

Characterization of *CagA* variable region of *Helicobacter pylori* isolates from Chinese patients

Yong-Liang Zhu, Shu Zheng, Qin Du, Ke-Da Qian, Ping-Chu Fang

Yong-Liang Zhu, Qin Du, Ke-Da Qian, Department of Gastroenterology, Second Hospital of Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China
Shu Zheng, Cancer Institute, Second Hospital of Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China
Ping-Chu Fang, Department of Microbiology, Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China
Supported by the National High Technology Research and Development Program of China, No. 2001AA227111
Correspondence to: Professor Shu Zheng, Jiefang road 88#, Hangzhou 310009, Zhejiang Province, China. zhengshu@zju.edu.cn
Telephone: +86-571-87783868
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Abstract

AIM: To characterize the *CagA* variable region of *Helicobacter pylori* isolates from Chinese patients.

METHODS: DNA fragments in *CagA* variable region were amplified and sequenced respectively from genomic DNA of 19 isolates from patients with gastric cancer and 20 isolates from patients with chronic gastritis. The tendency of phosphorylation in tyrosine(s) of *CagA* proteins was evaluated subsequently by phosphorylation assay *in vivo* and *in vitro* respectively.

RESULTS: About 97.44% (38/39) *H pylori* isolates possessed *CagA* gene. *CagA*⁺ strains contained 2-4 tandem five-amino-acid motifs EPIYA but only one EPIYA had repeated sequence in *CagA* variable region in different isolates. There was no significant difference between the number of EPIYA motifs in *H pylori* from patients with different diseases. However, only tyrosine site in EPIYA within repeated sequence could be phosphorylated by AGS cells *in vivo* although all tyrosine sites in EPIYA could be phosphorylated *in vitro*.

CONCLUSION: *CagA* in Chinese has no functional difference in perturbing cellular signal pathway among different *H pylori* isolates.

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Key words: *Helicobacter pylori*; *cag* pathogenicity island; *CagA*; Tyrosine phosphorylation

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INTRODUCTION

Helicobacter pylori (*H pylori*) has been recognized as the causative agent of chronic gastric inflammation, which can progress further to a variety of diseases such as peptic ulcer and mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma^[1-3]. Type I isolates of *H pylori* possess a major disease-associated genetic component, the *cag* pathogenicity island (*PAI*) which encodes a type IV secretion apparatus and virulence factors such as the immunodominant *CagA* protein^[4,5]. Recent studies have demonstrated that the *CagA* protein is translocated from the bacterium into the host cell and induces reorganization of the host cell actin cytoskeleton, activation of Rho GTPases Rac1 and Cdc42, recruitment of transcription factors NF-KB and AP-1, activation of proto-oncogenes *c-fos* and *c-jun* and release of cytokines and chemokines^[6-10]. Stein *et al*^[11] have confirmed that the C-terminal half of *CagA* (namely *CagA* variable region) is phosphorylated in tyrosine sites by *c-src/lyn* protein kinase family once translocated into eukaryotic cells. Higashi and coworkers^[12] found a src homology 2 (SH2) containing tyrosine phosphatase 2 (SHP-2) was an intracellular target of *CagA* protein. SHP-2 is an important mediator molecule in several cell signal transductions and rearrangement of the actin cytoskeleton which plays a crucial role in inducing the scattering ('hummingbird') phenotype in gastric epithelial cells (AGS)^[12,13]. Phosphorylated *CagA* disturbs signal transductions by binding to SHP-2 in host cells^[10]. However, *CagA* protein varies in size in different strains, this size variation raises an intriguing possibility that the biological activity of *CagA* can vary from one strain to the next, which may influence the pathogenicities of different strains^[14-16]. China is one of the nations where the infection incidence of *H pylori* is among the highest in the world and more than 90% isolated strains possess *PAI* gene, the biological activity of *H pylori* virulence factor *CagA* isolated from Chinese remains unclear so far^[17,18]. In the present report, we cloned and sequenced the variable region of *CagA* DNA fragments in 19 strains from patients with gastric cancer and 20 strains from patients with chronic gastritis. Further, we evaluated the ability of *CagA* protein to phosphorylate in tyrosine(s).

MATERIALS AND METHODS

A total of 39 *H pylori* clinical strains including 19 strains from patients with gastric cancer and 20 strains from patients with chronic gastritis were isolated from the biopsy specimens in the Second Affiliated Hospital of Zhejiang University College of Medicine between October 2000 and

April 2002. The diagnosis obtained by endoscopy and histology was recorded for all patients from whom the strains were isolated. There were 22 males and 17 females, with their age ranging 36-68 years (median age 48.6 years).

Isolation and culture of *H pylori* strains

Gastric mucosa supernatant was directly cultured in the PYL plate (Biomerux Company, France) and incubated in microaerophilic atmosphere for 4 d at 37 °C. Bacterial clones were identified by both biochemical (including rapid urease reaction, peroxidase test and Gram's staining) and serological reactions. Following primary isolation, the bacteria were inoculated into Columbia agar (Biomerux Company, France) with 50 mL/L frozen-melting sheep blood, 100 mL/L fetal bovine serum, and Skirrow's antibiotic supplement (Biomerux Company, France) in a microaerophilic atmosphere for 5 d at 37 °C, washed 3 times with 100 mmol/L PBS, pH7.4. *H pylori* genomic DNA was extracted according to the instructions of bacterial genome extraction kit (Sangon Engineering Biotechnology Company, Shanghai, China).

Amplification of DNA fragments of *CagA* variable region in clinical isolates

A total of 39 DNA samples were amplified by high fidelity PCR master (Roche Company, Germany) with upstream primer: 5'-GACGAGCCTATTTATGCT-3' and downstream primer: 5'-GCCTCATCAAATCAATTGT-3'. The reaction mixture was incubated in a thermal cycler. PCR conditions were as follows: denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 30 s, and extension at 72 °C for 30 s, with a final extension reaction for 7 min^[14]. Samples were analyzed on an 1% agarose gel in 1×Tris base-EDTA buffer. DNA fragments were excised and purified from gel with kit for DNA sequence respectively.

Construction of pcDNA3.1(+) and pET15b expression vectors containing *CagA* DNA fragments with 2, 3 and 4 EPIYAs as well as their mutants respectively

Genomic DNA containing *CagA* fragments with 2, 3 and 4 EPIYAs from the above clinical isolates were used as templates, respectively. Two subfragments (about 800 bp) from *CagA* variable region in each strain could be amplified by high fidelity overlapped PCR with primers containing *Bam*H I or *Xba* I^[19]. The upstream primer was 5'-TCGGATCCAATTGGAGAGCAGAA-3' and the downstream primer: 5'-GCTAGGCTCCAAAGCGGCCGCTCTTGCTTCCTTACTAG-3' for construction 1. The upstream primer was 5'-ATGCCTGGACCAAGAGCGGCCGCTTTCGATC-3' and the downstream primer was 5'-GACTCGAGTTAAGATTTTGGAAACCAC-3' for construction 2. After ligation at *Not* I site, the PCR products were digested with double restriction endonuclease *Bam*HI/*Xba* I, and a 1 509 bp *CagA* fragment was cloned into pcDNA3.1(+) mammalian expression vector (Invitrogen Company, USA). The mutants (referred to as EPIFA) of *CagA* variable region in which tyrosine in the repeated sequence was mutated to phenylalanine were produced by using a Chameleon site-directed mutagenesis kit (Stratagene Company, USA, data

not shown), including EPIFA with 1, 2 and 3 EPIYAs, respectively. The procedure was strictly performed under the manufacturer's instructions. pcDNA3.1(+) containing *CagA* or mutants was constructed as template, wild and mutated *CagA* fragments with EPIYA(s) were subsequently subcloned into *Nde* I/*Bam*HI sites of pET15b expression vectors (Novagen Company, USA) and BL21 DE3 plysS *E. coli* component cells (Novagen Company, USA) were transformed. The recombinant proteins were induced by IPTG at the concentration of 0.4 mmol/L for 2 h, and purified by the His.Bind purification kit (Novagen Company, USA) for evaluation of the phosphorylation at tyrosine site (s) of *CagA* protein *in vitro*.

Phosphorylation assay

Phosphorylation assay *in vivo* Human AGS gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen Company, USA) before transfection. Cells were transfected with pcDNA3.1(+) expression vectors containing *CagA* or mutants with 2-4 EPIYA motifs respectively by Lipofectamine 2000 reagent (Invitrogen Company, USA) according to the manufacturer's protocols. Fifty milligrams of plasmid was transfected into AGS cells by 40 µL of Lipofectamine 2000 reagent. Cells were harvested 72 h after transfection and lysed in lysis buffer containing 1 mmol/L EDTA, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µmol/L leupeptin, 1 µmol/L pepstatin. The cell lysate was mixed with anti-*CagA*-antibody-sepharose 4B gel (a generous gift from Dr. Yi-Xiong Wang in Right Biotechnology, China) at 4 °C for 60 min, gently mixed, washed 3 times with 100 mmol/L PBS, pH7.4, denatured with Tris base-chloride buffer containing 100 mmol/L dithiothreitol, centrifuged at 13 000 g for 20 min. The supernatant collected was subjected to electrophoresis and transferred to a nitrocellulose membrane filter (Hybond Company, USA), blocked with 5% milk powder, incubated in at 1:250 diluted monoclonal antibodies against phosphorylated tyrosine (Y99, Santa Cruz Company, USA) at 37 °C for 2 h, washed 5 times. Then 1:100 diluted biotinylated sheep antibodies against mouse (Maxim Biotechnology Company, USA) were added and washed 5 times, and incubated with streptavidin-peroxidase conjugate (Maxim Biotechnology Company, USA) for 60 min. Finally, the color was developed by ECL substrate.

Phosphorylation assay *in vitro* Nonidet P40-soluble fraction of AGS cells was performed as described by Stein *et al*^[11]. Ten milligrams of *CagA* or mutant recombinant proteins with EPIYAs was incubated with 10 µL of cell lysate in a total reaction volume of 40 µL 250 mmol/L Tris-Cl buffer (pH7.4) containing 100 Ci gamma-³²P ATP (Perkin Elmer company, USA), 62.5 mmol/L MnCl₂, 312.5 mmol/L MgCl₂, 5 mmol/L EGTA, 1 mmol/L sodium-o-vanadate. Phosphorylation was carried out at 30 °C for 10 min and the mixed solution was subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was exposed at 4 °C for 24 h, respectively.

RESULTS

Amino acid sequence of *CagA* variable region

Thirty-eight of 39 isolates possessed *CagA* gene and the positive rate of *CagA*⁺ was 97.44% (38/39). The nucleotide sequences of *CagA* were quite similar among the 38 strains with *CagA*⁺. However, the variation in the number of repetitions of the 5-amino acid EPIYA sequence in the 3' region of *CagA* was observed. Amino acid sequences were found to be 2-4 tandem five-amino-acid motifs EPIYA between each of which, there was an interval of 13-44 amino acids in *CagA* variable region. The case of *CagA* protein with 3 tandem EPIYAs was most common in all clinical isolates. There were 3 types of *CagA* variable region among the 38 isolates, namely *CagA* with 2 EPIYAs, *CagA* with 3 EPIYAs and *CagA* with 4 EPIYAs (Figure 1). To explore the relationship between the number of EPIYAs in isolates and disease outcomes, we adopted χ^2 test to evaluate the correlation and found that there was no significant difference in the number of tandem five-amino-acid motif EPIYAs in isolates from gastric cancer and chronic gastritis (Table 1), $P>0.05$. It implied that the variation of the number of EPIYAs might not relate to the disease outcome (gastric cancer *vs* chronic gastritis). Subsequently the analysis of sequences showed that each *CagA*⁺ strain contained only one repeated sequence in the C-terminal half of *CagA* protein, namely genetic structure of EPIYA-TIDDL-FPLKRHDKVEDLSKV (this EPIYA was usually referred to as D1 region, TIDDL as D2 region, FPLKRHDKVEDLSKV as D3 region^[11]). There were two obviously differences in this structure compared with Western standard strain NCTC11637. One difference was found in the number of repeated sequences. There was only one repeated sequence in each Chinese isolate compared with three repeated sequences in NCTC11637. The other was found in the key amino acid sequences in D2 region of repeated sequences. The amino acid sequence in D2 region was DFD in each *H pylori* from Chinese patients, and was DDL in NCTC11637 and other Western isolates^[11,14]. The variation of two amino acids in D2 region conferred Chinese *CagA* protein to more perfectly match the SHP-2 consensus motif (PY-S/T/A/V/I-X-V/I/L-X-W/F) than the Western strains^[20]. Hence, we speculated that Chinese *CagA* protein might be more able to activate SHP-2 molecules.

Tyrosine phosphorylation sites in EPIYA

We used the phosphorylation assay *in vivo* and *in vitro* to identify whether and which tyrosine site(s) in EPIYA could be phosphorylated. In phosphorylation assay *in vitro*, *CagA* recombinant proteins with 2, 3 and 4 EPIYAs as well as their mutants EPIFA with 1, 2 and 3 EPIYAs could be labeled with ³²P respectively. Moreover, *CagA* protein with 4 EPIYAs became more marked with ³²P than that with 2 or 3 EPIYAs and their mutants with 1, 2 and 3 EPIYAs (Figure 2: A-F). It implied that the tyrosine phosphorylation site in EPIYA both within and outside the repeated sequences of *CagA* recombinant proteins could be phosphorylated by AGS cell lysate *in vitro*, and *CagA* protein with 4 EPIYAs became more phosphorylated than that with 2 or 3 EPIYAs and their mutants. However, only one tyrosine site in EPIYA within the repeated sequences could be phosphorylated and no tyrosine in EPIYA outside the repeated sequences could

CZU-1: EPIYAQVNKKKAGQVARINKIASAGKGVGGFGVGRSASPE
 CZU-2: EPIYAQVNKKKAGQVASPEEPIYAQVARKVSVKIDQLNEAT
 CZU-3: EPIYAQVNKKKTGQATSPEEPIYAQVNKKKTGQVASPE-----
 NCTC11637:EPIYAKVNKKKAGQAASLEEPIYA-----

CZU-1: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
 CZU-2: SAINRKIDRINKIASAGKGVGGFGVGRSASPEEPIYAQOVAKK
 CZU-3: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
 NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKV-----
 Repeat1 D1 D2 D3

CZU-1: -----
 CZU-2: VSAKIDQLNEATSAINRKIDRINKIASAGKGVGGFGVGRSASPE
 CZU-3: -----
 NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKVG-----
 Repeat2 D1 D2 D3

CZU-1: -----
 CZU-2: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
 CZU-3: -----
 NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKV-----
 Repeat3 D1 D2 D3

Figure 1 Amino acid sequence characteristics of *CagA* variable region. CZU-1,2,3: Isolates from Chinese patients stand for the three types of *CagA* variable region respectively; CZU-1: *CagA* with 2 EPIYAs; CZU-2: *CagA* with 3 EPIYAs and CZU-3: *CagA* with 4 EPIYAs. The red letters stand for the deletion of amino acids in CZU-1 and the letters underline the mean extra amino acids in CZU-3 when compared to CZU-2. NCTC11637 was a Western standard strain.

Table 1 Comparison of the number of tandem five-amino-acid motif EPIYAs in strains from gastric cancer and gastritis

Case	EPIYA (n)			
	2	3	4	
Gastritis	20	4	13	3
Gastric cancer	18	3	12	3

χ^2 test, $\chi^2 = 0.301, P>0.05$.

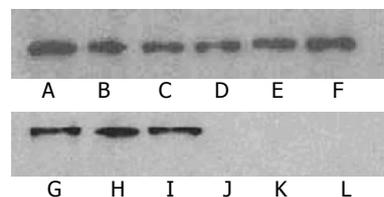


Figure 2 Tyrosine phosphorylation sites in EPIYA *in vitro* and *in vivo*. A-F: tyrosine phosphorylation *in vitro*. Lane A: *CagA* with 4 EPIYAs; lane B: *CagA* with 3 EPIYAs; lane C: *CagA* with 2 EPIYAs; lane D: EPIFA with 1 EPIYA; lane E: EPIFA with 2 EPIYAs; lane F: EPIFA with 3 EPIYAs. lane G-L: tyrosine phosphorylation *in vivo*. G: *CagA* with 4 EPIYAs; H: *CagA* with 3 EPIYAs; I: *CagA* with 2 EPIYAs; lane J: EPIFA with 3 EPIYAs; lane K: EPIFA with 2 EPIYAs; lane L: EPIFA with 1 EPIYA.

be phosphorylated *in vivo* by AGS cells when transfected pcDNA3.1(+) mammalian expression vectors containing *CagA* with 2, 3 and 4 EPIYAs as well as EPIFA with 1, 2 and 3 EPIYAs, since the mutants of EPIFA with 1, 2 and 3 EPIYAs could not react with antibody to phosphorylated tyrosine in transfected AGS cells (Figure 2: G-L). Moreover, *CagA* protein with 4 EPIYAs did not become more phosphorylated than that with 2 or 3 EPIYAs. The outcomes were similar to that of infection of AGS cells with *H pylori* bacteria containing *CagA* with 2, 3 and 4 EPIYAs, respectively

(data not shown). Hence, we speculated that the number of EPIYA motifs might not relate to the ability of tyrosine to phosphorylate on *CagA* protein *in vivo*, which could only be determined by the number of EPIYA repeated sequences. As a result, this outcome raised an intriguing possibility that the ability of Chinese *CagA* to phosphorylate in tyrosine was equal to one another in different isolated strains of type I because each *CagA* protein contained only one repeated sequence in clinical isolates from Chinese patients with either gastric adenocarcinoma or gastritis.

DISCUSSION

The correlation between *H pylori* as a class I carcinogen and gastric adenocarcinoma is not completely clarified and has attracted much attention so far. *CagA* gene as a marker for *cag* pathogenicity island associated with more virulent *H pylori* strains may be one of the most important contributors in the process of gastric carcinogenesis, because the antibody titer of *CagA* is much higher in gastric carcinoma than in non-gastric carcinoma and the detectable rate of *CagA* antibody in human beings is significantly higher in high occurrence region of gastric cancer than in low occurrence area. *CagA*⁺ strains can significantly increase the risk for developing severe gastritis and gastric carcinoma compared with *CagA*⁻ *H pylori* strains^[21-23]. A research suggests that the development of more prominent gastritis and severe atrophy in *CagA*⁺ patients is an indicator for the importance of *CagA* rather than *H pylori* load^[24], indicating that *CagA* protein plays an important role in pathogenesis of *H pylori*. In general, *CagA* from different *H pylori* has a significant difference because of the number of repeated sequences of EPIYA in C-terminal half of *CagA* although N-terminal of *CagA* protein is quite conserved. Hence, the highlight in study of *CagA* is whether this diversity of *CagA* is correlated with disease outcomes. Azuma *et al.*^[15] found that there were more EPIYAs in *H pylori* from atrophic gastritis and gastric cancer than in *H pylori* from gastritis. The frequencies of genotypes of *CagA* with more than 4 EPIYAs were significantly higher in atrophic gastritis than in duodenal ulcer. One-third of strains with more than 4 EPIYAs were in gastric cancer. They concluded that *H pylori* infection with the *CagA* genotype with more than 4 EPIYAs might correlate with the pathogenesis of atrophic gastritis and gastric cancer^[15]. In contrast, *CagA* protein varied in the number (from 1 to 3) of repeated sequences of EPIYA in different strains isolated from Western countries^[11,16]. We demonstrated in this work that each Chinese *CagA* variable region contained 2-4 tandem EPIYA motifs and only one EPIYA had repeated sequences. There was no significant difference between the number of EPIYA motifs in *CagA* variable regions in *H pylori* isolated from patients with either gastritis or gastric carcinoma. There were three types of *CagA* variable region among the 38 isolates, namely *CagA* with 2 EPIYAs, *CagA* with 3 EPIYAs and *CagA* with 4 EPIYAs. But no *CagA* with more than 4 EPIYAs or deletion of *cagE* was observed in these Chinese *H pylori* isolates. It appears that there is a difference in the 3'-variable region of *CagA* of *H pylori* isolates from Chinese and Japanese^[15].

The ability of *CagA* protein to phosphorylate in tyrosine

(s) is the premise of *CagA* protein perturb cellular signal pathway and induction of cellular response. Higashi and coworkers^[12] have found that a tyrosine phosphatase -2 containing c-src homology 2 is an intracellular target of tyrosine-phosphorylated *CagA* protein. The phosphorylase activity of SHP-2 by binding to tyrosine-phosphorylated *CagA* protein has a close relationship with the "hummingbird" phenotype of AGS cells. Besides SHP-2 as an intracellular target of tyrosine-phosphorylated *CagA* protein, *CagA* protein could interact with Grb2 and phosphorylate *CagA* protein which is indispensable for the Grb2 binding^[25], resulting in the activation of the Ras/MEK/ERK pathway and cell proliferation. Hence, the ability of phosphorylated *CagA* protein could reflect the level of biological function of *CagA* and might play a key role in the pathogenicity of *H pylori*. Except that one tyrosine site in repeated sequences is phosphorylated after *CagA* DNA is transfected into gastric epithelial cells, no other tyrosine sites in EPIYA outside the repeated sequences could be phosphorylated *in vivo* although all the tyrosine sites in EPIYA could be phosphorylated *in vitro*, indicating that sequence diversity of tyrosine phosphorylation motifs on *CagA* is not related to the presence of tyrosine phosphorylation. The reason is that the membrane tethering of *CagA* protein is necessary for the tyrosine-phosphorylated EPIYA in host cells. The other obvious difference that might influence the phosphorylation ability of *CagA* is the amino acid sequence in D2 region in repeated sequences^[16]. Each Chinese isolate is DFD which could more perfectly match the SHP-2 consensus motifs than DDL in NCTC11637 and other Western isolated strains. It implies that Chinese *CagA* has a much stronger ability to perturb cell signal pathway. Above all, the fact raises an intriguing possibility that Chinese *CagA* has no functional difference in perturbing cellular signal pathway among different isolates of type I although there is a diversity in C-terminal half of *CagA*.

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