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ORIGINAL ARTICLE

Variant of *Helicobacter pylori* CagA proteins induce different magnitude of morphological changes in gastric epithelial cells

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Abstract

Infection with *Helicobacter pylori cagA*-positive strains is associated with gastroduodenal diseases. The CagA protein is injected into gastric epithelial cells and supposedly induces morphological changes termed the 'hummingbird phenotype', which is associated with scattering and increased cell motility. The molecular mechanisms leading to the CagA-dependent morphological changes are only partially known. The present study was carried out to investigate the effect of CagA variants on the magnitude of gastric epithelial cell morphological changes. Recombinant 3' terminal domains of *cagA* were cloned and expressed in a gastric epithelial cell line and the hummingbird phenotype was quantified by microscopy. The 3' region of the *cagA* gene of Malaysian *H. pylori* isolates showed six sub-genotypes that differed in the structural organization of the EPIYA repeat sequences. The percentage of hummingbird cells induced by CagA increased with duration of transfection. The hummingbird phenotype was observed to be more pronounced when CagA with 4 EPIYA motifs rather than 3 or 2 EPIYA motifs was produced. The activity of different CagA variants in the induction of the hummingbird phenotype in gastric epithelial cells depends at least in part on EPIYA motif variability. The difference in CagA genotypes might influence the potential of individual CagAs to cause morphological changes in host cells. Depending on the relative exposure of cells to CagA genotypes, this may contribute to the various disease outcomes caused by *H. pylori* infection in different individuals.

Keywords: Helicobacter pylori, hummingbird phenotype, CagA EPIYA

INTRODUCTION

Infection with *Helicobacter pylori* is known to be associated with the development of chronic atrophy, peptic ulcers and gastric adenocarcinoma. However, the molecular mechanisms that underlie the development of *H. pylori*-associated gastroduodenal diseases remain to be elucidated. The search for *H. pylori* virulence factors related to outcome of infection has been hampered by the fact that there appear to be important differences in the predominant strains circulating in different geographic regions.[1] Thus, conclusions derived from data of a single geographic region may not apply to strains from other geographic regions. It is therefore important to define whether *H. pylori* variants are associated with clinical outcomes of the infection. This is particularly interesting as existing data have shown that the prevalence of peptic ulcer and gastric cancer is much higher in for instance Chinese patients rather than other ethnic groups.[2,3]

It has been reported that the CagA protein displays C-terminal variability which involves different types and/or numbers of repeat sequences.[4] Diversity in CagA may lead to functional differences of the protein and, thus, distinct clinical outcomes. To date, there is no established data with regard to the pattern of *H. pylori cagA* genotypes associated with various gastroduodenal diseases in the Malaysian population. Since *cagA* is considered an important pathogenicity marker and as information on the pathogenesis of disease inflicted in different ethnic groups are still lacking, this study was

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carried out to clarify the effect of peptide repeat-mediated CagA variation on the morphological changes of gastric epithelial cells.

MATERIALS AND METHODS

Construction of recombinants of the cagA gene
Twenty-one *H. pylori cagA* sequences were cloned in pCR2.1 selected on the basis of CagA repeat variants, ethnicity of the patients and severity of disease (Table 1). Oligonucleotide primers as described previously[5] were used to amplify the *H. pylori* 3' region of *cagA* gene. A *Bam*HI site was incorporated into the 5' end of the cag1 primer and a *Hind*III site was incorporated into the 5' end of the cag3 primer, which facilitated cloning of PCR products into pTriEx-4 vector (Novagen, USA). The primers sequence used were cag1_*Bam*HI: 5'-CGTGGATCCGACCCTAGTCGGTAATGGGTTA-3' and cag3_*Hind*III: 5'-TCGAAGCTTGTAATTGTCTAGTTTCGC-3'. Underlined are the sequences of the restriction site. The region amplified corresponds with amino acid position 852 to 1063 in the *H. pylori* 26695 strain. Due to the repeat variation in this region the length of the PCR products varied from 175 to 248 codons.

The PCR mixture of 25 µl volume that contained 1x PCR buffer, 0.2 mM dNTP, 10 pmol of each primer, 0.5 µl of template and 1U of *Pfu*Ultra High Fidelity Taq polymerase (Stratagene, USA) were prepared. Amplified product was cloned into pJET1 vector (Fermentas, USA) and digested with *BamHI* and *HindIII* restriction enzyme. Digested product was then cloned into pTriEx-4 expression vector (Novagen, Germany). The orientation and the sequence of the cloned gene were confirmed by sequencing.

Cell culture and transfection
The human gastric adenocarcinoma cell line, AGS (ATCC CRL 1739), was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. pTriEx-4_CagA recombinant plasmids (5 μg) were transfected into 5 x 10^5 AGS cells in 60 mm tissue culture dish with 15 μl of GeneJuice transfection reagent (Novagen, Germany). All transfection experiments were performed in triplicate with incubation at 16, 20 and 24 hours. To avoid differences in transfection efficiency the number of cells used, the incubation volume and the amount of plasmid was strictly standardized.

Analysis of AGS cell hummingbird phenotype formation
Cells that showed elongation phenotype (hummingbird cell) were observed after 16, 20, 24 hours of transfection using a phase contrast microscope Olympus CKX41 (Olympus, Japan) with 10x objective. Ten different areas in each dish were observed for each replicate. Photographs of AGS cells were captured using IC Capture 1.1 Software (The Imaging Source Europe GmBH). The percentage of hummingbird cells phenotype were calculated on the basis of the mean of the three replicates.

Western Blotting
After examination of cell morphology, the cells were harvested and then lysed in RIPA lysis buffer. Total cell lysates were subjected to 12% SDS-PAGE and the proteins were transferred to nitrocellulose membrane. The membranes were probed with anti-CagA antibody (Austral Biological, USA) and then visualized using WesternBreeze Chromogenic Western Blot Immunodetection kit (Invitrogen, Carlsbad, California).

Statistical analysis
Statistical analysis was performed with a two-tailed Student's test. *p* values of less than 0.05 were considered statistically significant.

RESULTS

Amplification and sequencing of cagA variants
PCR amplification of the *cagA* 3' region among 21 clinical isolates of *H. pylori* showed differences in the fragment length which varies from around 550 to 750 bp. Deduced amino acid of the nucleotide sequences of the C-terminal of CagA protein revealed the presence of six subgenotypes based on the EPIYA sequence as previously described.[6] Table 1 showed that three strains had 2 EPIYA motifs (one with A-C motifs and 2 with A-B motifs), 13 had 3 EPIYA motifs (7 with A-B-D motifs and 6 with A-B-C motifs) and 5 had 4 EPIYA motifs (1 with A-B-B-D motif and 4 with A-B-C-C motifs). The characteristics of patients and their diseases from which different CagA subtypes were isolated are as shown in Table 1.

Expression CagA protein in AGS cells
As depicted in Figure 1, immunoblotting of the cell lysates with anti-CagA antibody showed that CagA proteins were expressed in AGS after transfection. The proteins showed differences

TABLE 1. Characteristics of patients and diseases with different *H. pylori CagA* subtypes.

Strain	CagA EPIYA type	Patients' ethnicity	Patients' disease
HP601	A-C	Indian	Gastritis
HP586	A-B	Malay	Gastritis
HP280	A-B	Malay	Duodenitis
HP295	A-B-D	Indian	Intestinal metaplasia
HP361	A-B-D	Malay	Gastric ulcer
HP167	A-B-D	Chinese	Gastric ulcer
HP65	A-B-D	Chinese	Gastric ulcer
HP529	A-B-D	Chinese	Gastritis
HP210	A-B-D	Chinese	Intestinal metaplasia
HP644	A-B-D	Chinese	Duodenal ulcer
HP507	A-B-C	Chinese	Gastritis
HP486	A-B-C	Malay	Gastritis
HP102	A-B-C	Chinese	Duodenal ulcer
HQ362	A-B-C	Chinese	Duodenal + gastric ulcer
HP464	A-B-C	Indian	Gastritis
HQ192	A-B-C	Chinese	Duodenal + gastric ulcer
HQ326	A-B-B-D	Chinese	Gastric ulcer
HQ279	A-B-C-C	Malay	Gastritis
HP594	A-B-C-C	Indian	Gastritis
HP372	A-B-C-C	Indian	Intestinal metaplasia
HP491	A-B-C-C	Indian	Gastritis

in the molecular weight which varies from 29 to 37 kDa. No product was identified in AGS lysates that is not transfected (AGS control) and AGS transfected with pTriEx-4 without insert (pTriEx-4 control). The expression of CagA in AGS cells resulted in morphological changes with hummingbird phenotype as shown in Figure 2.

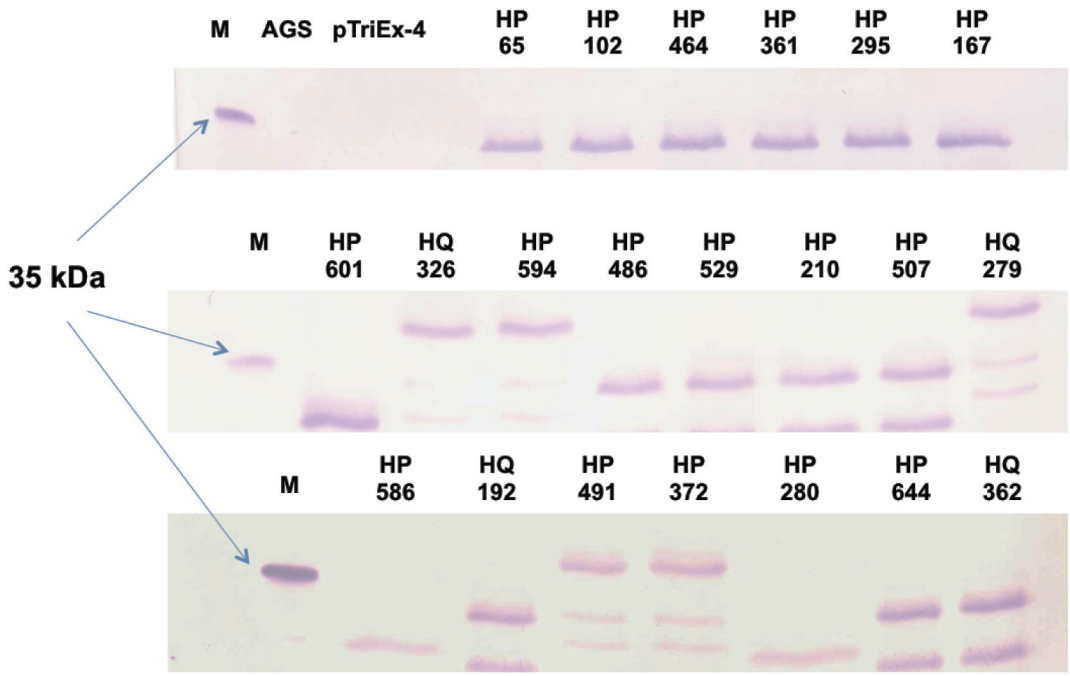

FIG. 1: Expression of recombinant CagA fragments cloned in pTriEx-4 vector and transfected into AGS cells. M; Western blot protein marker. AGS cells were transfected with no DNA (AGS cells alone), vector pTriEx-4 alone (vector with no insert) or vector with recombinant CagA.

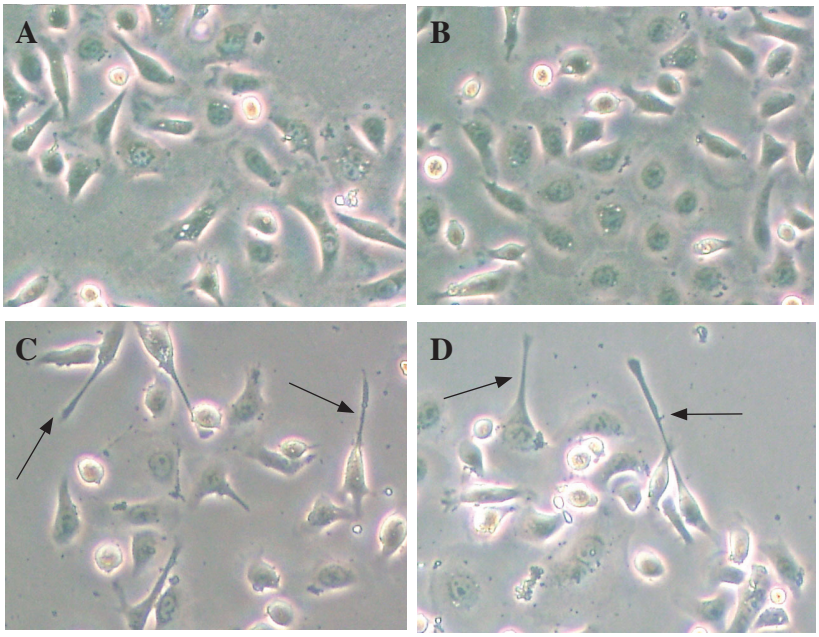

FIG. 2: AGS cells were transiently transfected with control and CagA. A. AGS cells alone, B. AGS cells transfected with pTriEx-4 without insert, C and D. AGS cells transfected with pTriEx-4_CagA. Hummingbird phenotype was characterized by spreading and elongation of the cells (as shown by an arrow).

Induction of AGS cell hummingbird phenotype by different variants of CagA

To characterize the extent of this phenotype effect, the morphology of CagA-expressing cells with hummingbird phenotype was monitored at three different transfection incubation times (Figure 3). At 16 hours after transfection, 5 – 8% of the cells exhibited the hummingbird phenotype compared to 1 – 1.5% of control cells. After 24 hours transfection, trend of AGS cells

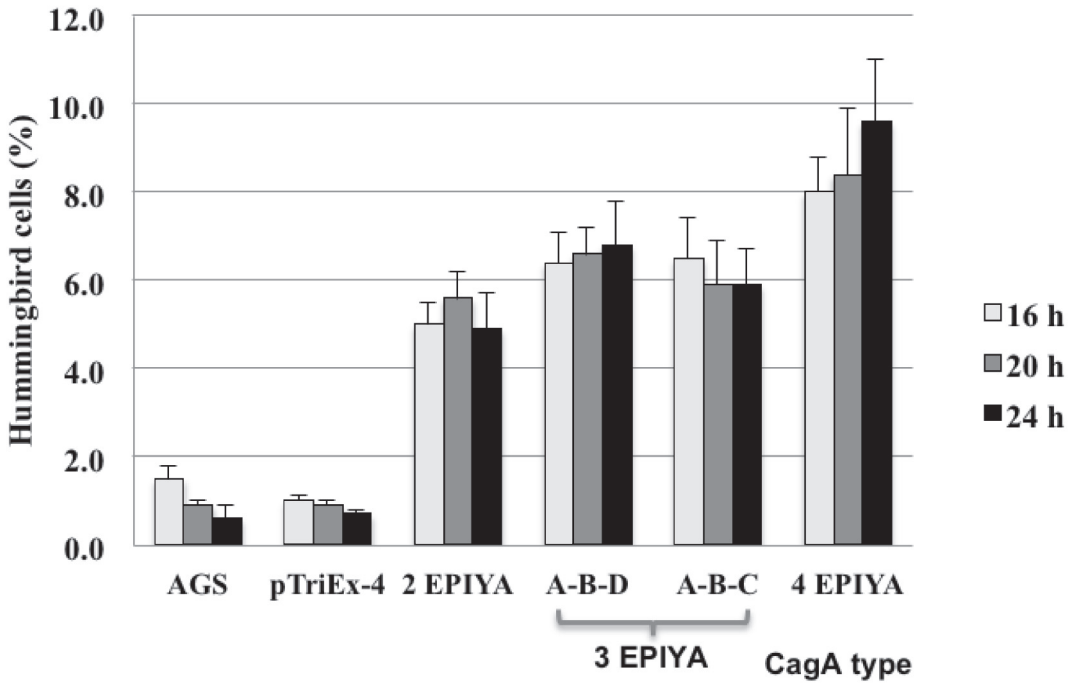

FIG. 3: Percentage of cells showing humming phenotype after CagA transfection from 16 to 24 hours.

showing hummingbird phenotype depend on the numbers and type of CagA EPIYA motif. In CagA containing 3 EPIYA and CagA containing 4 EPIYA motifs, the percentage of AGS cells showing hummingbird phenotype increases from 6.4 to 6.8% and 8.0 to 9.6%, respectively. Cells transfected with CagA containing 3 EPIYA motifs of A-B-C type showed a decreasing trend in the percentage of hummingbird phenotype from 6.5 to 5.9%. However, the trend of hummingbird phenotype formation in cells transfected with CagA containing 2 EPIYA motifs was not consistent with yields of 5.0% after 16 hours, 5.6% after 20 hours and then decreased to 4.9% after 24 hours.

Further analysis of the hummingbird phenotype caused by different variants of CagA and at different transfection times is shown in Table 2. After 16 and 20 hours of transfection, the statistical analysis shows that percentages of hummingbird cells formation were not significantly different between cells transfected with variant of CagA proteins. However, after 24 hours transfection there was a significant difference in the percentage of hummingbird cells formation among cells transfected with CagA containing 4 EPIYA motifs compared to those with CagA containing 3 EPIYA motifs of the A-B-C type ($p = 0.011$), and also cells transfected with CagA containing 4 EPIYA motifs compared to those transfected with CagA containing 2 EPIYA motifs ($p = 0.028$). There is no significant difference observed between cells transfected with CagA containing A-B-D motifs with CagA containing A-B-C motifs or CagA with 4 EPIYA motifs. CagA with an A-B-D motif and CagA with an A-B-C motif showed no significant difference in the induction of morphological changes in AGS cells. CagA containing 4 EPIYA motifs including one with EPIYA-D (A-B-B-D motif) showed similar strength in inducing morphological changes compared to CagA containing 4 EPIYA with A-B-C-C motif. Cells transfected with CagA containing 2 EPIYA with A-C motif (HP601) yielded higher percentage of hummingbird phenotype compared to CagA with A-B motif. However, the differences are not statistically significant (HP601 *vs* HP586; $p = 0.09$, HP601 *vs* HP280; $p = 0.08$).

TABLE 2. The percentage of AGS cells with hummingbird phenotype after transfection with variant of CagA proteins at 16 h, 20 h and, 24 h. Data were expressed as mean ± standard error of the mean from triplicates.

Transfected cells	% of hummingbird cells		
	16 h	20 h	24 h
AGS control	1.5 ± 0.3	0.9 ± 0.1	0.6 ± 0.3
pTriEx-4 control	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
2 EPIYA-CagA	5.0 ± 0.5	5.6 ± 0.6	4.9 ± 0.8
3 EPIYA-CagA;			
A-B-D motif	6.4 ± 0.7	6.6 ± 0.6	6.8 ± 1.0
A-B-C motif	6.5 ± 0.9	5.9 ± 1.0	5.9 ± 0.8
4 EPIYA-Caga	8.0 ± 0.8	8.4 ± 1.5	9.6 ± 1.4

Statistical analysis: Two-tailed Student's t test (*p value is significant):

A-B-D type *vs* A-B-C type;	16 hours, p = 0.943
	20 hours, p = 0.439
	24 hours, p = 0.408
A-B-D type *vs* 4 EPIYA CagA;	16 hours, p = 0.242
	20 hours, p = 0.158
	24 hours, p = 0.078
A-B-C type *vs* 4 EPIYA CagA;	16 hours, p = 0.221
	20 hours, p = 0.053
	24 hours, p = 0.011*
4 EPIYA CagA *vs* 2 EPIYA CagA;	16 hours, p = 0.090
	20 hours, p = 0.138
	24 hours, p = 0.028*

DISCUSSION

CagA is translocated from *H. pylori* into cells and is thought to play a crucial role in the pathogenesis of *cagA*-positive infection. However, the molecular mechanism by which CagA deregulates intracellular signaling is not fully understood. We decided to look at the effect of the expression of CagA proteins in AGS cells using *H. pylori* cell-free system. We constructed recombinant plasmids of variants *cagA* which are representatives of the major *cagA* types differing in amino acid sequences, EPIYA types and numbers. We show that products of CagA with 4 EPIYA motifs yielded greater levels of hummingbird phenotype compared to 3 or 2 EPIYA motifs, thus indicating the importance of numbers of EPIYA in influencing the changes to the epithelial cells' morphology. Other studies[7,8] have only shown that CagA protein containing 3 EPIYA motifs induce more hummingbird phenotype than CagA protein with 2 EPIYA motifs. However, there was no study showing the importance of variants EPIYA types in clinical isolates in inducing the formation of hummingbird phenotype.

In our previous report[6], analysis of *cagA* gene variation with ethnicity showed that CagA with A-B-D motifs was predominantly detected in isolates from Chinese patients who had shown to have a high risk of severe gastroduodenal disease. The data also showed that CagA with 4 EPIYA motifs is predominantly detected in isolates from Indians and Malays carrying the A-B-C-C type. We now demonstrate that the strength of activity of an individual CagA variant to induce morphological changes of AGS cells is dependent on the presence of EPIYA-C or EPIYA-D and will be enhanced with the presence of EPIYA-A or EPIYA-B. This suggests that ethnic preferences and hence invasiveness of *H. pylori* strains may be EPIYA repeat dependent.

Apparently, biological diversities among different CagA proteins are at least in part caused by the variations in the number and sequences of EPIYA motifs of the molecule. The observed distribution may help to explain some of the differences in *cagA*-positive *H. pylori*-associated diseases throughout the world. Transfection of (domains of) CagA into gastric epithelial cells provides a direct means for assessing the role of the CagA protein in morphological changes of gastric epithelial cells. The hypothesis to explain differences in the association of CagA types with patients' ethnicity as well as the data of transfection study suggest that strains colonizing the stomach of patients may change their abilities to induce epithelial-cell response, as part of their adaptation to the changing condition with the host milieu or host genetic factors. However, this analysis was done on the same host background i.e. gastric epithelial cell line. This excludes the effect of interaction between CagA protein with different host genetic factors.

In conclusion, the results of this study showed that the activity of CagA to induce hummingbird phenotype is dependent on its EPIYA numbers and EPIYA type. Different forms of CagA display differences in induction of hummingbird phenotype. The differences in CagA phenotypes may represent important differences in the pathogenic potentials of strains infecting different individuals.

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