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Analysis of the 3' Variable Region of Cytotoxin-Associated Gene A (*cagA*) in *Helicobacter pylori* Isolates in China

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ABSTRACT

Infection by the bacterium *Helicobacter pylori* is a putative cause of various gastric disorders, including gastric adenocarcinoma. Incident rates are associated with variants of the *H. pylori* virulence factor cytotoxin-associated gene A protein (CagA), encoded by the gene *cagA*. However, these variants have not been characterized in China, where gastric cancer is common. We investigated the diversity of CagA variants in *H. pylori* strains isolated from a Chinese population. The 3' variable region of *cagA* genes from 66 clinical isolates in China were amplified by polymerase chain reaction, sequenced, aligned, and analyzed. All 66 *H. pylori* strains were CagA-positive, of which 93.9% were East Asian type and the tyrosine phosphorylation motifs (TPMs) were EPIYA-ABD. The remainder was Western type, in which TPMs were EPIYA-ABC. Interestingly, two of sixty-two strains (3.2%) of the East Asian type were mutated into ESIYA-B, whereas all four Western type (100%) strains were mutated into EPIYT-B. Both of the two strains with Western-type CagA obtained from gastric cancer patients contained a distinguished mutation on the first residue following the EPIYA site in the EPIYA-A motif. The predominant CagA type in these *H. pylori* strains isolated from Chinese patients in China was East Asian, with TPMs EPIYA-ABD, and there existed mutations in both the East Asian and Western type CagA.

Keywords: *Helicobacter pylori*; CagA; 3' Variable Region; China; Gastritis; Gastric Cancer

1. Introduction

Helicobacter pylori, a spiral microaerophilic gram-negative bacterium, colonizes the stomachs of 50% or more people worldwide [1]. It is the causative agent of many peptic disorders, including gastritis, gastric and duodenal ulcers, and gastric mucosa-associated lymphoid tissue lymphoma. *H. pylori* infection is also a recognized risk factor for gastric adenocarcinoma [2,3]. However, not all people with *H. pylori* infections will suffer these diseases—about 30% of the population of Western countries is infected but the rate of gastric cancer is only 0.1% - 1%. In East Asian countries such as China, Japan, and Korea, the prevalence of *H. pylori* infection is 60% - 88%, and *H. pylori*-associated gastric cancers occur more frequently than in Western countries [4-6]. Many epidemiological studies have suggested that progression from *H. pylori* infection to adverse effects is controlled by combinations of genetic variants in the host, environmental factors, and *H. pylori* gene polymorphisms [7-9].

An *H. pylori* virulence marker found in most isolates in high-risk areas of the world is a 40-kb cytotoxin-associated gene (cag) pathogenicity island, which forms a type IV secretion system for delivering bacterial gene products into host gastric epithelial cells. One of these products is the oncoprotein CagA (encoded by the gene *cagA*), putatively responsible for most malignancies associated with the bacterium [10]. CagA has a variable C-terminus containing structural tyrosine phosphorylation motifs (TPMs) within repetitive amino acid sequences of Glu-Pro-Ile-Tyr-Ala (EPIYA) [11]. There are four known EPIYA motifs (EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D), differentiated by specific flanking amino acids [12,13].

Two types of CagA, Western and East Asian, have been described. Western CagA has EPIYA-A and EPIYA-B segments followed by (1 to 3) EPIYA-C segments. In the East Asian form, a EPIYA-D segment substitutes for the EPIYA-C segments of the Western CagA. When a CagA molecule is injected into the host cell, its tyrosines are phosphorylated and it binds to the N- and C-SH2

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domains of a SHP-2 tyrosine phosphatase of the host, initiating a cascade of abnormal enzymatic responses and changes in cellular phenotype. The greater binding affinity of East Asian CagA to SHP-2 makes East Asian CagA more virulent than the Western type; the degree of pathogenicity of Western CagA is associated with the number of EPIYA-C motifs [12-15].

Variations in the TPMs of the 3' region of CagA have been reported in Western countries, and these variations are associated with different peptic disorders. Yet to the best of our knowledge, there have been no systematic studies of CagA variants in China or their association with diseases, although China is representative of the East Asian area and gastric cancer is common [16], with incidence rates in Shanghai (the latest statistics available) reported at 34.2 and 17.3 per 100,000 men and women, respectively [17]. It is not known if the EPIYA motifs of CagA in Chinese *H. pylori* strains are East Asian, or whether TPM types in China are associated with gastric cancer.

In this study, we investigated the diversity of CagA found in *H. pylori* strains isolated from a Chinese population. We amplified the 3' region of *cagA* genes from 66 *H. pylori* clinical isolates, and then sequenced, aligned and compared the amino acid sequences of corresponding regions.

2. Materials and Methods

2.1. Patients

Gastric tissue biopsy samples from the antrum were obtained via endoscopy of 66 patients (46 men and 20 women; mean age 49.8 y, range 20 - 82 y) at three hospitals in Shandong, China. These patients were diagnosed with gastritis (n = 31), gastric ulcer (n = 11), duodenal ulcer (n = 6), or gastric cancer (n = 18), respectively.

2.2. Strain Culture and DNA Extraction

To examine the gastric biopsy specimens for the presence of *H. pylori*, the tissues were ground and then cultured on Campylobacter agar base plates containing 10% sheep blood and vancomycin (6 μg/mL), trimethoprim (5 μg/mL), polymyxin B (4 μg/mL), amphotericin B (2.5 μg/mL) under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 72 h. The bacteria were harvested and genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the instructions of the manufacturer.

2.3. Detection of CagA and EPIYA Motifs by PCR

The 3' variable region and TPMs of *cagA* were amplified by PCR using the primers shown in **Table 1**. All the primers were designed using "Primer Premier 5.0" soft-

Table 1. PCR primers and the expected size of PCR products.

Primer	Sequence (5'-3')	Size (bp)
cagF	CCTAGTCGGTAATGGGTTAT	
cagR[a]	TTAATGCGTGTGTGGCTG	659
cagA[a]	CTTGTCCTGTTTTCTTTTTATT	172
cagB[a]	GCATTTACCTTTTTAGCAACT	224
cagC[a]	CCGAGATCATCAATCGTAG	380
cagD[a]	TGCTTGATTTGCCTCATC	396

[a]With the forward primer cagF.

ware based on the homologous nucleotide sequence of *cagA* gene among strains ATCC26695 and F32, representative strains of Western and East Asian types, published on the PubMed. Primers cagF and cagR were used to amplify the full-length of the 3' variable region; primers cagA, cagB, cagC and cagD together with cagF were used to detect the EPIYA-A, -B, -C and -D motifs, respectively.

A reaction mixture contained 2.5 μL of each primer, 5 μL of genomic DNA, 25 μL 2 × Taq PCR MasterMix, and ddH2O to a total volume of 50 μL. PCR conditions consisted of pre-denaturation for 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. Five μL of each PCR product was then resolved via electrophoresis on a 1% agarose gel and observed under UV light after staining with ethidium bromide.

2.4. Alignment and Analysis of the Amino Acid Sequences

The PCR products of the cagF and cagR primers were sequenced by Shanghai Majorbio. Alignment and analysis of the sequences in the 3' variable region were carried out using DNAStar 5.0 software.

3. Results

The gene amplification and sequencing protocols were successful, and all the *H. pylori* strains detected in this study were *cagA*-positive (*cagA*[+]). Representative PCR products are shown in **Figures 1(a)** and **(b)**.

The prevalence of East Asian CagA (62/66, 93.9%,) was significantly higher than that of Western CagA (4/66, 6.1%; **Table 2**). Among the strains isolated from gastric cancer patients specifically, 88.9% (16/18) were East Asian CagA, and 11.1% (2/18) were Western CagA, while the strains from the remaining patients were 95.8% (46/48) East Asian and 4.2% (2/48) Western. All the TPMs in East Asian-type strains were EPIYA-ABD, while all the Western-type were EPIYA-ABC. No EPIYA-ABCC or -ABCCC TPMs were found. Interestingly, 3.2% of the East Asian-type strains (2/62, gastritis
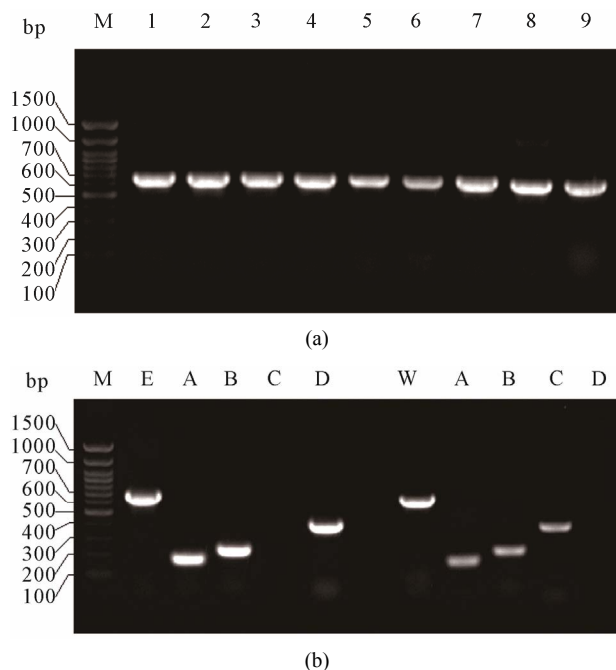
(a)

(b)

Figure 1. (a) Representative PCR products using the primers *cagF* and *cagR*. Lanes: M, molecular ladder (100 bp stepwise ladder; range 100 - 1500 bp); 1 to 6, 3' variable regions of East Asian-type strains; 7 to 9, 3' variable regions of Western-type strains; (b) Representative PCR products detected by multiple primer pairs. Lanes: M, molecular ladder (100 bp stepwise ladder; range 100 - 1500 bp); W: 3' variable region of Western CagA; E: 3' variable region of East Asian CagA; A: EPIYA-A motif amplified by primers cagF and cagA; B: EPIYA-B motif amplified by primers cagF and cagB; C: EPIYA-C motif amplified by primers cagF and cagC; D: EPIYA-D motif amplified by primers cagF and cagD.

Table 2. *H. pylori* CagA types detected in 66 isolates.

Group	East Asian	Western	Total
Gastric cancer	16	2	18
Gastritis	30	1	31
Gastric ulcer	11	0	11
Duodenal ulcer	5	1	6
Total	62	4	66

patients) were mutated into ESIYA-B, in which the P (proline) residue is replaced by S (serine), whereas all 4 of the Western-type strains (100%, 2 gastric cancers, 1 gastritis, and 1 duodenal ulcer) were mutated into EPIYT-B, in which the A (alanine) residue is replaced by T (threonine). Intriguingly, both of the gastric cancer patients with the Western CagA type harbored stains with a unique mutation, from K (lysine) to E (glutamate), on the first residue following the EPIYA site in the EPIYA-A motif (**Table 3**).

The alignment of the amino acid sequences in the 3'-variable region of CagA is shown in **Figure 2**.

Table 3. Diversity of TPMs in 3' variable region of CagA.

TPMs	East Asian	Western	Total
ABD	60	0	60
AB'D[*]	2	0	0
AB"C[*]	0	4	4
Total	62	4	66

[*]B' refers to ESIYA-B, in which the P residue is replaced by S; B" refers to EPIYT-B, in which the A residue is replaced by T.

4. Discussion

CagA is the most thoroughly studied virulence factor of *H. pylori*. Patients infected with CagA[+] strains of *H. pylori* have significantly increased levels of inflammation of gastric epithelial cells and gastritis severity [18-21]. The East Asian CagA is more virulent than the Western, because it has more affinity to bind SHP-2 [12,13,15]. The N- and C-SH2 domains of SHP-2 were highly related to its binding activity; the complement binding sequences for the two domains are pY-(S/T/A/V/I)-X-(V/I/L)-X-(W/F). Intriguingly, the consensus sequence of East Asian CagA, pY-A-T-I-D-F, perfectly matches the binding sequence, while in Western CagA the sequence is pY-A-T-I-D-D. Once the bacterium adheres to gastric epithelium, CagA is continually translocated into the host cells, establishing a sustained and deregulated stimulation to signal pathways, and finally leading to cell dysfunction and even carcinogenesis [22-24].

In the 66 gastric biopsy tissues obtained from Chinese patients in the present study, all contained *H. pylori* and all were CagA[+]. We found that the prevalence of East Asian CagA (93.9%, 62 of 66) was significantly higher than that of the Western type (6.1%, 4 of 66). We confirmed our sequence data by using multiple reverse primers to individually amplify EPIYA-A, -B, -C and -D motifs. Although China is an East Asian country, four Western CagA strains were found in our investigation. Taking the patients' age, gender and other demographic factors into account, we considered that this is probably own to that with the developing of the world economy, it was more fascinate and frequently for the exchange of the Eastern and Western countries, those patients who were diagnosed as Western type CagA might have been gone abroad and been infected with Western type *H. pylori*.

Yamazaki *et al*. [25] reported finding four types of mutations in the TPMs of the 3' variable region of CagA. Shunfu *et al*. [26] analyzed 539 *cagA* genes of 539 *H. pylori* isolates and found 14 mutations; the most common of which can be described as EPIYT-B and ESIYA-B. Even an EPTYA-B was reported by Reyes-Leon *et al*. [27]. According to the results of our present research, all the TPMs of the East Asian strains were EPIYA-ABD, including 2 strains mutated into ESIYA-B. Of the West-
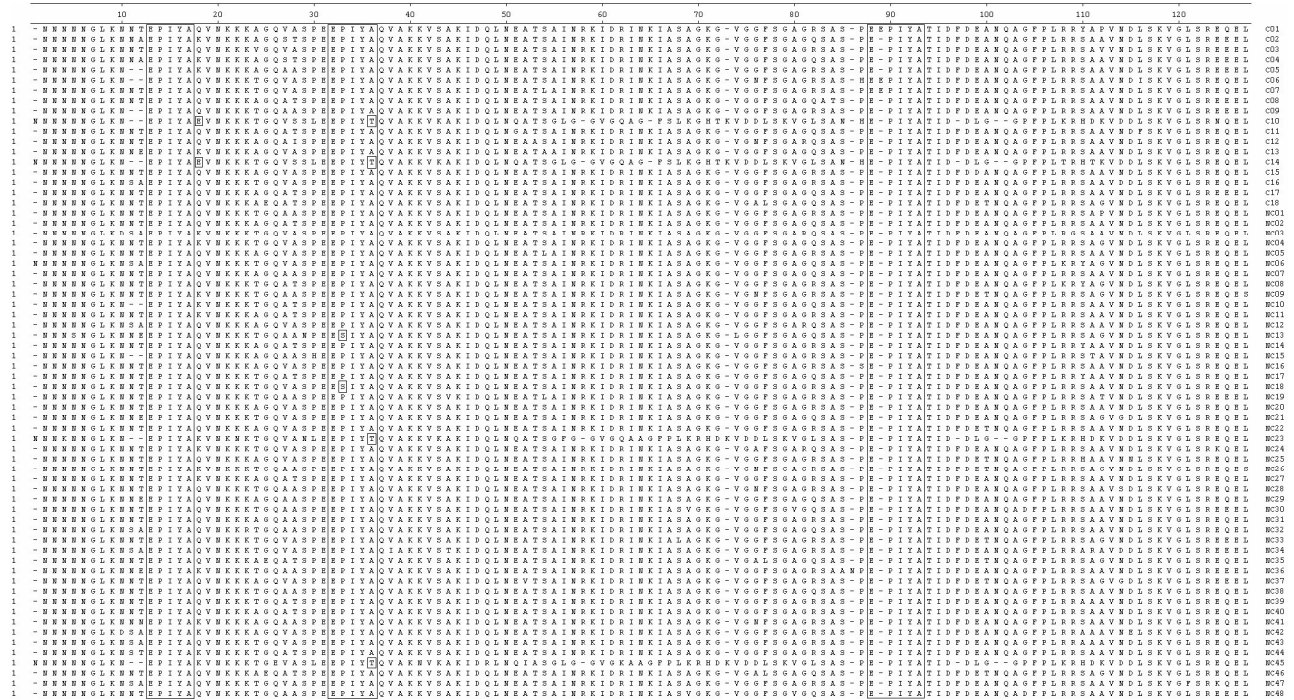
Figure 2. Alignment of the amino acid sequences in the 3' variable region of CagA.

ern CagA, all were EPIYA-ABC. No EPIYA-ABCC or EPIYA-ABCCC strains were found in our study. Interestingly, all 4 strains of the Western type were mutated into EPIYT-B.

In the present study, the amino acid sequence of the EPIYA-A motif, EPIYA (K/Q) VNKKK (A/T) GQ, was confirmed based on the classification defined by Higashi *et al*. [12] and Panayotopoulou *et al*. [28]. There were 2 Western-type strains isolated from the gastric cancer patients, and both contained a remarkable mutation, from lysine to glutamic acid, on the first residue following the EPIYA-A site. The isoelectric points of the basic amino acid lysine and the acidic glutamic acid are 9.74 and 3.22, respectively, and we speculate that differences in the isoelectric point values of the two residues cause a spatial configuration change in the mutated CagA protein molecule, which in turn strengthens the pathogenicity of Western-type strains. This is the first report of an increase in pathogenicity caused by a single amino acid change, and the mechanism of this phenomenon is still under investigation.

Of the 18 patients with gastric cancer in our study, 16 (88.9%) carried *H. pylori* with the East Asian CagA and the remaining 2 (11.1%) had the Western type. Those strains isolated from non-gastric cancer patients were 95.8% (46 of 48) East Asian CagA and 4.2% (2 of 48) were Western. The CagA type did not appear to influence clinical outcome in this study. Although many epidemiological studies have shown that CagA is associated with gastric cancer, these findings have been contra-

dicted by other researchers. Yamaoka *et al*. [29] reported no differences between the levels of CagA antibody in the serum of gastric cancer patients and the non-cancer group. Other studies in Japan [30-32] also suggested that there was no association between the presence of *H. pylori* and gastric cancer. However, it may be that the gastric mucosa of gastric cancer patients could no longer support colonization by *H. pylori,* and while the bacterium may have had a causative role it was no longer detectable. What is more, if the genes which encoded *cag*-PAI were mutated or the type IV secretion system was disabled, the protein would not be translocated into host cells to achieve its biological function even the strains were CagA[+]. Most important of all, gastric cancer is a process that is not only influenced by polygenic variations of the pathogen, but also factors associated with the host and environment.

Strain diversity among *H. pylori* isolates has been studied at the gene level. Studies have shown that different clinical outcomes could be linked with differences in *H. pylori* virulence factors, which play essential roles in pathogenesis [33,34]. Another study of Chinese, Indian, and Malay patients showed that the gene structure of *H. pylori* was related to the isolate's geographic location [35]. Studies of the relatedness of geography, disease, and *H. pylori* polymorphisms will contribute to the understanding of strain-host interaction and the pathogenicity level of each strain.

In summary, in this study conducted in China, we detected strains of *H. pylori* in all the gastric biopsy sam-

ples, all were CagA positive, and the prevalence of the East Asian CagA was considerably higher than that of the Western CagA. There was not sufficient evidence to indicate that the type of TPM was related to different clinical outcomes. EPIYA-ABD and EPIYA-ABC were the most common types found, and mutations existed in both the East Asian- and Western-type CagA.

5. Acknowledgements

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