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Tyrosine Phosphorylation of CagA from Chinese *Helicobacter pylori* Isolates in AGS Gastric Epithelial Cells

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***Helicobacter pylori* strains possessing the *cag* pathogenicity island (PaI) are associated with the development of gastroduodenal diseases, including gastric cancer. *cag* PaI products induce the secretion of interleukin-8 (IL-8) from epithelial cells and facilitate the translocation of CagA into the cell cytosol. In East Asia, where the incidence of gastric cancer is high, most strains possess the *cag* PaI. To date, however, no *cag* PaI phenotypic data have been provided for strains isolated in mainland China. Here we used 31 Chinese strains to determine the genotypic and phenotypic status of the *cag* PaI. All strains possessed *cagA* and *cagE*, and we observed a variation in the length of *cagA* variable regions. Nucleotide sequencing of the *cagA* variable region revealed that CagA was of two types, a short "Western" form with two tyrosine phosphorylation sites and a longer "East Asian" form with three tyrosine phosphorylation sites. Coculture of strains with AGS epithelial cells showed that strains could induce IL-8 secretion from the cells and that CagA with three phosphorylation sites became more phosphorylated than that with two and could induce significantly ($P < 0.001$) more cells to elongate. We hypothesize that the preponderance of the more active East Asian form of *cagA* may underlie the high rate of gastric cancer in China.**

Helicobacter pylori is a spiral, gram-negative, microaerophilic bacterium that is present in the stomachs of approximately half of the world's population. *H. pylori* has been identified as a causative agent of chronic gastritis and of gastric and duodenal ulceration, and infection with this organism is an important risk factor for the development of gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (12, 23). Several putative *H. pylori* virulence factors have been identified as additional risk factors involved in ulceration and gastric cancer, including the cytotoxin-associated gene (*cag*) pathogenicity island (PaI).

The *cag* PaI comprises a 40-kb DNA segment integrated into the *H. pylori* chromosome in some strains (9) and encodes a type IV secretory system, which is associated with enhanced virulence and increased mucosal inflammation. The *cagA* gene encodes the CagA protein and has been used as a marker for the presence of the *cag* PaI. The type IV secretory system facilitates the translocation of CagA into the cytosol of epithelial cells; CagA localizes to the inner surface of the plasma membrane (21) and subsequently undergoes tyrosine phosphorylation in the host cells (2, 7, 16, 19, 21) by Src family protein tyrosine kinases (2, 20, 22). The CagA tyrosine phosphorylation motifs (TPMs) have been mapped to the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present in the C-terminal region of the protein (3, 13, 15, 22). CagA proteins generally possess two or more EPIYA motifs within the variable region, which accounts for the size variation observed between CagA proteins isolated from different *H. pylori* strains. CagA proteins with greater numbers of TPMs become more phosphorylated and lead to enhanced formation of the "hummingbird" phenotype (1, 13). Analysis of *cagA* in *H. pylori* strains isolated from patients in East Asia, where the incidence of stomach cancer is high, has shown differences between the *cagA* variable region of those isolates and that of "Western" isolates (24) and also an association between the number of CagA TPMs and the development of atrophic gastritis and gastric cancer in Japan (5, 24).

In this study we looked at the CagA status of *H. pylori* strains from Chinese patients. Previously, Zhou et al. (25) showed that all 18 *H. pylori* strains isolated from the Hangzhou region of China possessed *cagA* and that 17 of 18 were of the "East Asian" type, although no sequence data were provided to show differences between *cagA* genes from different strains. Zhou et al. (26) showed that 79 of 82 Chinese *H. pylori* strains possessed *cagA* genes which, as determined by PCR amplification of the 3′ variable regions, were of three types, although no data were provided to show the composition of each of these variable regions. Neither study presented phenotypic data regarding the expression and tyrosine phosphorylation of CagA from these strains. We therefore decided to look at the *cag* PaI status and *cagA* variable region in *H. pylori* strains isolated from eastern China in terms of the numbers of CagA TPMs, tyrosine phosphorylation, and hummingbird phenotype formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strains were isolated from 31 Chinese patients, living in Jiangsu Province of mainland China, with chronic gastritis alone (12 cases), duodenal ulcers (11 cases), gastric ulcers (5 cases), both duodenal and gastric ulcers (2 cases), and gastric cancer (1 case), and were grown

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on horse blood agar plates (Oxoid, Basingstoke, United Kingdom) at 37°C in a microaerobic environment.

PCR amplification of *cagA* and *cagE* genes. *H. pylori* genomic DNA was extracted from minimally passaged strains as previously described (4). PCR amplification of the *cagA* 3′ variable region with primers cag2 and cag4 as previously described for South African and European strains (18) was unsuccessful. The reverse primer was therefore redesigned as either cagA31 (5′-CGGCTATGCTCAACCTGGTGGAAAACTTGAACG) or cagA26R (5′-GCTTTAGCTTCTGATACC), and the PCR was performed under otherwise identical conditions. For nucleotide sequencing of *cagA* variable regions, PCR-amplified products (with primers cag2 and cagA26R) were purified with a QIAquick purification kit (Qiagen, Crawley, United Kingdom) before sequence analysis by the Biopolymer Synthesis and Analysis Unit, Queen's Medical Centre, University of Nottingham, United Kingdom. PCR amplification of *cagE* was carried out with primers PBRTF (5′-AAGGGTAAAGAAATGGGACTGAAT) and PBRTR (5′-GGAAGCGTGATAAAAGAGCAATGT). A reaction mixture containing a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.4 nM concentration of each primer, 0.05 U of *Taq* DNA polymerase (Roche Diagnostics, Penzberg, Germany) per μl, and 1 μl of genomic DNA, in buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride), was incubated at 95°C for 90 s; followed by 35 cycles of 95°C for 30 s, 56°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 5 min.

Infection of AGS cells with *H. pylori* strains and preparation of cell lysates. AGS human gastric epithelial cells were seeded into 25-cm^2 flasks (10^6 cells/flask) in Ham F-12 nutrient mixture and incubated at 37°C in a 5% CO_2–air humidified atmosphere until almost confluent. *H. pylori* strains were resuspended in Ham F-12 nutrient mixture and diluted to an optical density at 550 nm of 0.1 (multiplicity of infection, ~100) before addition to AGS cells for 6 h at 37°C in a 5% CO_2–air humidified atmosphere. Infected AGS cells were washed twice with phosphate-buffered saline (PBS), and the cells were scraped from the flasks in 5 ml of PBS containing 1 mM sodium vanadate, harvested by centrifugation at $1,000 \times g$ for 10 min, and then resuspended in 100 μl of PBS-vanadate and 50 μl of 4× sample loading buffer (0.2 M Tris-HCl [pH 6.8], 0.4 M dithiothreitol, 8% [wt/vol] sodium dodecyl sulfate, 40% [vol/vol] glycerol, 0.4% [wt/vol] bromophenol blue). Samples were heated at 100°C for 5 min before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antiphosphotyrosine monoclonal antibodies (clone PY99; Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-CagA polyclonal antibodies (Austral Biologicals, San Ramon, Calif.). Quantification of the degree of CagA phosphorylation was performed by densitometry with a Bio-Rad (Hemel Hempstead, United Kingdom) GS-800 calibrated densitometer and Quantity One software and was expressed as a ratio of phospho-CagA to total CagA.

Analysis of AGS cell hummingbird phenotype formation. AGS cells were seeded into six-well dishes at a density of 2×10^5 cells/well and incubated at 37°C in a 5% CO_2–air humidified atmosphere for 24 h. *H. pylori* strains were cocultured with AGS cells as described above, except that cells were incubated for 1 to 2 days, before the cells were examined for hummingbird phenotype formation (1) by microscopy of randomly chosen fields with a Nikon Eclipse TE200 microscope. Protrusions characteristic of the hummingbird phenotype (at least 100) were measured by using Lucia G (version 3.5) software. Statistical analysis was performed with a two-tailed Student's *t* test.

Nucleotide sequence accession numbers. The nucleotide sequences of the *cagA* variable regions have been entered into the EMBL nucleotide sequence database under accession numbers AJ832140 to AJ832149.

RESULTS

PCR amplification of *cagA* and *cagE* genes. PCR amplification of *cagA* from Chinese *H. pylori* strains, using primers cag2 and cagA31, revealed that all (31 of 31) strains possessed this gene, although there was size variation in the lengths of products generated. Most strains possessed a *cagA* fragment of ~890 bp, whereas three of the strains possessed smaller fragments of ~840 bp (strain Z4) and ~770 bp (strains Z11 and Z33) (Fig. 1). Amplification of *cagE* revealed that all (31 of 31) strains also possessed this gene.

Sequencing of the *cagA* variable region. As we found size variation within *cagA* between strains, we decided to perform nucleotide sequence analysis of the *cagA* variable regions from 10 strains (the three with shorter *cagA* genes and seven of those with longer *cagA* genes). The *cagA* variable region was amplified by PCR with primers cag2 and cagA26R prior to nucleotide sequencing. We found that CagA variable regions from these Chinese isolates fell into two types: a short one possessing two EPIYA motifs and a longer one possessing three EPIYA motifs (Fig. 2). Of the three shorter *cagA* genes, we found that one (strain Z4) possessed three variable-region TPMs but had a deletion between the second and third EPIYA motifs, whereas the other two (strains Z11 and Z33) possessed only two EPIYA motifs. We also found that the variable region possessing two TPMs had a Western-type sequence, whereas the variable region with three TPMs had an East Asian-type sequence, characterized by the EATSAINRKIDRINKIASAGKGVGGFSGA pattern following the second EPIYA motif (Fig. 2).

Expression and tyrosine phosphorylation of CagA. We next went on to look at the expression of CagA by Chinese *H. pylori* strains and whether they became phosphorylated within AGS

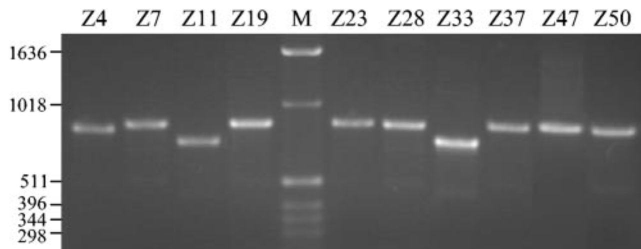

FIG. 1. *cagA* variable regions display size variation by PCR. PCR amplification of the 3′ *cagA* variable regions from Chinese *H. pylori* strains with primers cag2 and cagA31 is shown. Lane M, size markers (in base pairs).

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Z4    FSDIRKELNEKLFGNS-NNNNNGLKNNTEPIYAQVNKKKTGQVASPEEPIYA
Z7    FSDIRKELNEKLFGNS-NNNNNGLKNNEEPIYAQVNKKKTGQATSPEEPIYA
Z11   FSDIKKELNEK-FKNFNNNNNNGLEN--EPIYAKVNKKKAGQAANPEEPIYT
Z19   FSDIRKELNEKLFGNS-NNNNNGLKNNTEPIYAQVNKKKTGQAASPEEPIYA
Z23   FSDVRKELNEKLFGNS-NNNNNGLKNNTEPIYAQVNKKKTGQATSPEEPIYA
Z28   FSDIRKELNEKLFGNS-NNNNNGLKNNTEPIYAKVNKKKTGQATSPEEPIYA
Z33   FSDIKKELNEK-FKNFNNNNNNGLKN--EPIYAKVNKNKTGQVANLEEPIYT
Z37   FSDIRKELNEKLFGNS-NNNNNGLKNSTEPIYAKVNKKKAGQVASPEEPIYA
Z47   FSDIRKELNEKLFGNS-NNNNNGLKNNEEPIYAQVNKKKTGQVASPEEPIYA
Z50   FSDIRKELSEKLFGNS-NNNNNGLKNNTEPIYAQVNKKKAGQAISPEEPIYA

Z4    QVAKKVSA------------------------------------KIDRINKIAS
Z7    QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z11   QVAKKVKEKIDRLDQIASGLGGVGQA-GFSLKGHT-------------------
Z19   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z23   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z28   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z33   QVAKKVKAKIDQLNQATSGFGGVGQAAGFPLKRHD-------------------
Z37   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z47   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS
Z50   QVAKKVSAKIDQL----------------------NEATSAINRKIDRINKIAS

Z4    AGKGVGGFSGAGRSASPEEPIYATIDFDEANQAGFPLRRYAPVNDLSKVGLSR
Z7    AGKGVGGFSGAGQSASP-EPIYATIDFDETNQAGFPLRRYAGVGDLSKVGLSR
Z11   -----------------------------------------KVGDLSKVGLSR
Z19   AGKGVGNFSGAGQSASP-EPIYATIDFDEANQAGFPLRRSAAVNDLSKVGLSR
Z23   AGKGVGGFSGAGRSTSP-EPIYATIDFDEANQAGFPLRRSAAVNDLSKVGLSR
Z28   AGKGVGGFSGAGRSASP-EPIYATIDFDEANQAGFPLRRYAGVGDLSKVGLSR
Z33   -----------------------------------------KVDDLSKVGLSR
Z37   AGKGVGGFSGAGRSASP-EPIYATIDFDEANQAGFPLRRSVAVNDLSKVGLSR
Z47   AGKGVGGFSGAGRSASP-EPIYATIDFDEANQAGFPLRRSAAVNDLSKVGLSR
Z50   AGKGVGGFSGAGRSASP-EPIYATIDFDEANQAGFPLRRSAAVNDLSKVGLSR
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FIG. 2. Deduced amino acid sequences of 10 CagA variable regions. *cagA* variable regions were amplified by PCR with primers cag2 and cagA26R, and the products were purified and subjected to nucleotide sequencing. The deduced amino acid sequences are shown.

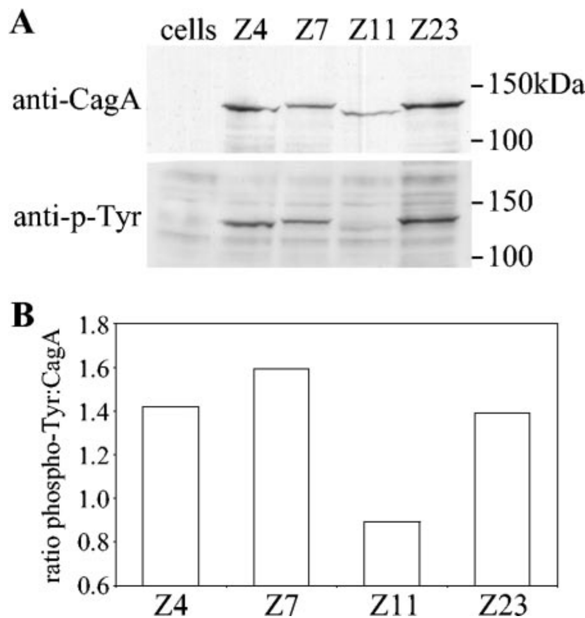

FIG. 3. CagA with two variable-region TPMs is less phosphorylated than CagA with three variable-region TPMs. (A) AGS cells were cocultured with *H. pylori* strains possessing two (strain Z11) or three (strains Z4, Z7, and Z23) variable-region EPIYA motifs for 6 h at 37°C before the cells were lysed and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antiphosphotyrosine monoclonal antibodies (lower panel). The blots were then stripped and reprobed with anti-CagA polyclonal antibodies (upper panel). (B) Densitometric analysis of the degree of CagA phosphorylation, expressed as a ratio of phospho-Tyr intensity to CagA protein intensity. The data shown are representative of those from three separate experiments.

epithelial cells by coculture. We tested 18 of the strains and found that all of them expressed CagA, which became tyrosine phosphorylated within AGS cells (Fig. 3), and all of these strains induced interleukin-8 secretion from AGS cells (Fig. 4). As expected, CagA proteins with two TPMs were of lower

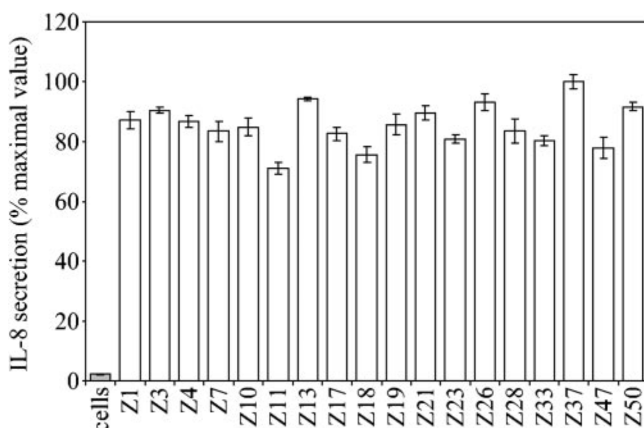

FIG. 4. Interleukin-8 (IL-8) secretion induced by *H. pylori* strains. Chinese *H. pylori* strains were cocultured with AGS cells for 6 h before the amount of secreted interleukin-8 was determined by enzyme-linked immunosorbent assay. The results are expressed as percentages of the maximal value (for strain Z37). Errors bars represent standard errors of the means.

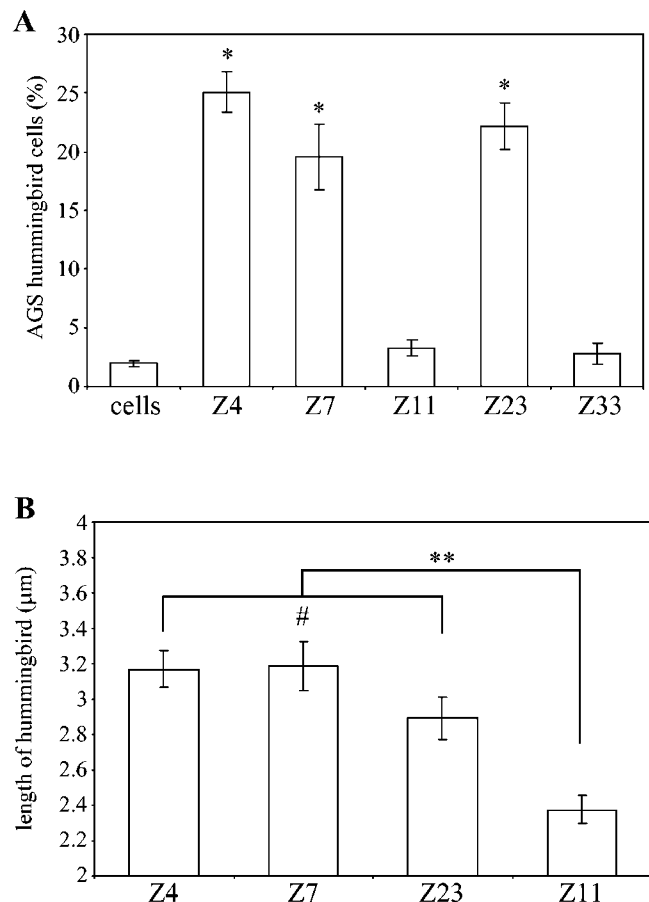

FIG. 5. AGS cell hummingbird phenotype formation is reduced by CagA with two variable-region TPMs. AGS cells were cocultured with *H. pylori* strains Z4, Z7, and Z23 (three variable-region EPIYA motifs) and with strains Z11 and Z33 (two variable-region EPIYA motifs) for 1 day before cells were visualized by microscopy. (A) Percentages of AGS cells displaying the hummingbird phenotype in randomly chosen microscopic fields. *, $P < 0.001$ (Z4, Z7, or Z23, compared to Z11, Z33, or cells alone). (B) Average lengths of protrusions characteristic of hummingbird phenotype of at least 100 randomly chosen hummingbird phenotype AGS cells cocultured with each strain. **, $P < 0.0001$; #, $P > 0.05$. Errors bars represent standard errors of the mean.

molecular weight than those with three TPMs, and densitometric analysis revealed that CagA proteins possessing three EPIYA motifs became more phosphorylated than those with only two EPIYA motifs (Fig. 3).

Induction of AGS cell hummingbird phenotype formation by CagA. CagA phosphorylation within epithelial cells leads to hummingbird phenotype formation, the extent of which appears to be increased in proportion to the number of EPIYA motifs (1). We found, following 24-h coculture of AGS cells with *H. pylori* strains, that hummingbird phenotype formation was significantly ($P < 0.001$) more pronounced in AGS cells incubated with strains expressing CagA with three variable-region EPIYA motifs (Fig. 5A). Only 3% of AGS cells displayed the hummingbird phenotype when cocultured with strains expressing CagA with two variable-region EPIYA motifs, compared to an average of 16% for those with three TPMs. We also found that the average lengths of protrusions

characteristic of the hummingbird phenotype induced were significantly ($P < 0.0001$) shorter for the CagA proteins with two variable-region EPIYA motifs than for those with three EPIYA motifs (Fig. 5B).

DISCUSSION

H. pylori is recognized as a major causative agent of chronic gastritis and peptic ulcer disease and has been identified as a major risk factor for gastric cancer. In most studies, strains possessing *cagA* have been associated with an increased risk for the development of gastric cancer (8, 11), although some studies found no significant association (14, 17). This is especially true in East Asia, where more than 90% of strains possess *cagA*. As CagA shows size differences due to variations in the number of TPMs within the variable region of the protein, the degree of CagA phosphorylation may be a more important determinant of virulence than possession of *cagA* (1, 5, 24). Indeed, studies using *H. pylori* strains isolated from Japanese subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins with four or more variable-region EPIYA motifs were from patients with gastric cancer (1).

In this study we looked at the CagA status of *H. pylori* strains from Chinese patients. Most (28 of 31; 90%) of the strains possessed a longer form of *cagA*, but 3 of 31 strains possessed *cagA* genes with shorter variable regions, of which one was found to possess a deletion between the second and third EPIYA motifs. Sequencing analysis revealed that strains with longer *cagA* genes possessed three TPMs which were of the East Asian type, as they possessed the motif EATSA INRKIDRINKIASAGKGVGGFSGA (5), whereas the two *cagA* alleles with only two EPIYA motifs were of the Western type. The longer type of *cagA* variable region corresponded to the type I *cagA* described by Zhou et al. (26), which was found to occur in 67 of 71 Chinese strains (94%). Analysis of the degree of CagA phosphorylation by AGS cell coculture showed that CagA proteins with three TPMs became more phosphorylated than those with two TPMs. This also shows that CagA with only two EPIYA motifs can become phosphorylated, in agreement with other studies showing that all EPIYA motifs can become phosphorylated (3, 13, 15, 22).

Phosphorylation of CagA within the epithelial cell cytosol leads to hummingbird phenotype formation, which is dependent upon the degree of CagA phosphorylation. CagA proteins with greater numbers of EPIYA motifs become more phosphorylated and induce more AGS cells to display the hummingbird phenotype and increase the length of cellular protrusions (1, 13). Higashi et al. (13) showed, by mutagenesis of CagA from strain NCTC 11637, which has five EPIYA motifs (in an ABCCC pattern), that the C type of motif was responsible for binding SHP-2 and promoting hummingbird phenotype formation and that CagA with more C-type motifs caused more cellular rearrangements. It has also been shown that CagA proteins from East Asian *H. pylori* strains bind to SHP-2 phosphatase more strongly than those from Western strains, due to the presence of the pY-(S/T/A/V/I)-X-(V/I/L)-X-(W/F) SHP-2 phosphatase binding motif (10) in the C-type motif, EPI**YATIDF**D (6, 13). CagA proteins lacking this motif (or the EPIYATIDDL motif present in CagA proteins from Western strains) do not appear to bind the phosphatase (6). Coculture of AGS cells with the Chinese *H. pylori* strains used in this study revealed that the CagA proteins with three EPIYA motifs induce significantly more cells to undergo transformation into the hummingbird phenotype and induce longer protrusions than CagA proteins with only two EPIYA motifs, due presumably to lack of the third (C-type) TPM.

In summary, we show that, in *H. pylori* strains from Jiangsu Province in China, there are two allelic forms of *cagA*: the more commonly occurring form of East Asian origin and the infrequently occurring form of Western origin. Strains with these different forms of *cagA* display marked differences in CagA phosphorylation and induction of the hummingbird phenotype. As all strains possess *cagA*, the differences in Cag phenotypes may represent important differences in the pathogenic potentials of strains infecting different patients. Large studies are now needed to examine the association between the number of tyrosine phosphorylation sites and disease, especially among populations where *cagA* is virtually ubiquitous, such as that in China.

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