# RESEARCH ARTICLE

# *Helicobacter Pylori* CagA and Gastric Carcinogenesis

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## Abstract

 <u>Objectives</u>: This study aimed to demonstrate the tyrosine phosphorylation motif (TPM) and 3' region structure of the *Helicobacter pylori* CagA gene as well as its SHP-2 binding activity in AGS cells and relation to gastric carcinogenesis. <u>Methods</u>: Sixteen clinical isolate *H. pylori* strains from eight duodenal ulcer and eight gastric adenocarcinoma patients were studied for CagA repeat sequence EPIYA motifs, C-terminal structure, and western blot analysis of CagA protein expression, translocation, and SHP-2 binding in AGS cells. <u>Results</u>: Except for strain 547, all strains from the gastric adenocarcinoma patients were positive for CagA by PCR and had three EPIYA copy motifs. Western blotting showed that all strains were positive for CagA protein expression (100%), CagA protein translocation (100%), and SHP-2 binding (100%). CagA protein expression was significantly higher in the gastric adenocarcinoma patients than in the duodenal ulcer patients (P=0.0023). CagA protein translocation and SHP-2 binding in the gastric adenocarcinoma patients were higher than those in the duodenal ulcer patients, but no significant differences were found between the two groups (P=0.59, P=0.21, respectively). <u>Conclusions</u>: The TPMs and 3' region structures of the *H. pylori* CagA gene in the duodenal ulcer and gastric adenocarcinoma patients have no significant differences.

## Introduction

Helicobacter pylori colonises the stomachs of 50% of the world's population and is associated with the development of gastroduodenal diseases, such as peptic ulcer disease, atrophic gastritis, and distal gastric adenocarcinoma and lymphoma. Although *H. pylori* infection always results in histological gastritis, most people harbouring *H. pylori* are asymptomatic, and only the minority of infected subjects develop an associated clinical disease. Approximately 74% of *H. pylori* strains contain the CagA gene in Western countries (Apostolopoulos et al., 2002). However, most Japanese *H. pylori* strains are positive for CagA. The prevalence of both gastric cancer and atrophic gastritis is extremely high in Japan (Graham and Asaka, 2010). Murakita et al. reported that tox+ *H. pylori* isolates are more prevalent in patients with severe atrophic gastritis and that the cytotoxic activities in *H. pylori* isolates from patients with severe atrophic gastritis are much higher than those from patients with mild atrophic gastritis in Japan (Murakita et al., 1996). The lineage of *H. pylori* isolates infecting Japanese subjects may be different from that of isolates in other parts of the world, and a specific strain may have accumulated in the Japanese population. *H. pylori* strains possessing the CagA gene were linked with an increased risk of developing gastric cancer and peptic ulcer (Cavalcante et al., 2012; Epplein et al., 2012). The risk of developing gastric cancer in

*H. pylori* -infected CagA-positive subjects is sixfold higher than that in CagA-negative subjects (Yamaoka et al., 2002). However, this association is not absolute. Some studies failed to show any significant association between CagA status and clinical outcomes (Milehlke et al., 1996), especially studies from East Asia, where more than 90% of isolated *H. pylori* strains were found positive for CagA in Japan (Yamaoka, 2012). Thus, several scholars have focused on determining whether different levels of pathogenicity exist among CagA-positive strains. CagA is a gene in the Cag pathogenicity island (PaI), a 40-kilobase region of the *H. pylori* chromosome that contains 27 to 31 genes (Yamahashi and Hatakeyama, 2012). The Cag PaI encodes a type IV secretory system (TFSS) that forms a syringe-like structure that penetrates epithelial cells and delivers CagA into the host cytosol (Yamahashi and Hatakeyama, 2012). Within the cytosol, CagA is phosphorylated on tyrosine residues (Odenbreit et al., 2000; Backert et al., 2002; Gobert et al., 2012; Mueller et al., 2012) by Src family kinases (Stein et al., 2002; Hatakeyama, 2004; Xu et al., 2012) that recognise tyrosine phosphorylation motifs (TPMs). Phosphorylated CagA interacts with the phosphatase SHP-2 (Tegtmeyer et al., 2011; Yamahashi and Hatakeyama, 2012) causing dephosphorylation of cortactin (Argent et al., 2004) and cytoskeletal rearrangements that form the 'hummingbird' phenotype (Tegtmeyer et al., 2011). Phosphorylated CagA can also interact with C-terminal Src kinase, which

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attenuates SHP-2 signalling and inactivates Src kinases, thereby decreasing hummingbird cell formation and preventing further phosphorylation of CagA (Safari et al., 2011; Tegtmeyer et al., 2011). In addition to inducing hummingbird cell formation, CagA also promotes cell spreading or 'scattering' similar to the scattered phenotype induced by the scatter factor/hepatocyte growth factor on binding the c-Met receptor (Tegtmeyer et al., 2011; Yamahashi and Hatakeyama, 2012). Contact of the TFSS with the epithelial cell or possible translocation of a second unidentified factor also leads to the activation and nuclear translocation of nuclear factor-κB and the secretion of proinflammatory cytokines and chemokines such as interleukin-8 (Martínez et al., 2012; Xue et al., 2012). CagA is not needed for this effect. CagA is differently sized in different strains because of the presence of repeat sequences encoding TPMs within the 3' variable region of CagA (Yamaoka et al., 1998; Higashi et al., 2002; Higashi et al., 2002; Chomvarin et al., 2012; Mueller et al., 2012; Sahara et al., 2012). Yamaoka et al. and Azuma et al. found that *H. pylori* strains possessing more than three TPMs within the CagA variable region are significantly associated with gastric carcinoma and atrophic gastritis in Japan (Higashi et al., 2002; Chomvarin et al., 2012). Yamaoka et al. also showed that *H. pylori* strains with more than three CagA variable region TPMs are significantly associated with gastric mucosal atrophy and intestinal metaplasia in patients from Colombia, the United States, Italy, and South Korea (Yamaoka et al., 1999). However, most Japanese strains are positive for CagA with unknown TPM, SHP-2 binding activity, and gastric carcinogenesis. In this study, we investigated the number of EPIYA repeats and the structure of the 3' region of CagA in duodenal ulcer and gastric cancer strains, studied CagA protein expression, and examined the translocated CagA protein and the SHP-2 binding activity within AGS cells co-cultured with duodenal ulcer or gastric cancer strains.

## Materials and Methods

*Bacterial strains*

Sixteen *H. pylori* strains were clinically isolated from patients with duodenal ulcer (eight cases) and gastric adenocarcinoma (eight cases), which were biopsy specimens obtained during endoscopy examinations at Hokkaido University Hospital (Sapporo, Japan) and Yanda International Hospital (East Beijing, China) from 1990 to 2011. This study was conducted in accordance with the declaration of Helsinki and was approved by the Ethics Committee of Yanda International Hospital, East Beijing. Written informed consent was obtained from all participants. The bacteria were thawed and cultured for 72 h on a Columbia agar plate containing 5% sheep blood under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% N2) at 37 °C and then inoculated into brucella broth (Sigma, St. Louis, Missouri, USA) supplemented with 10% (Vol/Vol) foetal bovine serum (FBS) at 37 °C under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% N2) with shaking (150 rpm to 200 rpm) overnight. The bacterium was washed down from the 50 mL of liquid culture for further experiments.

*H. pylori genomic DNA*

Genomic DNA was extracted and purified using a sepa gene kit (Sankou Junyaku, Tokyo, Japan) according to the manufacturer's instructions.

*PCR*

The 3' region of the CagA gene primers were designed by *H. pylori* ATCC 53726 Gene Bank accession no. L117714 and amplified by PCR using the following primers.

Forward primer: 5'-GGAACCCTAGTCGGTAAT-3'
Reverse primer: 5'-TATCGTTTGTTCTAGATT-3'

Amplification conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The tubes were maintained at 72 °C for 7 min before storage at 4 °C. The PCR products were examined using 1.5% agarose gel electrophoresis and then purified with QIAquick PCR purification kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. DNA sequencing was performed through the dideoxynucleotide chain termination method using a Big Dye terminator cycle sequencing ready reaction mix (Applied Biosystems, Tokyo, Japan) in an ARI prism 310 genetic analyser (Applied Biosystems, Tokyo, Japan) using the same primers shown above. According to the manufacturer's protocol, reagent mixtures containing 5 μL of purified PCR product, 3.2 pmol of primer, 8 μL of terminator cycle sequencing ready reaction mix (A, C, G, T-DyeDeoxy Terminator dITP, dATP, dCTP, dTTP, Tris-HCl buffer, AmpliTaq DNA polymerase, FS), and 5 μL of sterilised distilled water were prepared. The reaction tube was placed in a thermal cycler, and thermal cycling was initiated under the following conditions: 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Nucleotide sequences were aligned and analysed using GENTYX-Mac version 10.0 (Software Development Co., Tokyo, Japan).

*CagA protein from H. pylori*

The bacterium was washed down thrice with phosphate-buffered saline (PBS) from the 50 mL liquid culture at 3000 rpm. A total of 1 mL 0.2 M glycine buffer was added for every 100 mg of bacterium pellet. The mixture was maintained at room temperature for 20 min and then centrifuged for 10 min at 3000 rpm. The supernatant was removed, neutralised using 1 N NaOH until pH 7, and then subjected to dialysis (Sankou Junyaku, Tokyo, Japan) for 24 h using pure water at 4 °C.

*AGS cell culture*

AGS cells (ATCC CRL 1739, a human adenocarcinoma epithelial cell line) were grown in 150 mm × 250 mm cell-cultured dishes containing Ham's F-12 (Sigma, St. Louis, USA) supplemented with 10% FBS in an atmosphere of 5% $CO_2$ at 37 °C until confluence. For co-culture experiments, *H. pylori* were grown in brucella broth with 10% FBS at 37 °C under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% N2) with shaking (150 rpm to 200 rpm) overnight. The final cell number used for the interaction experiment was $4 \times 10^6$ cells/well. The ratio of *H. pylori* and AGS cells (multiplicity of infection) was

100:1. *H. pylori* was suspended in culture medium and added to dishes attached with gastric epithelial cells. They were co-cultured in a cell culture incubator (5% $CO_2$/95% air) for 5 h.

*Preparation of cell lysates*

Infected cells were washed thrice with ice-cold PBS. The cells were scraped from the ice, transferred into a microfuge tube, and then pelleted at 1200 rpm for 5 min. The cell pellets were suspended in 200 μL of lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride) for 30 min and then centrifuged at 10000 rpm for 5 min.

*Western blot*

Cell lysates were mixed with equal amounts of 2× sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 25% glycerol), loaded per lane, and then boiled for 5 min. Proteins were separated using SDS-PAGE on 6% polyacrylamide gels and then blotted onto pure nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using standard protocol. The membranes were blocked in PBS with 3% bovine serum albumin (BSA) at 4 °C overnight. CagA was detected using a polyclonal rabbit anti-CagA antibody (Austral Biologicals, California, USA) in a 1:1500 dilution and incubated in 3% BSA-PBS for 2 h at room temperature. SHP-2 was detected with a polyclonal rabbit anti-SHP-2 antibody (Santa Cruz Biotechnology, Inc, USA) in a 1:1000 dilution and incubated in PBS for 2 h at room temperature. The blots were washed thrice in T-PBS (0.05% Tween, PBS) and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibodies (dilution, 1:1000; Dako Cytomation, Glostrulp, Denmark) for 1 h. After three more washings, the membranes were visualised using an enhanced chemiluminescence-detection system according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Quantification was performed using density score with computer image processing and analysis by Wyne Rasband National Institutes of Health Research Service Branch, NIMH (Wayne@helix.nih.gov) version 1.63 soft.

*Statistical analysis*

The Student's t-test was performed to determine differences in age, gender, grade of gastric mucosal atrophy, distribution of gastritis, activity, inflammation, metaplasia, and density score between the gastric cancer and duodenal ulcer patients.

# Results

*General data*

The characteristics of the patients according to the Updated Sydney System are shown in Table 1. No significant differences were observed between the two groups in terms of age and sex. Antrum activity, inflammation, and corpus inflammation score were significantly higher in the duodenal ulcer patients than in the gastric adenocarcinoma patients (P=0.0034, P=0.0008, and P= 0.01, respectively). The corpus atrophy score was

**Table 1. Characteristics of the DU and GCa Patients**

| Item | DU | GCa | P |
|---|---|---|---|
| Age | 44.3 | 60 | 0.059 |
| Sex | | | |
| Male | 3 | 4 | NS |
| Female | 5 | 4 | NS |
| Antrum(GC) | | | |
| Activity | 1.75±0.46 | 0.63±0.74 | 0.0034 |
| Inflammation | 2.00±0.00 | 0.63±0.74 | 0.0008 |
| Atrophy | 0.66±0.83 | 0.75±0.71 | 0.82 |
| Metaplasia | 0.13±0.35 | 0.63±0.76 | 0.12 |
| Corpus (GC) | | | |
| Activity | 1.00±0.53 | 0.63±0.76 | 0.28 |
| Inflammation | 1.38±0.52 | 0.5±0.53 | 0.01 |
| Atrophy | 0.25±0.46 | 0.91±1.13 | 0.16 |
| Metaplasia | 0.00±0.00 | 0.66±1.06 | <0.01 |
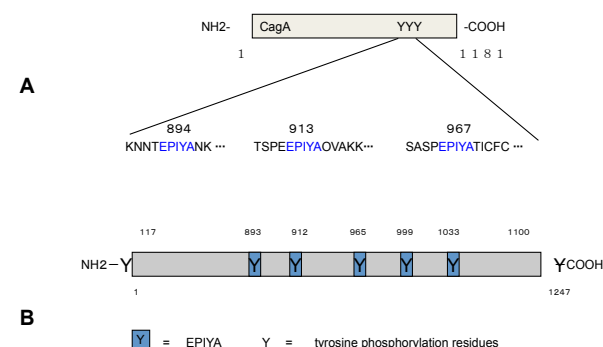


**Figure 1. A: Schematic Presentation of the EPIYA Repeats in the CagA Protein are Potential Src Phosphorylation Sites and CagA Protein from Strain ATCC 53726, and the Position of the C Terminus EPIYA Repeats, B: The NCTC11637-derived CagA Protein Possesses Seven Potential Tyrosine Phosphorylation Sites, Residues -117, -893, -912, -965, -999, -1033, -1100. Of these, Residues -893, -912, -965, -999, and -1033 Constitute Five Copies of the EPIYA Sequences**

higher in the gastric cancer patients than in the duodenal ulcer patients, but no significant differences were found between the two groups. The corpus metaplasia score was significantly higher in the gastric adenocarcinoma patients than in the duodenal ulcer patients (P<0.01).

*PCR*

Among the 16 *H. pylori* strains, 8 were from duodenal ulcer patients and 8 were from gastric adenocarcinoma patients. All strains from the patients with duodenal ulcer (8 strains) and gastric adenocarcinoma (8 strains) were positive for CagA (100%) by PCR using the above primers.

*TPMs and structure*

The EPIYA repeats in the CagA protein are potential Src phosphorylation sites. The CagA protein from strain ATCC 53726 and the position of the C-terminus EPIYA repeats are shown in Figure 1A. Strain ATCC 53726 possesses three potential tyrosine phosphorylation sites (i.e., residues -894, -913, and 967) and three EPIYA motifs, all of which are in the C-terminus. The NCTC 11637-derived CagA protein possesses seven potential tyrosine phosphorylation sites (residues -117, -893, -912, -965, -999, -1033, and -1100), among which residues -893, -912, -965, -999, and -1033 constitute five copies of the
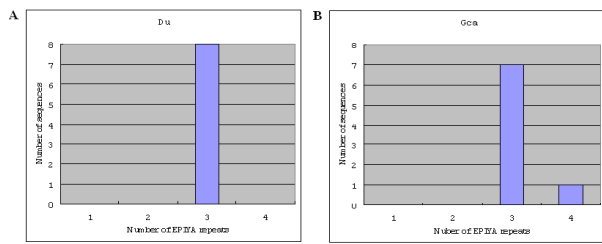
Figure 2. A: CagA Proteins from Different *H.pylori* trains of the Duodenal Ulcer Patients and EPIYA Repeats in the C Terminus. B: CagA Proteins from Different *H. pylori* strains of the Gastric
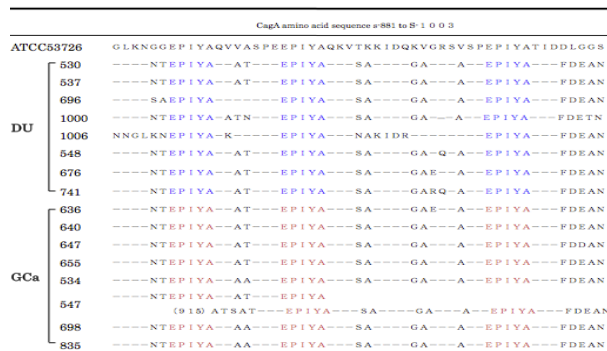


Figure 3. Analysis of Amino Acid Sequence of CagA Tyrosine Phosphorylation Motifs,Y-894,Y-913 and y-967 in Helicobacter Pylori Isolates

EPIYA sequences (Figure 1B). Our results showed that all EPIYA repeat positions are in the C-terminus and that most strains possess three copies of the EPIYA sequences. These findings are consistent with those reported by Matthias et al. (2002).

*EPIYA copies*

With the exception of strain 547 from the gastric adenocarcinoma patients, all strains possess three copies of the EPIYA (Figsure 2A and 2B). The EPIYA copies in the duodenal ulcer patients and the gastric adenocarcinoma patients are not significantly different.

*Analysis of sequence diversity of TPMs*

Strain 547 from the gastric adenocarcinoma patients possesses TPMs Y-894, Y-913, Y-949, and Y-1003. Except for strain 547, all strains possess TPMs Y-894, Y-913, and Y-967. No association was found between the sequence diversity of TPMs in the strains from the duodenal ulcer and gastric cancer patients. Except for strain ATCC 53726, no significant difference was found between the two groups in terms of the amino acid sequences from Y-894 to Y-967. Only one strain (1006, from the patients with duodenal ulcer) was found to be different from the sequence of other clinical strains and strain ATCC 53726 (Figure 3).

*Western blot*

Argent et al. reported that although *H. pylori* strains tested positive for CagA by PCR, only 77.3% was expressed as CagA protein (Argent et al., 2004). Thus, we determined whether they expressed the CagA protein through western blot analysis of the duodenal ulcer and
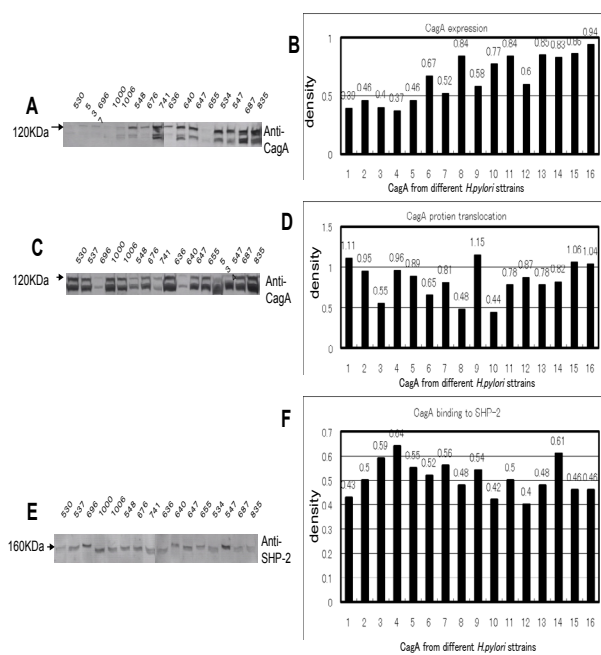


Figure 4. A: CagA Protein Expression of *H.pylori* strains from Different Duodenal Ulcer Patients and From Different Gastric Adenocarcinoma Patients, B: Density Score of CagA Expression by *H.pylori* strains. Lane 1-8 with *H. pylori* strains with Duodenal Ulcer Patients; Lane 9-16 with *H. pylori* strains with Gastric Adenocarcinoma Patients, C: Western Blotting Analysis of AGS whole Cell Lysates: Lane 1-8 with *H.pylori* strains with Duodenal Ulcer Patients; Lane 9-16 with *H.pylori* strains with Gastric Adenocarcinoma Patients, D: Density Score of CagA Protein Translocation into AGS Cells. Lane 1-8 with *H.pylori* strains with Duodenal Ulcer Patients; Lane 9-16 with *H. pylori* strains with Gastric Adenocarcinoma Patients Individuals, E: Western Blotting Analysis of AGS whole Cell Lysates: Lane 1-8 with *H. pylori* strains with Duodenal Ulcer Patients; Lane 9-16 with *H.pylori* strains with Gastric Adenocarcinoma Patients and F: Density Score of CagA Binding to SHP-2. Lane 1-8 with *H. pylori* strains with Duodenal Ulcer Patients; Lane 9-16 with *H.pylori* strains with Gastric Adenocarcinoma Patients Individuals

gastric cancer patients with anti-CagA antibodies. Among the 16 *H. pylori* strains, 16 expressed the CagA protein (100%) by western blot (Figure 4A). The density score showed that the CagA protein expression by the *H. pylori* strains from the gastric adenocarcinoma patients was significantly higher than that by the *H. pylori* strains from the duodenal ulcer patients (P = 0.0023, Figure 4B).

All *H. pylori* strains from the duodenal ulcer patients and gastric cancer patients translocated the CagA protein into the AGS cells (Figure 4C). The density score showed that the CagA protein translocation by the *H. pylori* strains from the gastric adenocarcinoma patients was higher than that by the *H. pylori* strains from the duodenal ulcer patients, but no significant differences were found between the two groups (P = 0.59, Figure 4D).

All *H. pylori* strains showed CagA binding to SHP-2 in the AGS cells (Figure 4E). The density score showed that the CagA binding to SHP-2 by the *H. pylori* strains from the gastric cancer patients was higher than that by the *H. pylori* strains from the duodenal ulcer patients, but no significant differences were found between the two groups (P = 0.15, Figure 4F).

## Discussion

Compared with uninfected patients, individuals infected with *H. pylori* are more likely to develop chronic gastritis, peptic ulcer, and gastric malignancies. The prevalence of both gastric cancer and atrophic gastritis is extremely high in Japan. Murakita et al. reported that tox+ *H. pylori* isolates are more prevalent in patients with severe atrophic gastritis and that the cytotoxin activities in patients with severe atrophic gastritis are higher than those in patients with mild atrophic gastritis in Japan (Murakita et al., 1996). Our results showed that all gastric cancer patients had atrophic gastritis and scored higher than the duodenal ulcer patients. Among those infected, the risk of disease is further increased if the strains possess CagA (Cavalcante et al., 2012; Epplein et al., 2012). However, the association is unclear so far. Lars-Erik Hanssion et al. (1996) reported that some factors in duodenal ulcer disease seem to act against the development of gastric cancer. Thus, we studied the correlation between duodenal ulcer (low risk of developing gastric cancer) and gastric adenocarcinoma patients with *H. pylori* infection. Argent et al. (2004) reported that 44 South African strains were found positive for CagA by PCR; however, 10 of these 44 strains did not express the CagA protein. By contrast, the present study found that all strains (16 strains: 8 from duodenal ulcer patients and 8 from gastric adenocarcinoma patients.) expressed the CagA protein. The expression of the CagA protein in the gastric adenocarcinoma patients was significantly higher than that in the duodenal ulcer patients ($P = 0.0023$, Student's t-test). The Cag PaI encodes a TFSS that forms a syringe-like structure that penetrates epithelial cells and delivers CagA into the host cytosol (Yamaoka, 2012). Within the cytosol, CagA is phosphorylated on tyrosine residues (Odenbreit et al., 2000; Backert et al., 2002; Gobert et al., 2012; Mueller et al., 2012) by Src family kinases (Stein et al., 2002; Hatakeyama, 2004; Gobert et al., 2012) that recognise TPMs. Phosphorylated CagA interacts with the phosphatase SHP-2 (Xu et al., 2012), causing dephosphorylation of cortactin (Xu et al., 2012) and cytoskeletal rearrangements that form the 'hummingbird' phenotype (Xu et al., 2012). Phosphorylated CagA can also interact with C-terminal Src kinase that attenuates SHP-2 signalling and inactivates Src kinases, thereby decreasing the hummingbird cell formation and preventing further phosphorylation of CagA (Tsutusmi et al., 2003). Our results showed that CagA, through the TFSS into the AGS cells within the cytosol, becomes phosphorylated and binds to SHP-2 to form the 'hummingbird' phenotype. All the strains delivered CagA, bound to SHP-2, and formed 'hummingbird' in the AGS cells. The density score for the gastric carcinoma patients was higher than that for the duodenal ulcer patients in both CagA translocation and SHP-2 binding. The absence of significant differences between the two groups indicates the need for more experiments on clinical isolates.

In conclusion, all strains were PCR-positive for the CagA gene. The CagA protein expression in the gastric adenocarcinoma patients was significantly higher than that in the duodenal ulcer patients. The TPMs and 3' region structure of the *H. pylori* CagA gene showed no significant differences between the two groups. All *H. pylori* strains expressed the CagA protein, and the expression in the gastric cancer patients was significantly higher than that in the duodenal ulcer patients. All *H. pylori* strains from the duodenal ulcer and gastric adenocarcinoma patients enabled CagA protein translocation and SHP-2 binding within the AGS cells. The *H. pylori* strains from the gastric cancer patients exhibited higher CagA expression, higher CagA translocation, and higher SHP-2 binding than those from the duodenal ulcer patients, and these strains were more closely associated with gastric carcinogenesis.

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