Eur J Clin Microbiol Infect Dis (2010) 29:651–660
DOI 10.1007/s10096-010-0909-z

ARTICLE

Clinical relevance of *cagPAI* intactness in *Helicobacter pylori* isolates from Vietnam

L. T. Nguyen · T. Uchida · Y. Tsukamoto · T. D. Trinh · L. Ta · H. B. Mai · H. S. Le · D. Q. D. Ho · H. H. Hoang · T. Matsuhisa · T. Okimoto · M. Kodama · K. Murakami · T. Fujioka · Y. Yamaoka · M. Moriyama

Received: 20 November 2009 / Accepted: 8 March 2010 / Published online: 7 April 2010

Abstract The purpose of this paper is to investigate the relationship between clinical outcome and the intactness of *cagPAI* in *Helicobacter pylori* strains from Vietnam. The presence or absence of 30 *cagPAI* genes was investigated by polymerase chain reaction (PCR) and dot-blotting. *H. pylori*-induced interleukin-8 secretion and hummingbird phenotype, and *H. pylori* adhesion to gastric epithelial cells were examined. The serum concentration of pepsinogen 1, pepsinogen 2, and gastrin was also measured in all patients. *cagPAI* was present in all 103 Vietnamese *H. pylori* isolates, of which 91 had intact *cagPAI* and 12 contained only a part of *cagPAI*. Infection with the partial *cagPAI* strains was less likely to be associated with peptic ulcer and chronic gastric mucosal inflammation than infection with strains possessing intact *cagPAI*. The partial *cagPAI* strains lacked almost all ability to induce interleukin-8 secretion and the hummingbird phenotype in gastric cells. Their adhesion to epithelial cells was significantly decreased in comparison with intact *cagPAI* strains. Moreover, for the first time, we found an association between *cagPAI* status and the serum concentration of pepsinogens 1 and 2 in infected patients. *H. pylori* strains with internal deletion within *cagPAI* are less virulent and, thus, less likely to be associated with severe clinical outcomes.

L. T. Nguyen · T. Uchida · Y. Tsukamoto · M. Moriyama (✉)
Department of Molecular Pathology, Faculty of Medicine,
Oita University,
Yufu City, Oita 879-5593, Japan
e-mail: mmoriyam@med.oita-u.ac.jp

L. T. Nguyen · T. Okimoto · M. Kodama · K. Murakami · T. Fujioka
Department of Gastroenterology, Faculty of Medicine,
Oita University,
Oita, Japan

T. Uchida
Department of Human Environmental and Social Medicine,
Faculty of Medicine, Oita University,
Oita, Japan

T. D. Trinh · L. Ta · H. B. Mai · H. S. Le
108 Hospital,
Hanoi, Vietnam

D. Q. D. Ho · H. H. Hoang
Cho Ray Hospital,
Ho Chi Minh, Vietnam

T. Matsuhisa
Department of Gastrointestinal Endoscopy,
Tama-Nagayama Hospital, Nippon Medical School,
Tokyo, Japan

Y. Yamaoka
Department of Environmental and Preventive Medicine,
Faculty of Medicine, Oita University,
Oita, Japan

Introduction

Helicobacter pylori is a spiral bacterium that chronically colonizes the human stomach, and is currently recognized to be the etiologic factor responsible for gastritis, gastroduodenal ulcer, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma [1, 2]. Infection with *H. pylori* almost always results in chronic gastritis, but severe diseases such as peptic ulcer and gastric cancer occur in only a small proportion of infected patients, suggesting that clinical outcomes are determined by the interaction of bacterial virulence, host, and environmental factors [2, 3]. To date, several *H. pylori* virulence factors associated with severe clinical outcome have been reported, including *vacA*, *babA*, *iceA*, *oipA*, *dupA*, and, most notably, *cagPAI* [2, 4–9].

cagPAI is a cluster of about 30 genes spanning approximately 40 kb that have been acquired through horizontal transmission from unknown extraneous sources and integrated into the *H. pylori* chromosome (Fig. 1a) [6, 10]. *cagPAI* contains several genes encoding component proteins of the type IV secretion system (TFSS), a syringe-like structure responsible for the translocation of CagA protein and peptidoglycan from *H. pylori* into the host cell [6, 10–13]. Moreover, *cagPAI* is involved in the induction of many proinflammatory cytokines, including interleukin (IL)-8, from gastric epithelial cells [6, 14, 15]. Systematic mutagenesis studies have revealed that 17 out of 27 genes examined in *cagPAI* are essential for CagA translocation, while 14 genes are indispensable for the full induction of IL-8 [6, 14, 15], indicating the importance of *cagPAI* intactness in the pathogenesis of *H. pylori*-associated diseases. Therefore, it can be speculated that partial deletions within *cagPAI* are likely to affect bacterial virulence and, thus, clinical outcome.

H. pylori is categorized as *cagPAI*-negative or -positive, based mostly on the presence or absence of the *cagA* gene as a marker of the whole *cagPAI* stretch [6]. However, *cagPAI* is usually subject to internal deletions [16], suggesting that *cagPAI*-positive strains can be further divided into intact *cagPAI* (i.e., those with full-length *cagPAI*) and partial *cagPAI* groups (i.e., those lacking one to several genes). It is accepted that strains possessing *cagPAI* are more toxic and more associated with severe diseases than those lacking it [2, 3, 17]. Nevertheless, the virulence of *H. pylori* containing partial *cagPAI* and its association with clinical outcome has not been extensively studied. Moreover, there is currently no information about the *cagPAI* status of *H. pylori* from Vietnam. Therefore, the present study was performed to investigate the relationship between clinical outcome and the *cagPAI* status of *H. pylori* strains isolated from Vietnamese patients.

Materials and methods

Patients

A total of 103 Vietnamese patients (47 males and 56 females), aged 14 to 83 years (mean age, 45 years) were enrolled. Local ethical approval and written informed consent from all participants were obtained before the study. During gastroduodenoscopy, five biopsy samples were taken from each patient, including two from the antrum, two from the corpus, and one from the upper part of the lesser curvature. Twenty-five patients were endoscopically diagnosed as having peptic ulcer (13 with duodenal ulcers, six with gastric ulcers, and six with gastroduodenal ulcers) and 78 had chronic gastritis as determined by histological examination.

After endoscopy, blood samples from each patient were collected on the same day for the measurement of serum pepsinogen 1, pepsinogen 2, and gastrin.

Histology

Three biopsy specimens from the antrum, corpus, and upper part of the lesser curvature of each patient were examined by an experienced pathologist (T.U.), who was unaware of the characteristics of the *H. pylori* strains. For each biopsy specimen, the grades of neutrophil infiltration, mononuclear cell infiltration, atrophy, intestinal metaplasia, and *H. pylori* density were scored on the basis of the updated Sydney System (0, none; 1, mild; 2, moderate; 3, severe) [18].

H. pylori culture and genomic DNA extraction

Two biopsy specimens (one from the antrum and one from the corpus) were homogenized and inoculated onto Mueller Hinton II Agar medium (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 7% horse blood without antibiotics. The culture plates were incubated for up to 10 days at 37°C under microaerophilic conditions (10% O_2, 5% CO_2, and 85% N_2). Isolated strains were stored at −80°C in Brucella broth (Difco, Franklin Lakes, NJ, USA) containing 10% dimethylsulfoxide and 10% horse serum.

For DNA extraction, *H. pylori* was subcultured from the stock. Colonies on agar plates were harvested, and genomic DNA was extracted as described previously [19].

For in vitro experiments, we also used *H. pylori* strain *TN2GF4* (here denoted *TN2*) and its isogenic mutants, *ΔcagA*, *ΔcagE*, *ΔcagG*, *ΔcagPAI*, *ΔoipA*, and *ΔbabA*, which were used in our previous studies [20–24]. The strain *TN2* isogenic *cagY* mutant (*TN2ΔcagY*) was generated from the parental strain as described previously [25]. Briefly, genes upstream (*hp525–526*) and a gene downstream (*hp528*) of the *cagY* gene (*hp527*) were amplified and the corresponding polymerase chain reaction (PCR) products were cloned into pT7Blue vector (Novagen, Madison, WI, USA) to generate pT7hp525–526 and pT7hp528, respectively. A chloramphenicol (cm) cassette was inserted into pT7hp525–526, resulting in pT7hp525–526-cm. Next, the fragment hp525–526-cm was cut out from pT7hp525–526-cm and inserted into pT7hp528, resulting in the plasmid pT7hp525–526-cm-528. Finally, this plasmid was used to inactivate *cagY* by natural transformation. Inactivation of *cagY* was confirmed by both PCR and Southern blotting.

Detection of *cagPAI* genes

The presence or absence of 30 *cagPAI* genes was investigated by PCR using 32 sets of primers listed in Table 1. Unless

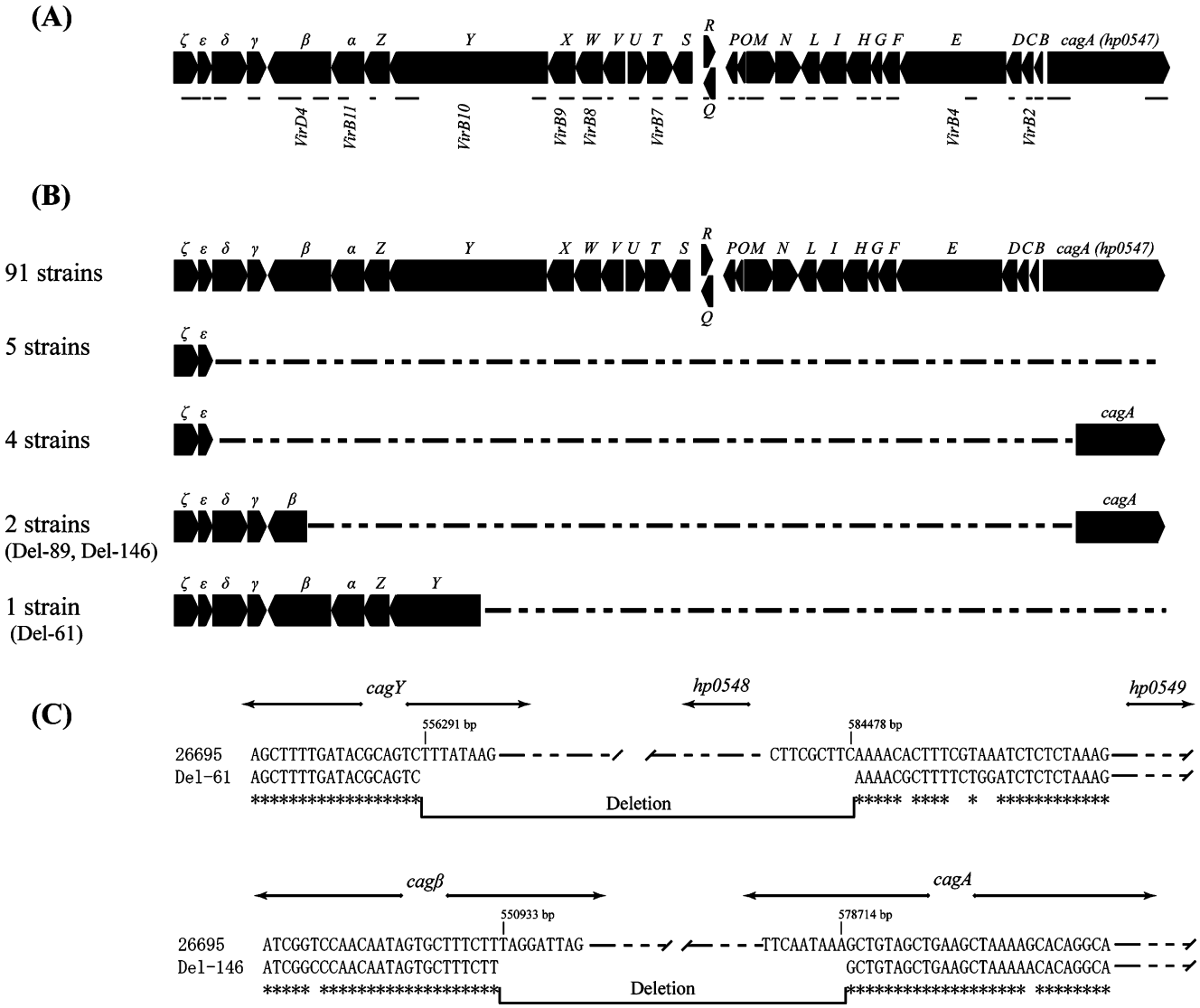

Fig. 1 *cagPAI* structure in *Helicobacter pylori* isolates from Vietnam. **a** *cagPAI* consists of about 30 genes (from *cagζ* to *cagA*), several of which are homologous with *vir* genes of *Agrobacterium tumefaciens*. Relative locations of the probes used in this study are presented as small lines under each gene. **b** *cagPAI* status of 103 Vietnamese *H. pylori* strains and the internal deletion patterns in 12 strains. **c** The endpoints of deletion were elaborated in two representative strains, Del-61 and Del-146, by alignment with the sequence of *H. pylori* 26695

stated otherwise, all primers were designed based on the nucleotide sequence of strain NCTC 11638 (GenBank accession numbers AF282852.1 and AF282853.1) using FastPCR software (downloaded from http://www.bio center.helsinki.fi/bi/Programs/fastpcr.htm).

To avoid false-negative results of PCR due to variations in the primer annealing sites, dot-blotting was performed as described previously [26]. Briefly, 300 ng of sample DNA was mixed with denaturing buffer (0.8 N NaOH; 1.5 M NaCl) and transferred to a Hybond N+ membrane (Amersham Biosciences, Buckinghamshire, UK) using a 96-well Bio-Dot apparatus (Bio-Rad, Ivry-sur-Seine, France). DNA of the reference strain ATCC43504 and human DNA were used as positive and negative controls, respectively. The probes were generated from genomic DNA of ATCC43504 by PCR using the corresponding primer sets and purified with an Illustra GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). With the use of the ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Biosciences), the probes were labeled with horseradish peroxidase, hybridized to the membranes at 42°C overnight, and, finally, exposed to Hyperfilm ECL.

A strain was defined as lacking a given gene if the results of PCR and dot-blotting were both negative, whereas the presence of a given gene required at least one positive result.

Nucleotide sequencing

The genomic regions of interest were initially amplified with primer set *cagEmpty* (Table 1) [27]. The amplicons were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence alignment was generated using the ClustalX program (http://clustalw.ddbj.nig.ac.jp/top-e.html).

Analysis of *H. pylori*-induced IL-8 production in gastric cell lines

The experiments were performed twice independently, as described previously [8]. Briefly, the gastric epithelial cell line MKN45 was seeded into 24-well plates and grown overnight in RPMI 1640 medium supplemented with 10% FBS. *H. pylori* was harvested from the agar dishes and washed twice with PBS before being added to the culture wells with a bacterium-to-cell ratio of 50:1. The plates were incubated at 37°C for the indicated periods of time in an environment containing 5% CO_2 and 95% air. The concentration of IL-8 in the cell culture supernatant was measured with the CXCL8/IL-8 ELISA Kit (R & D Systems, Minneapolis, MN, USA).

Analysis of *H. pylori*-induced hummingbird phenotype in AGS cells

The gastric epithelial cell line AGS was maintained and infected with clinical *H. pylori* strains as described above. After 36 h of co-culture, formation of the hummingbird phenotype was examined microscopically in five randomly chosen fields.

Quantification of *H. pylori* adhesion to gastric cell lines

Adhesion of *H. pylori* to gastric epithelial cells was measured by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), as described previously [28]. Briefly, *H. pylori* was added to culture wells containing MKN45 cells, as described above, and incubated for 3 h at 37°C. After vigorous washing three times with PBS to remove unbound bacteria, the cells were harvested and incubated with polyclonal rabbit anti-*H. pylori* antibody (DAKO, Glostrup, Denmark) (diluted 1:50) for 2 h at 4°C. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulins (Invitrogen, Eugene, OR, USA) (diluted 1:200) for 2 h at 4°C. Finally, the cells were washed, resuspended in PBS, and immediately subjected to flow cytometry. The experiments for negative control samples were done exactly as described above, except that *H. pylori* was not used.

Flow cytometric data from 2×10^4 cells were collected and analyzed with WinMDI software (downloaded from http://facs.scripps.edu/software.html). Cellular debris, non-adherent bacteria, and cell clumps were excluded from analysis by gating. The results were expressed as mean fluorescence value ± standard error of the mean (SE) from four independent experiments.

Measurement of serum pepsinogen 1 (PG1), pepsinogen 2 (PG2), and gastrin

Serum PG1, PG2, and gastrin were quantified using ARCHITECT pepsinogen I.II (Abbott, Tokyo, Japan) and Gastrin RIA Kit (Kyowa, Tokyo, Japan), in accordance with the manufacturers' instructions.

Statistical analysis

The quantitative data were expressed as mean ± SE. Fisher's exact test, the Mann–Whitney rank sum test, independent *t*-test, one-way analysis of variance (ANOVA) test, and Spearman's correlation were used. Differences at $p<0.05$ were regarded as statistically significant.

Results

cagPAI status in *H. pylori* strains from Vietnam

Based on the results of PCR and dot-blotting, we found that 91 out of 103 strains had all of the *cagPAI* genes (regarded as intact *cagPAI*), 12 contained only part of *cagPAI* (regarded as partial *cagPAI*), and none were *cagPAI*-negative (Fig. 1b).

In the 12 strains possessing partial *cagPAI*, four patterns of deletion were observed: deletion from *cagδ* through *cagA* (five strains), deletion from *cagδ* to the left half of *cagA* (four strains), deletion from the right half of *cagβ* to the left half of *cagA* (two strains), and deletion from the right half of *cagY* through *cagA* (one strain) (Fig. 1b).

By sequencing, we clarified the endpoints of *cagPAI* deletion in two representative strains, Del-61 and Del-146. Alignment with the sequence of *H. pylori* strain 26695 (GenBank accession number AE000511) revealed that the deletion in strain Del-61 spanned the stretch from nucleotide 556291 (corresponding to nucleotide 2716 of *cagY*) to nucleotide 584478 (located in the intergenic region between *hp0548* and *hp0549*). In strain Del-146, the deletion extended from nucleotide 550933 (corresponding to nucleotide 717 of *cagβ*) to nucleotide 578714 (corresponding to nucleotide 1794 of *cagA*) (Fig. 1c).

Table 1 List of primers used in this study

cagPAI genes	Primer	Primer sequence (5′–3′)	PCR product size (bp)
cagA/hp0547[a]	cagA-f1	AACAGGACAAGTAGCTAGCC	701
	cagA-r1	TATTAATGCGTGTGTGGCTG	
	cagA-f2	GTGGGTAAAAATGTGAATCG	730
	cagA-r2	CTGCAAAAGATTGTTTGGCAGA	
cagB	cagB-f	TGTCCCAACCATTTTTCTTTCT	233
	cagB-r	GGGAAACAAAATGGAAAACAA	
cagC/hp0546 (***VirB2***)	cagC-f	GCAAAAACAGTCGCCTGACCTCT	158
	cagC-r	GCCAGTCCTACAGAAGGCGTCACT	
cagD/hp0545	cagD-f	TTTGAGAGACTCTACGGTGCT	152
	cagD-r	CGACCAACAAACAACGCTGCTT	
cagE/hp0544 (***VirB4***)	cagE-f	GCGATTGTTATTGTGCTTGTAG	329
	cagE-r	GAAGTGGTTAAAAAATCAATGCCCC	
cagF/hp0543	cagF-f	CTTGCGTCATTTTTCCCACT	417
	cagF-r	GAAACAAAATTTGCGTGAACAA	
cagG/hp0542	cagG-f	TCGGTGGTAAAAACGATGAA	381
	cagG-r	TTGCTTGGTGTCTTATCATTGG	
cagH/hp0541	cagH-f	GCTATCAAGGGAATCGGCTGT	207
	cagH-r	ACCACCCCTAAAAATCCCAGTGC	
cagI/hp0540	cagI-f	AGATCGCAACTTCTATGAGCGCAT	452
	cagI-r	AGGAAGTTCTTCTGACAACGCT	
cagL/hp0539	cagL-f	GAAGCTGTACCTCCAATGTTGC	312
	cagL-r	CAAAAAAGCGGCCATTGCTT	
cagN/hp0538	cagN-f	AAAGCGGCTAAGCACAAAGG	437
	cagN-r	CTCTTCTCACAAATAGCGCACG	
cagM/hp0537	cagM-f	TTTGTCGGCTAATGTGGAGCAGTT	494
	cagM-r	ACACACCCATTTGAAGCAAAGCCT	
cagO	cagO-f	CAAAGAATACCCCACTTGCTG	189
	cagO-r	TTGTATGCGGCTTGTTGGTA	
cagP/hp0536	cagP-f	CCAATTTTGCCATTGAGTCAT	188
	cagP-r	GGGCAGGGGTGATTTTAGTT	
cagQ/R/hp0535	cagQ/R-f	TTACCAACCAAAGCAGATCCCA	220
	cagQ/R-r	CTTTGTTGGGTGGCGGAACA	
cagS/hp0534	cagS-f	GCGTTTTGCACTCCTTTTTC	393
	cagS-r	CATGAAAGATTATCGCGCTGT	
cagT/hp0532 (***VirB7***)	cagT-f	CCATGTTTATACGCCTGTGT	301
	cagT-r	CATCACCACACCCTTTTGAT	
cagU/hp0531	cagU-f	CAATTCGCTAAACGCTCCAT	296
	cagU-r	TCCCACCCATACACAATCCT	
cagV/hp0530	cagV-f	TGATAGCTTCTGCTCGGACT	185
	cagV-r	AGCCGTTGTGGATAGTGCAACTTC	
cagW/hp0529 (***VirB8***)	cagW-r	AACTAGGAGCGTTGTTGCCA	906
	cagW-r	CATCTATTGGTGCATCAGGGAGT	
cagX/hp0528 (***VirB9***)	cagX-f	TTGAGCGCATTAGCTTGAGC	619
	cagX-r	TAGAAGCAGCAGCACTTGAC	
cagY/hp0527 (***VirB10***)	cagY-f1	CCGTTCTTTCAGCAATCGGT	565
	cagY-r1	TGGCAGTCTAGCAGACAAGT	
	cagY-f2	CCTTTCACTTCTGCCTTTGC	914
	cagY-r2	ACCGCAAAACCTTTGACAAC	
cagZ/hp0526	cagZ-f1	CTGAAACGCCACGCCCATCA	154
	cagZ-r1	TCTGCAACAAACTCTCCAAGCGTT	
cagα/hp0525 (***VirB11***)	cagα-f	TCTATCAGGCCGCATTCTCA	258
	cagα-r	GAACAAGCGATCAGTGCGAT	
cagβ/hp0524 (***VirD4***)	cagβ-f1	CAATGCTTCGGCTCACATCT	896
	cagβ-r1	GGCTAAGTTGGTGTTCCCTGA	

Table 1 (continued)

cagPAI genes	Primer	Primer sequence (5′–3′)	PCR product size (bp)
	cagβ-f2	TGTTAGGCGTGATGAGCTTG	627
	cagβ-r1	TGGAAGACTTTTTGTATAACACCTT	
cagγ/hp0523	cagγ-f	GCCTATCCATGCCAAAAGGT	414
	cagγ-r	AGCGCCATTAGGGTTGTGTT	
cagδ/hp0522	cagδ-f	AGCAACCGCTGTATCGCTCATAGG	396
	cagδ-r	ATGAGATCCGCATAAGTCCCCA	
cagε/hp0521	cagε-f	GCTGTAAGGGCGTTTTACGA	252
	cagε-r	AAATTCCATTGCATTTCAAGGT	
cagζ/hp0520	cagζ-f	ATAGCGTTTTGTGCATAGAA	877
	cagζ-r	ATCTTTAGTCTCTTTAGCTT	
cagEmpty[b]	cagEmpty-f	ACTTTCACGCCCTTTCCCTCC	
	cagEmpty-r	TTGCATGCGTTATTATTTCAC	

[a] Ikenoue et al. [30]

[b] Björkholm et al. [27]

cagPAI status and clinical outcomes

Twenty-five (27.5%) of 91 patients infected with intact *cagPAI* strains developed peptic ulcer, whereas none of the 12 patients infected with partial *cagPAI* strains had such disease. This difference was statistically significant ($p<0.05$) (Table 2).

We analyzed the relationship between *cagPAI* status and the histological scores in 78 gastritis patients. Except for mononuclear cell infiltration and *H. pylori* density in the antrum, other histological scores were significantly higher in patients infected with intact *cagPAI* strains than in those infected with partial *cagPAI* strains, irrespective of the biopsy site ($p<0.05$) (Fig. 2). Intestinal metaplasia was rare, and was, therefore, excluded from the analysis.

Taken together, these data suggested that *H. pylori* strains containing partial *cagPAI* were associated with less severe disease and milder gastritis than those with intact *cagPAI*.

Association of serum PG1 and PG2 with *cagPAI* status

Patients infected with intact *cagPAI* strains had significantly higher serum concentrations of PG1 and PG2 than those infected with partial *cagPAI* strains (77.3±5.5 vs. 54.6±4.2 ng/ml and 19.7±1.6 vs. 10.9±1.0 ng/ml, $p<0.05$), whereas there was no difference in serum gastrin between the two groups (143.4±8.3 vs. 120.8±14.5 pg/ml). Interestingly, we also observed correlations between PG1 level and neutrophil infiltration score in the corpus ($p=0.018$) and between PG2 level and neutrophil infiltration score in both the antrum and the corpus ($p=0.023$ and $p<0.001$, respectively).

As expected, the PG1/PG2 ratio, which reflects gastric mucosal atrophy, was significantly lower in patients infected with intact *cagPAI* strains than in those infected with partial *cagPAI* strains (4.2±0.14 vs. 5.1±0.28, $p<0.05$).

Table 2 Association between *cagPAI* status and clinical outcomes

cagPAI genotype	Diseases	
	Gastritis	Peptic ulcer
Partial ($n=12$)	12	0
Intact ($n=91$)*	66	25

*$p=0.036$, Fisher's exact test

Analysis of *H. pylori*-induced IL-8 production and hummingbird phenotype in gastric cell lines

We randomly selected nine intact *cagPAI* strains and ten partial *cagPAI* strains and compared their abilities to induce IL-8 secretion and the hummingbird phenotype in gastric epithelial cells. As shown in Fig. 3a, b, the intact *cagPAI* strains always induced much higher production of IL-8 and a higher rate of the hummingbird phenotype than the partial *cagPAI* strains or mock at any time point ($p<0.01$). In contrast, there was no significant change in hummingbird phenotype formation or IL-8 secretion from cells infected with partial *cagPAI* strains as compared to mock, indicating that the *cagPAI* of these strains was nonfunctional.

H. pylori adhesion to gastric epithelial cells is associated with *cagPAI* status

Because *cagPAI* encodes the type IV secretion system, which targets the α5β1 receptor of the host cell [29], we examined whether internal deletions within this locus affect the ability of *H. pylori* to bind to the gastric epithelium.

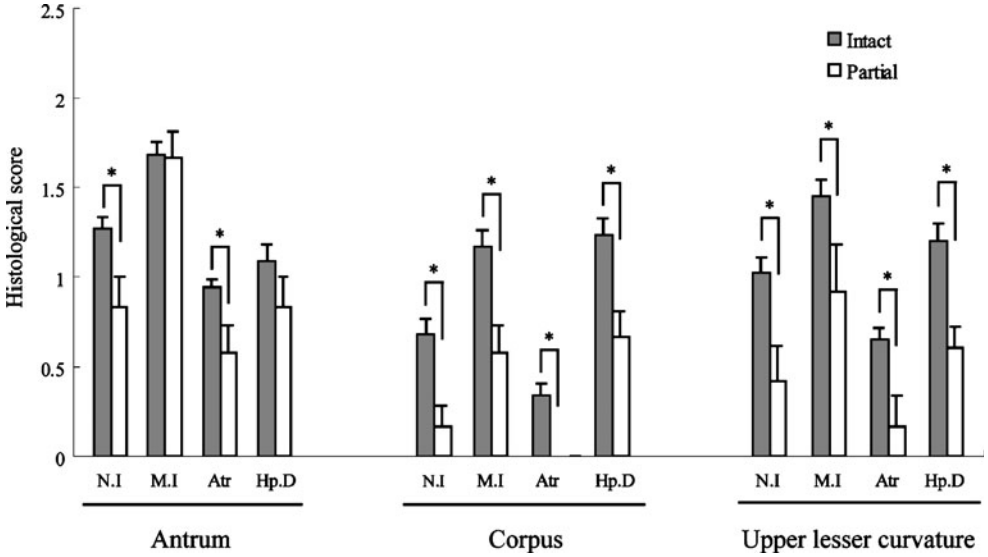

Fig. 2 *cagPAI* status and chronic gastritis. Generally, the histological scores in patients infected with intact *cagPAI* strains were higher than in patients infected with partial *cagPAI* strains, irrespective of the biopsy site (antrum, corpus, and upper lesser curvature). *$p<0.05$, Mann–Whitney rank sum test; *N.I* neutrophil infiltration; *M.I* mononuclear cell infiltration; *Atr* atrophy; *Hp. D H. pylori* density

Flow cytometry experiments with the 19 clinical isolates of *H. pylori* mentioned above showed that the intact *cagPAI* strains bound to gastric epithelial cells more strongly than the partial *cagPAI* strains (Fig. 4a). To confirm this observation, we repeated the experiments with *H. pylori TN2* and its various mutant strains. We found that deletion of the *cagA* gene did not significantly affect the binding ability of *H. pylori*. In contrast, the adhesion of *TN2ΔcagG* to gastric epithelial cells was significantly lower than that of *TN2*, but was still higher in comparison with *TN2ΔcagE*, *TN2ΔcagY*, or *TN2ΔcagPAI*. Knock-out of the *cagE* or *cagY* gene reduced adhesion to the level of *TN2ΔcagPAI*. However, all of the mutant strains still retained considerable adhesion ability compared to the negative control, indicating the involvement of other factors in the binding activity of *H. pylori*. In fact, as expected, the deletion of *oipA* and *babA*, which are well-known adhesion factors, also decreased the *H. pylori* binding ability significantly (Fig. 4b).

Discussion

The aim of this study was to characterize the *cagPAI* structure of Vietnamese *H. pylori* isolates, which have never been investigated before. To overcome the weaknesses of previous studies examining only a limited

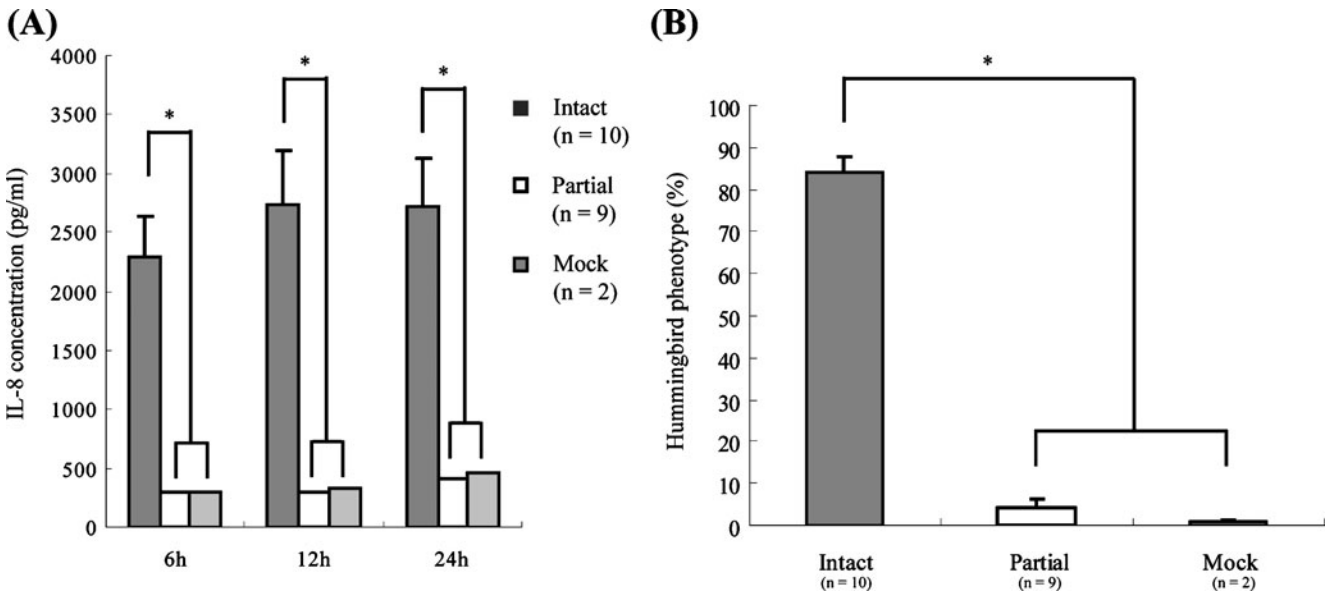

Fig. 3 *cagPAI* status and *H. pylori*-induced IL-8 production and hummingbird phenotype in gastric epithelial cells. The intact *cagPAI* strains induced much higher production of IL-8 at 6 h, 12 h, and 24 h (**a**) and higher rate of the hummingbird phenotype at 36 h (**b**) compared to the partial *cagPAI* strains or mock, but there was no significant difference between the partial *cagPAI* strains and mock (**a** and **b**). *$p<0.01$, independent *t*-test

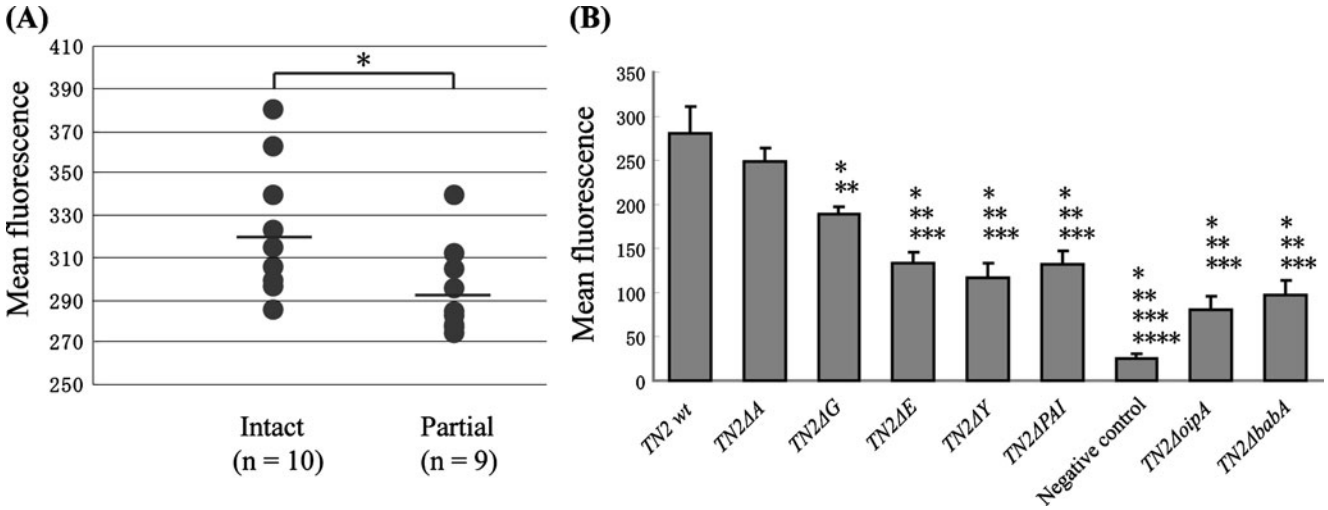

Fig. 4 *cagPAI* status and *H. pylori* adhesion to gastric epithelial cells. **a** In 19 clinical isolates selected randomly, the adhesion of partial *cagPAI* strains to gastric epithelial cells was significantly lower than that of intact *cagPAI* strains. *$p<0.05$, Mann–Whitney rank sum test. **b** Except for *TN2ΔA*, other mutant strains showed decreased adhesion compared with the wild type. *, **, ***, ****$p<0.05$ compared with *TN2 wt*, *TN2ΔA*, *TN2ΔG*, and *TN2ΔE* or *TN2ΔY*, respectively, one-way analysis of variance (ANOVA) and independent *t*-test

number of *cagPAI* genes, we used up to 32 probes covering all 30 genes located in this locus. Moreover, the combination of PCR and dot-blotting allowed us to avoid any false-negative results of PCR due to variations in the primer annealing sites. We found that *cagPAI* was present in all of the Vietnamese *H. pylori* isolates, the majority of which (91/103) had a complete set of *cagPAI* genes, while the remaining 12 possessed only part of the locus, indicating that *cagPAI* was obviously not a uniform, highly conserved structure. Our results suggest that *cagA* should not be regarded as an absolute marker for intact *cagPAI* in Vietnam because the presence of *cagA* predicted an intact *cagPAI* status correctly in only 88% of cases (91/103). Moreover, deletion within the *cag* island spanned quite a long DNA stretch containing several genes, suggesting that *cagPAI* should be investigated using several sets of primers spanning the locus, rather than focusing on certain genes.

Consistent with previous reports [30, 31], we found that *H. pylori* strains with partial *cagPAI* were less likely to cause peptic ulcer diseases, and were associated with milder gastritis, indicating that these strains were less virulent than those possessing intact *cagPAI*. These findings were in agreement with the in vitro experiment results showing that partial *cagPAI* strains lacked almost all ability to induce IL-8 secretion and the hummingbird phenotype in gastric cell lines. Their adhesion to epithelial cells was also significantly decreased. The intactness of *cagPAI* is a prerequisite for assembly of the type IV secretion system, by which *H. pylori* sticks to the host cell surface, disrupts cell junctions by translocating CagA into the cytoplasm, and induces the secretion of inflammatory cytokines [6, 11, 12, 14, 29, 32]. These various events allow *H. pylori* to avoid mechanical clearance, escape the acidic environment of the stomach lumen, gain nutrients, and, finally, to survive, replicate, and effectively colonize the gastric mucosa [33–36]. In fact, our histological scoring data showed that the density of *H. pylori* strains with intact *cagPAI* in biopsy specimens was generally higher, suggesting that these strains had a better capacity to colonize the gastric mucosa, compared with those possessing partial *cagPAI*.

The adhesion of *H. pylori* to epithelial cells is reported to be mediated by several bacterial factors, especially outer membrane proteins such as BabA, OipA, and SabA [25, 37–39]. Our data support the role of *oipA* and *babA* in adhesion, and also indicate the significance of *cagPAI* in the adhesion of *H. pylori* to gastric epithelial cells. It has been reported that the pilus of *H. pylori* TFSS is able to bind to the integrin α5β1 receptor on gastric epithelial cells via the RGD motif of CagL protein [29]. Because TFSS is encoded by several genes in *cagPAI*, deletions inside this locus are likely to affect the TFSS structure, leading to a reduction in binding activity. As both *cagE* and *cagY* are essential for the formation of TFSS [14], the deletion of these genes abolishes the *cagPAI*-dependent binding activity, as our data showed. However, the deletion of *cagG* appeared to have only a minor effect on the adhesion ability of *H. pylori*. Interestingly, *cagG* has been reported to be unnecessary for the induction of IL-8 secretion from host cells [14]. This result, together with ours, suggests that the CagG protein may play only a supplementary role in the activity of the TFSS. However, it should be noted that these in vitro data may not actually reflect what happens in the in vivo condition because *H. pylori* can adapt well to the

gastric environment. Further in vivo binding studies are required to address this issue.

The pepsinogens are digestive proenzymes that are specifically secreted by the gastric mucosa. Pepsinogens are divided into two distinct types, PG1 and PG2, which have different immunological and biochemical properties. PG1 is secreted by chief and mucous neck cells of the corpus [40, 41]. PG2 is produced also by these cells and others such as the cells in the cardiac and pyloric glands, as well as Brunner's glands in the duodenum [40, 41]. The PG1/PG2 ratio has long been used as a non-invasive method for the evaluation of gastric mucosal atrophy [40, 41]. Consistent with the histological scores, the PG1/PG2 ratio in patients infected with intact *cagPAI* strains was lower, suggesting that the gastric mucosal atrophy in these patients was more advanced than in patients infected with partial *cagPAI* strains. Moreover, we found that the serum concentration of PG1 and PG2 in *H. pylori*-positive patients was associated with *cagPAI* status, and this is the first time that such a phenomenon has been reported. In in vitro experiments, *H. pylori* was shown to directly stimulate pepsinogen secretion from gastric cells independently of *cagPAI* [42], a finding that seemed to be contradictory to ours. However, it should be noted that *H. pylori* might be able to affect pepsinogen secretion indirectly by inducing gastric mucosal inflammation, which cannot be reflected in an in vitro experiment. Correlations between the serum concentration of PG1 and PG2 and neutrophil infiltration in the antrum and/or corpus were observed in our study, as well as in others [43, 44]. Therefore, it can be speculated that intact *cagPAI* strains, with their stronger ability to induce gastric mucosal inflammation, are likely to stimulate more secretion of PG1 and PG2 in vivo than partial *cagPAI* strains.

In conclusion, our study has found intact and partial *cagPAI* in about 88 and 12% of Vietnamese *H. pylori*, respectively. Partial *cagPAI* strains are biologically less virulent and, thus, less likely to cause a severe clinical outcome than intact *cagPAI* strains.

Acknowledgment We thank Mrs. Yoko Kudou for her technical assistance.

References

1. Peek RM Jr, Blaser MJ (2002) *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2:28–37
2. Suerbaum S, Michetti P (2002) *Helicobacter pylori* infection. N Engl J Med 347:1175–1186
3. Ernst PB, Peura DA, Crowe SE (2006) The translation of *Helicobacter pylori* basic research to patient care. Gastroenterology 130:188–206
4. Atherton JC, Peek RM Jr, Tham KT, Cover TL, Blaser MJ (1997) Clinical and pathological importance of heterogeneity in *vacA*, the vacuolating cytotoxin gene of *Helicobacter pylori*. Gastroenterology 112:92–99
5. Basso D, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, Schiavon S, Guariso G, Ceroti M, Nitti D, Rugge M, Plebani M, Atherton JC (2008) Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. Gastroenterology 135:91–99
6. Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A (1996) *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci USA 93:14648–14653
7. Gerhard M, Lehn N, Neumayer N, Borén T, Rad R, Schepp W, Miehlke S, Classen M, Prinz C (1999) Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci USA 96:12778–12783
8. Lu H, Hsu PI, Graham DY, Yamaoka Y (2005) Duodenal ulcer promoting gene of *Helicobacter pylori*. Gastroenterology 128:833–848
9. Yamaoka Y, Kikuchi S, el-Zimaity HM, Gutierrez O, Osato MS, Graham DY (2002) Importance of *Helicobacter pylori oipA* in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. Gastroenterology 123:414–424
10. Akopyants NS, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, Reece CA, Bukanov NO, Drazek ES, Roe BA, Berg DE (1998) Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. Mol Microbiol 28:37–53
11. Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M, Meyer TF (2000) Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell Microbiol 2:155–164
12. Odenbreit S, Püls J, Sedlmaier B, Gerland E, Fischer W, Haas R (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science 287:1497–1500
13. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL (2004) Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. Nat Immunol 5:1166–1174
14. Fischer W, Püls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R (2001) Systematic mutagenesis of the *Helicobacter pylori cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol Microbiol 42:1337–1348
15. Selbach M, Moese S, Meyer TF, Backert S (2002) Functional analysis of the *Helicobacter pylori cag* pathogenicity island reveals both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms. Infect Immun 70:665–671
16. Kauser F, Khan AA, Hussain MA, Carroll IM, Ahmad N, Tiwari S, Shouche Y, Das B, Alam M, Ali SM, Habibullah CM, Sierra R, Mégraud F, Sechi LA, Ahmed N (2004) The *cag* pathogenicity island of *Helicobacter pylori* is disrupted in the majority of patient isolates from different human populations. J Clin Microbiol 42:5302–5308
17. Cover TL, Blaser MJ (2009) *Helicobacter pylori* in health and disease. Gastroenterology 136:1863–1873
18. Dixon MF, Genta RM, Yardley JH, Correa P (1996) Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 20:1161–1181
19. Uchida T, Kanada R, Tsukamoto Y, Hijiya N, Matsuura K, Yano S, Yokoyama S, Kishida T, Kodama M, Murakami K, Fujioka T, Moriyama M (2007) Immunohistochemical diagnosis of the *cagA*-gene genotype of *Helicobacter pylori* with anti-East Asian CagA-specific antibody. Cancer Sci 98:521–528
20. Kudo T, Lu H, Wu JY, Ohno T, Wu MJ, Genta RM, Graham DY, Yamaoka Y (2007) Pattern of transcription factor activation in *Helicobacter pylori*-infected Mongolian gerbils. Gastroenterology 132:1024–1038

21. Lu H, Wu JY, Kudo T, Ohno T, Graham DY, Yamaoka Y (2005) Regulation of interleukin-6 promoter activation in gastric epithelial cells infected with *Helicobacter pylori*. Mol Biol Cell 16:4954–4966
22. Saito H, Yamaoka Y, Ishizone S, Maruta F, Sugiyama A, Graham DY, Yamauchi K, Ota H, Miyagawa S (2005) Roles of *virD4* and *cagG* genes in the *cag* pathogenicity island of *Helicobacter pylori* using a Mongolian gerbil model. Gut 54:584–590
23. Tabassam FH, Graham DY, Yamaoka Y (2008) OipA plays a role in *Helicobacter pylori*-induced focal adhesion kinase activation and cytoskeletal re-organization. Cell Microbiol 10:1008–1020
24. Tabassam FH, Graham DY, Yamaoka Y (2009) *Helicobacter pylori* activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. Cell Microbiol 11:70–82
25. Yamaoka Y, Kwon DH, Graham DY (2000) A M(r) 34,000 proinflammatory outer membrane protein (*oipA*) of *Helicobacter pylori*. Proc Natl Acad Sci USA 97:7533–7538
26. Occhialini A, Marais A, Alm R, Garcia F, Sierra R, Mégraud F (2000) Distribution of open reading frames of plasticity region of strain J99 in *Helicobacter pylori* strains isolated from gastric carcinoma and gastritis patients in Costa Rica. Infect Immun 68:6240–6249
27. Björkholm B, Lundin A, Sillén A, Guillemin K, Salama N, Rubio C, Gordon JI, Falk P, Engstrand L (2001) Comparison of genetic divergence and fitness between two subclones of *Helicobacter pylori*. Infect Immun 69:7832–7838
28. Clyne M, Drumm B (1993) Adherence of *Helicobacter pylori* to primary human gastrointestinal cells. Infect Immun 61:4051–4057
29. Kwok T, Zabler D, Urman S, Rohde M, Hartig R, Wessler S, Misselwitz R, Berger J, Sewald N, König W, Backert S (2007) *Helicobacter* exploits integrin for type IV secretion and kinase activation. Nature 449:862–866
30. Ikenoue T, Maeda S, Ogura K, Akanuma M, Mitsuno Y, Imai Y, Yoshida H, Shiratori Y, Omata M (2001) Determination of *Helicobacter pylori* virulence by simple gene analysis of the *cag* pathogenicity island. Clin Diagn Lab Immunol 8:181–186
31. Maeda S, Yoshida H, Ikenoue T, Ogura K, Kanai F, Kato N, Shiratori Y, Omata M (1999) Structure of *cag* pathogenicity island in Japanese *Helicobacter pylori* isolates. Gut 44:336–341
32. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S (2003) Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. Science 300:1430–1434
33. Blaser MJ, Atherton JC (2004) *Helicobacter pylori* persistence: biology and disease. J Clin Invest 113:321–333
34. Mimuro H, Berg DE, Sasakawa C (2008) Control of epithelial cell structure and developmental fate: lessons from *Helicobacter pylori*. Bioessays 30:515–520
35. Mimuro H, Suzuki T, Nagai S, Rieder G, Suzuki M, Nagai T, Fujita Y, Nagamatsu K, Ishijima N, Koyasu S, Haas R, Sasakawa C (2007) *Helicobacter pylori* dampens gut epithelial self-renewal by inhibiting apoptosis, a bacterial strategy to enhance colonization of the stomach. Cell Host Microbe 2:250–263
36. Tan S, Tompkins LS, Amieva MR (2009) *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. PLoS Pathog 5(5):e1000407
37. Dossumbekova A, Prinz C, Mages J, Lang R, Kusters JG, Van Vliet AH, Reindl W, Backert S, Saur D, Schmid RM, Rad R (2006) *Helicobacter pylori* HopH (OipA) and bacterial pathogenicity: genetic and functional genomic analysis of *hopH* gene polymorphisms. J Infect Dis 194:1346–1355
38. Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Borén T (1998) *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 279:373–377
39. Mahdavi J, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T (2002) *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 297:573–578
40. di Mario F, Cavallaro LG (2008) Non-invasive tests in gastric diseases. Dig Liver Dis 40:523–530
41. Miki K, Urita Y (2007) Using serum pepsinogens wisely in a clinical practice. J Dig Dis 8:8–14
42. Lorente S, Doiz O, Trinidad Serrano M, Castillo J, Lanas A (2002) *Helicobacter pylori* stimulates pepsinogen secretion from isolated human peptic cells. Gut 50:13–18
43. Di Mario F, Cavallaro LG, Moussa AM, Caruana P, Merli R, Maini A, Bertolini S, Dal Bó N, Rugge M, Cavestro GM, Aragona G, Plebani M, Franzé A, Nervi G (2006) Usefulness of serum pepsinogens in *Helicobacter pylori* chronic gastritis: relationship with inflammation, activity, and density of the bacterium. Dig Dis Sci 51:1791–1795
44. Wagner S, Haruma K, Gladziwa U, Soudah B, Gebel M, Bleck J, Schmidt H, Manns M (1994) *Helicobacter pylori* infection and serum pepsinogen A, pepsinogen C, and gastrin in gastritis and peptic ulcer: significance of inflammation and effect of bacterial eradication. Am J Gastroenterol 89:1211–1218