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Distribution of *Helicobacter pylori* virulence markers in patients with gastroduodenal diseases in a region at high risk of gastric cancer

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ABSTRACT

Abstract background: Helicobacter pylori (H. pylori) is a major human pathogen that is responsible for various gastroduodenal diseases. We investigated the prevalence of *H. pylori* virulence markers in a region at high risk of gastric cancer.

Methods: One hundred and sixteen *H. pylori* strains were isolated from patients with gastroduodenal diseases. *cagA*, the *cagA* 3′ variable region, *cag*PAI genes, *vacA*, and *dupA* genotypes were determined by PCR, and some amplicons of the *cagA* 3′ variable region, *cag*PAI genes and *dupA* were sequenced.

Results: cagA was detected in all strains. The *cagA* 3′ variable region of 85 strains (73.3%) was amplified, and the sequences of 24 strains were obtained including 22 strains possessing the East Asian-type. The partial *cag*PAI presented at a higher frequency in chronic gastritis (44.4%) than that of the severe clinical outcomes (9.7%, $p < 0.001$). The most prevalent *vacA* genotypes were s1a/m2 (48.3%) and s1c/m2 (13.8%). Thirty-six strains (31.0%) possessed *dupA* and sequencing of *dupA* revealed an ORF of 2449-bp. The prevalence of *dupA* was significantly higher in strains from patients with the severe clinical outcomes (40.3%) than that from chronic gastritis (20.4%, $p = 0.02$).

Conclusion: The high rate of East Asian-type *cagA*, intact *cag*PAI, virulent *vacA* genotypes, and the intact long-type *dupA* may underlie the high risk of gastric cancer in the region.

1. Introduction

Helicobacter pylori (*H. pylori*) colonizes the surface area of the gastric mucosa in the human stomach, and is associated with an increased risk for diseases ranging from gastritis to peptic ulcer and gastric cancer. Although the mechanisms resulting in the disease development are poorly understood, a number of virulence factors in *H. pylori* have been identified and suggested to be involved in the pathogenesis of the diseases, including the *cag* pathogenicity island (*cag*PAI), the vacuolating cytotoxin (*vacA*), and the duodenal ulcer promoting gene A (*dupA*) [1,2].

The *H. pylori cag*PAI encodes components of a type IV secretion system (T4SS), which is used for the translocation of CagA protein into the gastric epithelium cells, and is clearly associated with an enhanced risk of developing gastritis, peptic ulcer and gastric cancer [3]. Notably the presence of intact *cag*PAI strains was found more frequently in patients with severe gastroduodenal disease, and partial deletions of the *cag*PAI appear to be sufficient to render the organism less pathogenic [4,5]. According to the sequence located in the 3′ region, *cagA* could be mainly classified into 2 types (East Asian-type and Western-type). It is interesting to note that both *in vitro and in vivo* (animal and human) data clearly show that East Asian-type CagA protein is more carcinogenic than Western-type CagA protein [6–8]. Virtually all *H. pylori* strains have a copy of *vacA*, which has been found to be associated with distinct gastrointestinal disorders [9]. The *vacA* gene contains at least two variable parts: the signal (s) region that is present as type s1 (subtype a, b and c) or type s2, and the middle (m) region occurs as the m1 or m2 allelic type. In general, type s1/m1 and s1/m2 strains produce high and moderate levels of toxin, respectively, whereas s2/m2 strains produce little or no toxin [10]. *dupA* located in the plasticity region of *H. pylori*, is typical of other virulence factor, such as *cagA* [11,12]. It has been supposed that *dupA* is a component of a new cluster of *vir* homolog genes that might form a type IV secretion system with other genes to induce the gastric epithelial cells to secrete IL-8, similarly to *cag*PAI [13,14].

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Although the high risk of gastric cancer was reported in a littoral region of Northeast China [15], there is currently no report explaining the mechanisms resulting in the phenomenon. In this study, we selected *H. pylori* strains to analyze the virulence markers status, and examine the association between virulence markers and severe clinical outcomes in the region. Furthermore, the *cag*PAI has a variable genetic structure [16], and *dupA* is commonly polymorphic [11,17]. We analyzed the sequence of *cag*PAI genes (*cagI, cagL, cagM, cagT, cagX)* and *dupA* in some *H. pylori* strains to consider whether their genetic structures are conserved in the region.

2. Materials and methods

2.1. Patients and sampling

Three hundred and eighty-six patients had abdominal symptoms and were clinically examined at the Department of Gastroenterology and further underwent upper gastrointestinal endoscopy in Weihai Municipal Hospital affiliated to Dalian Medical University from June 2010 to June 2013. None of the patients had recently been prescribed antibiotics and received nonsteroidal anti-inflammatory drugs. A total of six biopsies were obtained from each patient during the endoscopic procedure: two biopsies from the greater curvature of the gastric antrum, two from the greater curvature of the gastric fundic mucosa, and two from the margin of the lesion. One specimen each from the antral, fundic mucosae and the margin of the lesion was subjected to histological analysis. The other specimens were subjected to culture for *H. pylori*. Gastritis was defined as histological gastritis without peptic ulcer diseases or gastric malignancy, or erosive esophagitis. Peptic ulcers were identified endoscopically and histologically, and Gastric cancers were confirmed histologically.

Written and informed consent was obtained from all patients, and the study was conducted upon approval by the Ethical Committee of Weihai Municipal Hospital affiliated to Dalian Medical University.

2.2. Histological analysis

Biopsy specimens were stained with hematoxylin-eosin for histopathology. After the specimens were evaluated by an experienced histopathologist, the histological slides were then examined by an independent expert, who had no information on the patients, for confirmation of the diagnosis.

2.3. H. pylori cultivation and identification

After biopsies were taken, samples were collected in brain heart infusion broth (Oxoid, United Kingdom), and dispersed using a sterile tissue homogenizer within 2 h of collection. Every homogenate was inoculated onto Campylobacter agar (Oxoid, United Kingdom) with 8% sheep blood and *H. pylor*i selective supplement (Oxoid, United Kingdom) in microaerophilic condition (5% O_2, 10% CO_2 and 85% N_2) at 37 °C for 72 h. Small dew drop colonies were selected for the identification of *H. pylori* with the phenotypic characteristics and PCR based on 16SrRNA gene sequence as described previously [18].

Isolates with curved gram-negative rods, positive in all the three enzymes activity tests and 16SrRNA gene sequences having more 98% homology with *H. pylori* were identified as *H. pylori*. After bacteria were harvested, genomic DNA was isolated by using bacterial genomic DNA extraction kit (DV810A, Takara), and stored at −20 °C until PCR analysis and nucleotide sequencing.

2.4. Analysis of cagA status and 3′ variable region of cagA

PCR analyses were carried out to determine the presence or absence of *cagA* and to detect *cagA* 3′ variable region of *H. pylori* strains as described previously [19,20]. Primer sequences used in this study were listed in Table 1. After PCR, the amplified PCR products were electrophoresed in 2% agarose gels and examined under UV illumination. Some amplicons of *cagA* 3′ variable region were sequenced by Life Technologies Corporation using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.5. cagPAI genes PCR analysis and sequencing of cagPAI genes (cagI, cagL, cagM, cagT, cagX)

PCR analyses were carried out to amplify six different loci spread over the *cag*Iregion (*cagE, cagI, cagL, cagM*) and *cag*II region (*cagT, cagX*) to estimate whether the *cag*PAI was intact and likely to be functional *in vivo* using eight oligonucleotide pairs shown in Table 1 [21,22]. PCR tests were performed thrice, while *H. pylor*i NCTC 11637 was served as positive control and sterile distilled water was used as negative control. Where more than one primer pair was utilized, isolates positive for one primer pair were considered to be positive for that gene. In the present study, the *cag*PAI was classified as intact, partially or completely deleted according to the presence of the *cagA, cagE, cagI, cagL, cagM, cagT* and *cagX*.

Some amplicons of *cagI, cagL, cagM, cagT and cagX* were sequenced by Life Technologies Corporation using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The full-length amino acid sequences of each gene were constructed and translated from the nucleotide sequences using Primer 5.0 (Premier Biosoft International).

2.6. Detection of vacA genetype diversity

Genotyping of the *vacA* was performed by PCR amplification in the s-region and the m-region using the primers (Table 1) [23,24]. The amplified products were electrophoresed in 2% agarose gels and examined under UV illumination.

2.7. dupA status analysis and sequencing

Two sets of primers were employed for partial amplification of *jph0917* and *jhp0918* to screen for the presence of *dupA* as previously described [25]. Another set of primers (Table 1) was utilized to amplify the entire *dupA* for sequencing. Some amplicons of the entire *dupA* were sequenced and compared with deposited sequences in NCBI.

2.8. Statistical analysis

The statistical calculation was performed using SPSS version 13.0 (SPSS, Chicago, IL, USA). Chi-square test was used to check for presence of partial *cag*PAI and *dupA* between different clinical outcome groups. p value less than 0.05 was considered significant.

3. Results

H. pylori strains were isolated from 116 patients (30.1%, 116/386) with gastroduodenal diseases in this study. Diseases ($n = 116$) were diagnosed by pathological manifestations, including chronic gastritis (CG, $n = 54$), gastric ulcer (GU, $n = 25$), duodenal ulcer (DU, $n = 26$), and gastric cancer (GC, $n = 11$). A summary of the prevalence of individual virulence factors was presented in Table 2, including *cag*PAI, *vacA* and *dupA*.