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Helicobacter pylori with East Asian-type *cagPAI* genes is more virulent than strains with Western-type in some *cagPAI* genes



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ABSTRACT

The severity of *Helicobacter pylori*-related disease is correlated with the presence and integrity of a *cag* pathogenicity island (*cagPAI*). *cagPAI* genotype may have a modifying effect on the pathogenic potential of the infecting strain. After analyzing the sequences of *cagPAI* genes, some strains with the East Asian-type *cagPAI* genes were selected for further analysis to examine the association between the diversity of the *cagPAI* genes and the virulence of *H. pylori*. The results showed that gastric mucosal inflammatory cell infiltration was significantly higher in patients with East Asian-type *cagPAI* genes *H. pylori* strain compared with mosaicism *cagPAI* genes *H. pylori* strain ($p < 0.05$). *H. pylori* strains with the East Asian-type *cagPAI* genes were closely associated with IL-8 secretion *in vitro* and *in vivo* compared with *H. pylori* strains with the mosaicism *cagPAI* genes ($p < 0.01$). *H. pylori* strains with East Asian-type *cagPAI* genes are able to strongly translocate CagA to host cells. These results suggest that *H. pylori* strains with East Asian-type *cagPAI* genes are more virulent than the strains of *cagPAI* gene/genes that are Western type.

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium and is the main pathogenic factor of gastroduodenal diseases. *H. pylori* contains many virulence factors, including the *cag* pathogenicity island (*cagPAI*), that are involved in the pathogenesis of several diseases.^{1,2} *H. pylori* *cagPAI* consists of approximately 30 genes encoding for proteins of the type IV secretion system (T4SS), which transfers CagA and peptidoglycan into host epithelial cells, resulting in increased cellular

release of interleukin 8 (IL-8).^{3,4} Studies have also demonstrated that *H. pylori* strains with a functional T4SS are more frequently associated with the etiology of *H. pylori*.^{5,6}

H. pylori is a highly genetically diverse species that is attributed to its high rate of DNA recombination.^{7–9} It has been reported in Japan that the genetic diversity within the *cagPAI* may have modifying effects on the pathogenic potential of infecting strains.¹⁰ The similarity coefficient revealed that all *cagPAI* genes could be placed into two major groups, East Asian-type or Western-type. Most of *cagPAI* genes belong to the East Asian-type and is found associated with high risk

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of developing gastric cancer in some regions. Noticeably, in some regions, some strains of the Western-type of several *cagPAI* gene/genes are of East Asian-type of other *cagPAI* genes, potentially due to the mosaicism of *cagPAI* genes in *H. pylori* and genetic recombination.¹¹

Much work has been reported on the genetic diversity of *cagPAI*, particularly on the genotype of *cagA*.^{12,13} Minimal research has been conducted in applying *cagPAI* genotype analysis to evaluate the virulence of *H. pylori*. In some *H. pylori* strains, the two parts of the *cagPAI* genes are interposed by a segment called insertion sequence 605 (IS605): the upstream as *cagII* region, and the downstream as *cag I* region. In this study, some *cag PAI* genes in *cag II* (*cagT*, *cagX*) and *cagI* region (*cagI*, *cagL*, *cagM*) were selected for sequencing. The virulence of the strains with East Asian-type *cagPAI* genes was evaluated by comparing strains with mosaic *cagPAI* genes.

Materials and methods

Patients and sampling

Patients with abdominal symptoms were clinically examined, and biopsy samples were taken for *H. pylori* isolation and histological analysis in Weihai Municipal Hospital (affiliated with Dalian Medical University) from June 2010 to June 2014. None of the patients underwent antimicrobial therapy, or have taken proton pump inhibitors (PPIs) or non-steroidal anti-inflammatory drugs a month before their inclusion in the study. Written and informed consent was obtained from all patients, and the study was conducted upon approval by the *ad hoc* Ethical Committee of Weihai Municipal Hospital.

H. pylori cultivation and identification

Biopsy specimens were collected in brain heart infusion broth (Oxoid, United Kingdom), and dispersed by using the tissue homogenizer. Every homogenate was inoculated onto Campylobacter agar (Oxoid, United Kingdom) with 8% sheep blood and *H. pylori* selective supplement (Oxoid, United Kingdom) under microaerophilic condition (5% O₂, 10% CO₂ and 85% N₂) at 37 °C for 72 h. Small dew drop colonies of *H. pylori* were

selected for further phenotypic analysis by PCR of the 16SrRNA gene sequence as previously described.¹¹

Sequence analysis of *cagPAI* genes

The presence of five *cagPAI* genes, which spread over the *cag I* (*cagI*, *cagL*, *cagM*) and *cag II* regions (*cagT*, *cagX*) in *H. pylori* strains, was analyzed by PCR using five sets of primers listed in Table 1. The amplicons were selected for sequence analysis by Life Technologies Corporation. After the full-length amino acid sequences of each gene were translated from the nucleotide sequences by Primer 5.0, the phylogenetic trees of *cagPAI* proteins were constructed by the neighbor-joining method of Saitou and Nei by using a program called MEGA. All *cagPAI* genes in the isolates could be placed into two major groups. Most *H. pylori* strains from the region are either East Asian-type or Western-type. Three previously reported Western strains have been identified so far: 26695 (UK), J99 (USA), and NCTC11637 (Australia).

In the present study, the strains were classified based on the genotypes of *cagPAI* genes: East Asian-type *cagPAI* genes group (all five *cagPAI* genes), and mosaicism *cagPAI* genes group (less than five *cagPAI* genes identified as East Asian-type, and containing more than one Western-type *cagPAI* gene except *cagA*).

Analysis of *cagA* status and 3' variable region of *cagA*

PCR analyses were performed to analyze *cagA* status and *cagA* 3' variable region using specific primers (Table 1). After PCR, the amplified PCR products were electrophoresed in 2% agarose gels and examined under UV illumination. The amplicons of *cagA* 3' variable region were sequenced by Life Technologies Corporation.

Histological analysis

Stomach biopsy specimens from each patient were examined by an experienced pathologist. For each biopsy specimen, the grades of inflammatory cell infiltration, gastric mucosal atrophy, and *H. pylori* density were scored on the basis of

Table 1 – Polymerase chain reaction (PCR) primer pairs used for *cagPAI* genes in *Helicobacter pylori* isolates.

Region	Primer	Sequence	Amplicon size (bp)
<i>cagI</i>	f:	ATAGAATTCACAGAAGTAGTAATAACGCTTGAAC	1086
	r:	AGACTCGAGTTTGACAATAACTTTAGAGCTAG	
<i>cagL</i>	f:	GATGGATCCGAAGATATAACAAGCGGTTT	654
	r:	GCCCTCGAGTTTAAACAATGATCTTACTTGA	
<i>cagM</i>	f:	TAGGGATCCGAGCAGTTTGGTTCATTT	1149
	r:	CGCCTCGAGCTATTCAAAGGGATTATTC	
<i>cagT</i>	f:	TCCGATCCATGAAAGTGAGAGCAAGTGT	843
	r:	GCCAAGCTTTCACCTACCCTGAGCAAAC	
<i>cagX</i>	f:	GGAATTCATGGGGCAGGCATTTTTTA	1569
	r:	GGTTCGACTTATTATCTCTGACAAGAGGGAG	
<i>cagA</i> conservative region	f:	GATAGGGATAACAGGCAAGC	297
	r:	GGGGTTGTATGATATTTTC	
<i>cagA</i> 3' variable region	<i>cag2</i>	GGAACCCTAGTCGGTAATG	Uncertain

Table 2 – Histological differences among the two *cagPAI* groups.

Parameter	East Asian-type <i>cagPAI</i> genes group (n = 42)	Mosaicism <i>cagPAI</i> genes group (n = 26)
<i>H. pylori</i> density	1.52 ± 0.41	1.63 ± 0.52
Inflammatory cell infiltration	1.71 ± 0.68 ^a	1.03 ± 0.42
Atrophy	0.51 ± 0.17	0.47 ± 0.16

^a *p* < 0.05.

the updated Sydney System (0, none; 1, mild; 2, moderate; 3, severe).¹⁴

Mucosal IL-8 level analysis in biopsy samples

IL-8 levels in the biopsy specimens were measured after homogenization using an enzyme-linked immunosorbent assay (ELISA) (Invitrogen). Briefly, the supernatants from homogenized specimens were obtained by centrifugation (10,000 × *g* for 15 min), and the total proteins of the supernatants were measured by using the Bradford assay (Bio-Rad, Richmond, CA). IL-8 levels in samples were expressed as pg/mg of protein.

Determination of IL-8 secretion from GES-1 cells co-cultured with *H. pylori* strains

The strains were selected and co-cultured with GES-1 cells for analysis of IL-8 secretion. Briefly, GES-1 cells were seeded at a density of 8 × 10⁴ cells/well in 96-well plates. *H. pylori* was harvested from agar dishes and washed twice with PBS before being added to culture wells at a MOI of 100. The cell culture media were collected at 24 h. IL-8 levels were detected by ELISA (Invitrogen) according to the manufacturer's protocol. The experiments were performed twice independently.

CagA Translocation Assay

GES-1 cells infected with *H. pylori* (1:100) as described above were washed with PBS until no *H. pylori* were found adhered to cells as observed under a microscope. Cells were then scraped and centrifuged at 4000 rpm for 30 min. Cells were suspended in RIPA Lysis Buffer on ice for 30 min. After centrifuging at 13,500 rpm for 30 min, the supernatant was collected to analyze CagA by Western blotting. Colony-forming units (CFU) assay was performed to confirm that no *H. pylori* were detected on scraped cells.

Statistical analysis

Differences between the Asian-type strains group and the mosaic-type strains group were analyzed using Student's *t* test and Chi-square test. *p* values less than 0.05 was considered significant.

Results

After sequence analysis of *cagPAI* genes (Fig. 1), 76 strains with East Asian-type *cagPAI* genes were isolated from gastroduodenal diseases, including chronic gastritis (CG, *n* = 29), gastric

ulcer (GU, *n* = 21), duodenal ulcer (DU, *n* = 19), and gastric cancer (GC, *n* = 7). Twenty-six strains with mosaicism *cagPAI* genes were isolated from chronic gastritis (CG, *n* = 14), gastric ulcer (GU, *n* = 6), duodenal ulcer (DU, *n* = 4), and gastric cancer (GC, *n* = 2). Isolates with East Asian-type *cagPAI* genes were found at lower frequency in CG (38.2%, 29/76) than those with mosaicism of *cagPAI* genes in CG (53.8%, 14/26, *p* < 0.01).

Detection of *cagA* status and sequencing of the *cagA* 3' variable region

PCR products were obtained from all isolates of *H. pylori*. After nucleotide analysis, *cagA* 3' variable region were grouped as East Asian-type or Western-type. The East Asian-type (521 bp) possessed three EPIYA motifs, while the Western-type (502 bp) possessed two EPIYA motifs and one EPIYT (Fig. 2). All 76 strains with East Asian-type *cagPAI* genes harbored East Asian-type, and only 2 strains possessed Western-type in 26 strains with mosaicism *cagPAI* genes.

Histological analysis of *H. pylori* density, inflammatory cell infiltration, and atrophy in the biopsy specimens

Further histopathologic evaluations of biopsy specimens were performed on 42 patients with East Asian-type *cagPAI* genes *H. pylori* strains and 26 patients with mosaicism *cagPAI* genes *H. pylori* strains. The biopsy specimens of East Asian-type *cagPAI* genes group were selected randomly for histological analysis.

Gastric mucosal inflammatory cell infiltration was significantly higher in East Asian-type *cagPAI* genes group than mosaicism *cagPAI* genes group (*p* < 0.05) (Fig. 3). However, there were no significant differences in the grade of *H. pylori* density and gastric mucosal atrophy according to the diversity of *cagPAI* genes (Table 2). This suggested that the status of *cagPAI* genotype is associated with progression of gastric mucosal inflammatory cell infiltration.

IL-8 production in gastric mucosa (in vivo) and from GES-1 cells (in vitro)

We randomly selected 36 patients with East Asian-type *cagPAI* genes *H. pylori* strains and 26 patients with mosaicism *cagPAI* genes *H. pylori* strains to measure gastric mucosal IL-8 levels. The gastric mucosal IL-8 level in patients with East Asian-type *cagPAI* genes *H. pylori* strains was significantly higher (112.7 ± 33.1 pg/mg) than those containing mosaicism *cagPAI* genes *H. pylori* strains (75.8 ± 24.7 pg/mg, *p* < 0.01).

IL-8 production in GES-1 cells that were co-cultured with 20 *H. pylori* strains was also examined. These

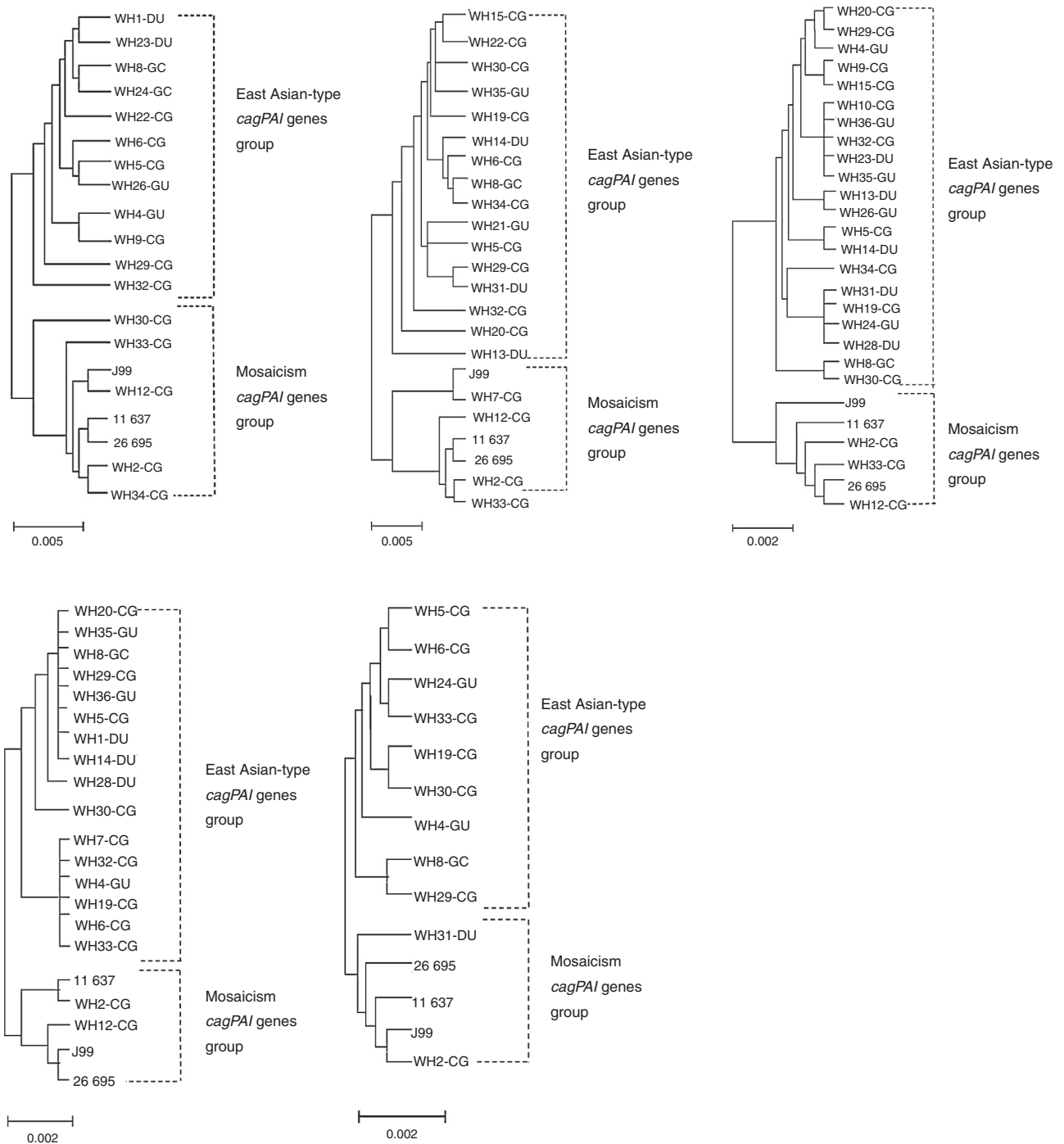


Fig. 1 – Phylogenetic tree of the complete amino acid sequences of *cagI*, *cagL*, *cagT*, *cagM* and *cagX* in some strains. Scale bars represent calculated distances. All three proteins were divided into two major groups: East Asian-group or Western-group.

included 10 strains with East Asian-type *cagPAI* genes and 10 strains with mosaicism *cagPAI* genes. IL-8 production (1423.9 ± 195.3 pg/mL) was significantly higher in strains with East Asian-type *cagPAI* genes than in those with mosaicism *cagPAI* genes (1113.9 ± 103.6 pg/mL, $p < 0.01$) (Table 3). These results suggested that the *cagPAI* genotype of *H. pylori* strain is important for inducing IL-8 production *in vivo* and *in vitro*.

CagA delivery to host cells

Ten *H. pylori* strains with East Asian-type *cagPAI* genes and 10 strains with mosaicism *cagPAI* genes were randomly selected for further analysis of CagA translocation. After infection of GES-1 cells by *H. pylori*, cells with translocated CagA were found more increased in East Asian-type *cagPAI* genes group than mosaicism *cagPAI* genes group. These results indicated

11637 FSDIKKELNEFKNFNNNNNNGLN----EPIYAKVNKKKTGQVASPEEPIYA
WH2 (CG) FSDIKKELNEFKNFNNNNNNGLKN----EPIYAKVNKKKTGQVASLEEPIYT
WH8 (GC) FSDIRKELNEFKNFNNNNNNGLKN----EPIYAKVNKKKTGQVASLEEPIYT
WH1 (CG) FSDIRKELNEKLF GNSNNNNNGLKNNTEPIYAQVNKKKTGQVASPEEPIYA
WH4 (GU) FSDIRKELNEKLF GNSNNNNNGLKNNTEPIYAQVNKKKVGQATSPEEPIYA
WH13 (DU) FSDIRKELNEKLF GNSNNNNNGLKNNTEPIYAQVNKKKAGQATSPEEPIYA

11637 QVAKKVNADIRLNQAASGLGGVQAGFPLKRHDKVDDLKSVGRSVSPEPIYA
WH2 (CG) QVAKKVNADIRLNQIASGLGDVGQAAGFPLKRHDKVDDLKSVGLSASPEPIYA
WH8 (GC) QVAKKVKAKIDRLDQIASGLGGVQAGFPLKRHDKVDDLKSVVLSAGPEPIYA
WH1 (CG) QVAKKVSADKIDQLNEATSAINRKIDRIN----KIASAGKGVGGFSGAGRSASPEPIYA
WH4 (GU) QVAKKVSADKIDQLNEATSAINRKIDRIN----KIASAGKGVGGFSGVGRSASPEPIYA
WH13 (DU) QVAKKVSADKIDQLNEATSAINRKIDRIN----KIASAGKGVGGFSGVGRSASPEPIYA

11637 TIDDLGGPFPLKRHDKVDDLKSVGRSVSPEPIYATIDDLGGPFPLKRHDKVDDLKSV
WH2 (CG) TIDDLGGPFPLKRHDKVDDLKSVGLSREQLKQKIDNLNQAVSEAK
WH8 (GC) TIDELGGPFPLKRHDKVDDLKSVGLSREQLKQKIDNLNQAVSEAK
WH1 (CG) TIDFDEANQAGFPLRRSAAVNDLSKVGLSREQLTRRIGDLNQAVSEAK
WH4 (GU) TIDFDEANQAGFPLRRSAAVNDLSKVGLSREQLTRIGDLNQAVSEAK
WH13 (DU) TIDFDEANQAGFPLRRSAAVNDLSKVGLSREQLTRRIGDLNQAVSEAK

11637 GRSVSPEPIYATIDDLGGPFPLKRHDKVDDLKSV

Fig. 2 – Deduced amino acid sequence of *cagA* 3' variable region from some strains.

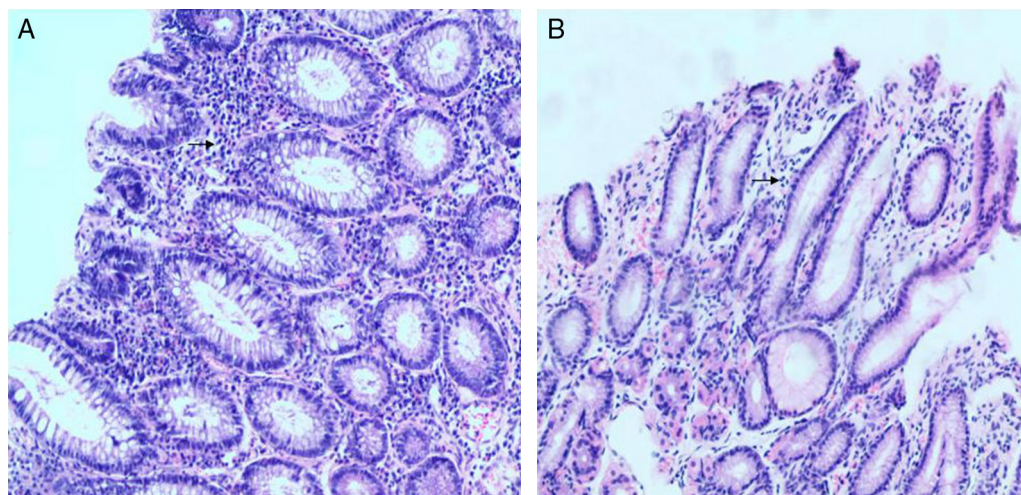


Fig. 3 – Histopathologic evolutions of biopsy specimens in two groups according to H&E stain (100×). (A) East Asian-type *cagPAI* genes group; (B) mosaicism *cagPAI* genes. Infiltration of inflammatory cells (arrows).

Table 3 – IL-8 levels in gastric mucosa (*in vivo*) and GES-1 cells (*in vitro*).

	East Asian-type <i>cagPAI</i> genes group	Mosaicism <i>cagPAI</i> genes group
Gastric mucosa (pg/mg)	112.7 ± 33.1 (n = 36) ^a	75.8 ± 24.7 (n = 26)
GES-1 cells (pg/mL)	1423.9 ± 195.3 (n = 10) ^a	1113.9 ± 103.6 (n = 10)

^a p < 0.01.

that the translocation of CagA to host cells was less effective in strains with mosaicism *cagPAI* genes (Fig. 4).

Discussion

H. pylori cagPAI is a major virulence determinant in *H. pylori*-related diseases. In comparison with *cagPAI*-negative strains, infection with *cagPAI*-positive *H. pylori* strains significantly increases the risk of developing severe gastric mucosal inflammation, duodenal ulcers, and gastric cancers.^{15,16} The *cagA*

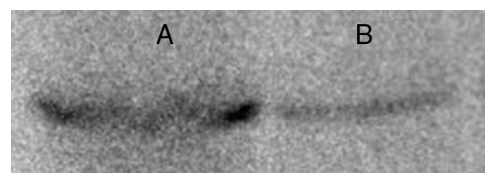


Fig. 4 – CagA translocation between two groups. (A) East Asian-type *cagPAI* genes group; (B) mosaicism *cagPAI* genes group.

gene is part of *cagPAI*, and CagA is the primary virulence factor of *H. pylori* that sufficiently induces tumorigenesis in cell and animal models. CagA has also been reported to be epidemiologically associated with higher risk of developing gastric cancer.^{17,18} Based on the sequence of the 3' region, *cagA* was classified into 2 types: East Asian-type and Western-type. Intriguingly, both *in vitro* and *in vivo* studies have clearly showed that East Asian-type CagA protein is more carcinogenic than Western-type CagA protein.¹⁹ In this region, most strains possess East Asian-type of *cagA* with three EPIYA motifs.

In a littoral region of Northeast China, nearly 100% of the strains possess *cagA*, and most isolates of *cagA* were included in East Asian-type.²⁰ Noticeably, a high incidence of atrophic gastritis and gastric cancer has also been reported in this region.²¹ Early studies showed that the distinct distribution of *cag PAI* diversity in this region may be involved in the development of atrophic gastritis, potentially increasing the risk of worsened outcome in different diseases based on phylogenetic analysis of *cagPAI* genes.¹¹ We hypothesize that the T4SS encoded by Eastern-type *cagPAI* genes in the region results in the ability of *H. pylori* to increase the risk of developing gastric cancer.

In the study, isolates with East Asian-type *cagPAI* genes were found at lower frequency in CG, compared to strains with mosaicism *cagPAI* genes in CG. Two *cagPAI* genotypes of *H. pylori* strains were selected based on the phylogenetic analysis of full-length CagPAI proteins, including East Asian-type *cagPAI* and mosaicism *cagPAI* genes strains. Some strains were selected to study their virulence, and to examine whether there is an association between the diversity of the *cagPAI* genes and the virulence of *H. pylori*. We found that gastric mucosal inflammatory cell infiltration was significantly higher in patients with East Asian-type *cagPAI* genes *H. pylori* than others with mosaicism *cagPAI* genes strains. These observations suggest that *cagPAI* genotype is associated with progression of gastric mucosal inflammatory cell infiltration. Meanwhile, we also found that *H. pylori* strains with the East Asian-type *cagPAI* genes are closely associated with IL-8 secretion compared with *H. pylori* strains with the mosaicism *cagPAI* genes. Intriguingly, *H. pylori* strains with the East Asian-type *cagPAI* genes can easily translocate CagA to host cells.

H. pylori exhibits extensive genetic diversity and rapid allelic diversification attributed to its high mutation rate and frequent recombination in different diseases.^{22–24} We believe that the mosaicism in *cagPAI* genes is best explained by genetic recombination. Thus, the genetic recombination in *cagPAI* between the west strain and East strain could increase the diversification and virulence of *H. pylori*.

In conclusion, *H. pylori* with the East Asian-type *cagPAI* genes is associated with gastric mucosal inflammatory cell infiltration and IL-8 secretion, which seems to be more virulent compared with other strains.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article.

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