4 repeats of R1, 1 repeat of R2 and 2 repeats of R3 was similar to that of type C reported by Yamaoka *et al*.[13]. Subtype II possessing 4 repeats of R1, 2 repeats of R2 and 1 repeat of R3 had the primary structure not similar to any types reported by Yamaoka *et al*[13] and was regarded as a newly found subtype of 3' region of *cagA* gene in *H pylori*. Comparison of the sequences of 5 *H pylori* strains presented subtype I with the corresponding sequences of a Japanese type A strain JK25 deposited in GenBank (GenBank accession number AF043487), showed a similarity of 95.0% (94.0-96.1%) for nucleotide sequences and 95.9% (94.9-97.4%) for deduced amino acid sequences. Comparison of the sequences of 2 *H pylori* strains presented subtype III (including a strain presented both subtypes I and III) with the corresponding sequences of a Japanese type C strain Jk269 deposited in GenBank (GenBank accession number AF043489), showed a similarity of 93.9% (90.8-96.9%) for nucleotide sequences and 93.2% (90.2-96.2%) for deduced amino acid sequences. Among the 6 *H pylori* strains presented subtype II in this study, the nucleotide and deduced amino acid sequences showed a similarity of 95.2% (94.1-96.5%) and 96.4% (93.8-97.9%), respectively. Alignments of the deduced amino acid sequences of 5 strains presented subtype I and 2 strains presented subtype III with the corresponding sequences deposited in GenBank are illustrated in Figure 3 A, B. Alignments of the deduced amino acid sequences of 6 strains presented subtype II are also illustrated in Figure 3 C.

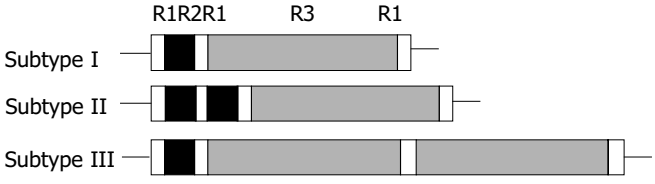

Figure 2 Primary structures of three subtypes of 3' region of *cagA* gene R1: 15 bp repeat region; R2: 42 bp repeat region; R3: 147 bp repeat region.

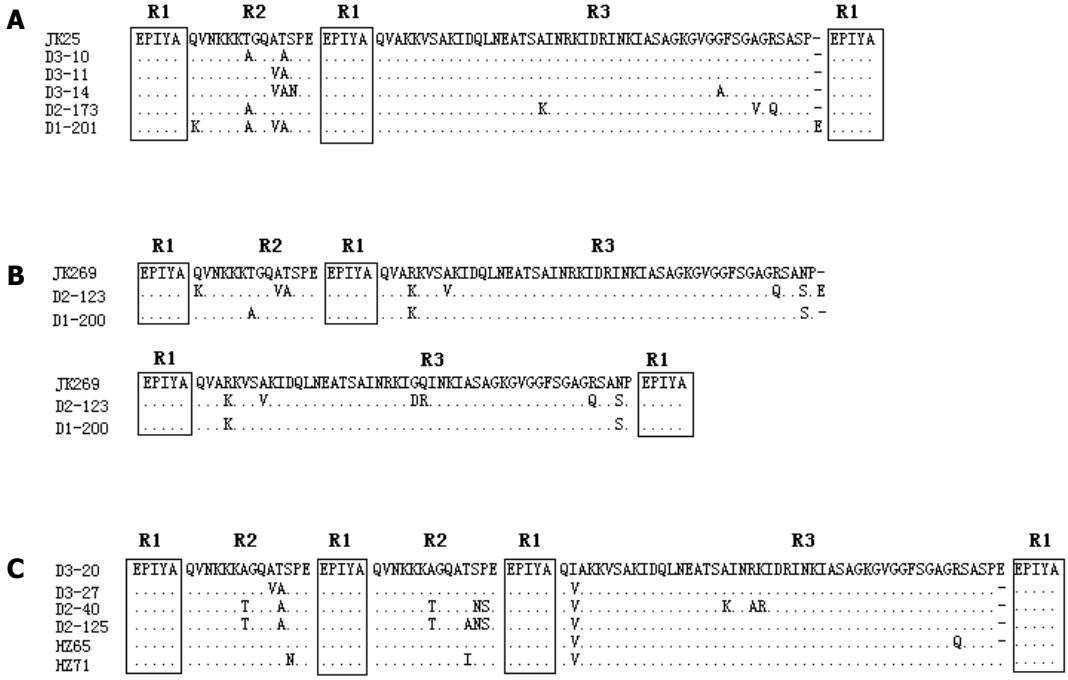

Figure 3 Alignment of amino acid sequences of subtypes of 3' region of *cagA* gene. A: Alignment of the deduced amino acid sequences of subtype I strains with the sequence of the corresponding region of a Japanese type A strain JK25 (GenBank accession number AF043487). Strain D2-173 was from a patient with chronic gastritis, and the remaining four strains were from patients with peptic ulcer. B: Alignment of the deduced amino acid sequences of subtype III strains with the sequence of the corresponding region of a Japanese type C strain JK269 (GenBank accession number AF043489). Strain D1-200 was from a patient with chronic gastritis, and strain D2-123 was from a patient with gastric ulcer. C: Alignment of the deduced amino acid sequences of six subtype II strains. Strain HZ65 and HZ71 were from patients with chronic gastritis, strain D2-40 was from a patient with gastric cancer, and the remaining three strains were from patients with peptic ulcer.

Relationship between subtypes of 3' region of cagA gene and gastroduodenal diseases

The distributions of the subtypes of 3' region of *cagA* gene in different groups of gastroduodenal diseases are demonstrated in Table 1. Statistical analysis showed that there were no significant differences among the subtypes of 3' region of *cagA* gene in different groups of gastroduodenal diseases ($\chi^2 = 11.544$).

Table 1 Relationship between subtypes of 3' region of *cagA* gene and different group of gastroduodenal diseases (*n*, %)

Group of diseases	Subtype I	Subtype II	Subtype III	Subtypes I and III	Total
Chronic gastritis	69 (95.8)	2 (2.8)	0 (0)	1 (1.4)	72
Peptic ulcer	53 (91.4)	3 (5.2)	1 (1.7)	1 (1.7)	58
Gastric cancer	1 (50.0)	1 (50.0)	0 (0)	0 (0)	2
Total	123 (93.2)	6 (4.5)	1 (0.8)	2 (1.5)	132

$\chi^2 = 11.544$, $P = 0.17 > 0.05$.

DISCUSSION

H pylori, a spiral shaped gastric organism, is the cause of chronic gastritis, and plays an important role in the pathogenesis of peptic ulceration, mucosa associated lymphoid tissue lymphoma, and gastric adenocarcinoma[17-20]. It has been reported that 50-60% of *H pylori* strains contain *cagA* gene and consequently produce CagA protein[21]. The CagA is a highly immunogenic outer membrane protein with a molecular weight of 120 to 140 ku. Variation in size of the protein has been correlated with the presence of a variable number of repeat sequences located in 3' region of the gene[22,23]. The biological importance of the repeat sequences in 3' region of *cagA* gene remains unknown. Because CagA is strongly immunogenic, these repeat sequences have been supposed to alter immunogenicity of the protein[24]. This alteration in the gene and its protein seems to correlate with clinical outcomes *in vivo*. The proportion of *cagA*-positive *H pylori* isolates varies from one geographic region to another. Studies from Japan, Korea, and China have shown that more than 90% of *H pylori* strains are *cagA*-positive[25-28], while in the United States of America, Canada, and Europe, these percentages are lower[24,29,30]. Therefore, *cagA* gene cannot be used as a marker for the presence of severe gastroduodenal diseases in those regions where the prevalence of *cagA*-positive *H pylori* strains is uniformly high. Since allelic variation in *cagA* exists and distinct *H pylori* subtypes may circulate in different regions, differences in *cagA* subtype might provide a marker for differences in virulence among *cagA*-positive *H pylori* strains[12].

Yamaoka *et al*.[13] reported that 3' region of *cagA* gene in *H pylori* isolated from Japanese patients could be classified into four types (types A, B, C, and D) depending on the types and number of repeat regions including R1 (15 bp), R2 (42 bp) and R3 (147 bp). The PCR products of type A ranged from 642 to 651 bp, and possessed 1 repeat of R2 and 1 repeat of R3. The PCR products of type B and type D were all 756 bp, but type B possessed 3 repeats of R2 and 1 repeat of R3, while type D had 2 repeats of R3 and no repeat of R2. Type C having 1 repeat of R2 and 2 repeats of R3 yielded PCR products of 813-815 bp and was associated with high levels of CagA antibody and severe degrees of atrophy. The same authors have also found that the sequences of the second repeat regions of 3' region of *cagA* gene in *H pylori* strains from East Asia are completely different from those in strains from non-Asian countries[31]. Non-Asian strains possess 102 bp second repeat regions, and East Asian strains possess 162 bp second repeat regions[31,32].

In the present study, we used the same PCR primers as those described by Yamaoka *et al*.[13] and found 132 (96.4%) of 137 strains yielded amplified products. The high prevalence of *cagA*-positive strains in Zhejiang Province was in accordance

with the result of our previous study[33] and the findings in other Chinese areas[11,27]. The PCR products could be classified into three subtypes according to their sizes and were named as subtype I, subtype II, and subtype III, respectively. The PCR products of subtype I ranged from 648 to 650 bp and possessed 1 repeat of R2 and 1 repeat of R3, so subtype I was equal to type A reported by Yamaoka *et al*.[13]. The predominance of subtype I strains (93.2%) in this study was in agreement with that of type A strains (93.5%) in Japanese patients[13]. The size and genetic structure of subtype III indicated that subtype III in this study was equal to type C in Japanese strains[13]. Three (including 2 multiple subtypes strains) of 132 *cagA*-positive strains presented subtype III, and the prevalence of this subtype (2.3%) was close to that of type C (4.5%). In contradiction to the two above-mentioned subtypes, subtype II having the size of PCR product of 705-707 bp and the structure of 2 repeats of R2 and 1 repeat of R3 has not been reported before. Because sequence analysis showed that the structure of subtype II still accorded with the characteristics of 3' region of *cagA* gene in Asian strains, subtype II in this study was regarded as a new subtype of 3' region of *cagA* gene in Asian *H pylori* strains. The fact that we did not find type B and type D reported by Yamaoka *et al*.[13], but discovered a new subtype revealed the diversity and randomicity of the assembling mode of repeat regions located at 3' region of *cagA* gene and the possibility that this assembling mode varied in *H pylori* strains isolated from different areas. In addition, 2 strains presenting more than one subtype were observed in this study and thought to be from the patients with multiple *H pylori* infection. Sequence analysis revealed high similarities between the sequences of 3' region of *cagA* gene in *H pylori* isolated from Zhejiang Province and those in *H pylori* strains from Japanese patients and high similarities among the 6 subtype II strains, so we could draw a conclusion that despite of the genetic diversity of 3' region of *cagA* gene in *H pylori* strains isolated from Zhejiang Province, the sequences of the same subtype were still conservative. There were no significant differences among the subtypes of 3' region of *cagA* gene in different groups of gastroduodenal diseases, so the subtypes of 3' region of *cagA* gene in *H pylori* isolated from Zhejiang Province seemed not to be correlated with the clinical outcomes of *H pylori* infection.

In conclusion, there are three subtypes of 3' region of *cagA* gene in *H pylori* strains isolated from Zhejiang Province. Subtype I is a predominant one and subtype II is a newly found subtype of Asian *H pylori* strains. The result of this study does not support the view that the subtypes of 3' region of *cagA* gene in *H pylori* isolated from Zhejiang Province are correlated with the clinical outcomes of *H pylori* infection.

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