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Polymorphism in the CagA EPIYA Motif Impacts Development of Gastric Cancer▽§

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Helicobacter pylori **causes diseases ranging from gastritis to peptic ulcer disease to gastric cancer. Geographically, areas with high incidences of *H. pylori* infection often overlap with areas with high incidences of gastric cancer, which remains one of the leading causes of cancer-related deaths worldwide. Strains of *H. pylori* that carry the virulence factor cytotoxin-associated gene A (*cagA*) are much more likely to be associated with the development of gastric cancer. Moreover, particular C-terminal polymorphisms in CagA vary by geography and have been suggested to influence disease development. We conducted a large-scale molecular epidemiologic analysis of South Korean strains and herein report a statistical link between the East Asian CagA EPIYA-ABD genotype and the development of gastric cancer. Characterization of a subset of the Korean isolates showed that all strains from cancer patients expressed and delivered phosphorylatable CagA to host cells, whereas the presence of the *cagA* gene did not strictly correlate to expression and delivery of CagA in all noncancer strains.**

Helicobacter pylori is a medically important pathogen, and although infection rates vary geographically, this bacterium colonizes more than 50% of the world's population globally (20, 28). This spiral-shaped, gram-negative, and microaerophilic bacterium chronically inhabits the unforgiving environment of the stomach and causes subclinical gastritis in the majority of patients. However, in some individuals, *H. pylori* colonization results in peptic ulcer disease; 75% of gastric ulcers and 90% of duodenal ulcers are attributed to *H. pylori* infection (19). At its most severe sequelae, *H. pylori* infection can lead to the development of two forms of gastric cancer, adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (11, 33, 34, 43). The association of *H. pylori* with stomach cancer led the World Health Organization to classify it as a class I carcinogen in 1994 (26). It currently remains the only bacterium to have obtained this perilous distinction.

Gastric cancer is the second most common cause of cancer deaths worldwide, and this fact could be reflective of the high incidence of *H. pylori* infection (18, 30, 32, 47). Interestingly, geographic areas with the highest levels of gastric cancer, which include most East Asian countries, also have the highest rates of *H. pylori* infection (1, 18, 47). Additionally, in East Asian countries, 90% of strains carry the cytotoxin-associated gene A (*cagA*) (27), which has emerged as a major contributor to disease severity. In fact, *cagA*-positive *H. pylori* strains are at least twice as likely to cause cancer as *H. pylori* strains without *cagA* (12, 22).

cagA is carried on the *cag* pathogenicity island (PAI), which carries genes that produce a type IV secretion apparatus that is used to directly inject CagA into host cells (15). Within the cells, CagA is phosphorylated by host cell kinases, forms a complex with SHP-2 (Src homology region 2-containing phosphatase 2) (25), and alters multiple host signaling pathways (23–25, 29, 36, 46). The phosphorylation of CagA occurs in the carboxy terminus of conserved tyrosine residues that are part of a repeated five-amino-acid sequence (Glu-Pro-Ile-Tyr-Ala) referred to as the EPIYA motif (24, 25).

Initial studies showed that CagA proteins from various *H. pylori* isolates migrated differently on denaturing gels (17). It was subsequently shown that a number of *cagA* alleles exist and that variation in the carboxy terminus of the protein is the major difference between the different alleles. Polymorphisms in the C terminus occur in the EPIYA region and typically involve changes in the amino acid sequences flanking the five-amino-acid repeat. The most-common motifs have been designated EPIYA-A, -B, -C, and -D (24) and are found in two distinct combinations by geographic location. Western CagA consists of a combination of EPIYA-A, -B, and -C motifs (up to five EPIYA-C motifs have been identified), whereas East Asian CagA contains a combination of EPIYA-A, -B, and -D motifs (4, 17, 24, 25, 31, 42).

EPIYA-C and -D serve as the primary CagA phosphorylation sites and are required for binding to SHP-2 (24). Among

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Western isolates, molecular epidemiological studies have indicated a correlation between disease severity and an increased number of EPIYA-C motifs (5, 24, 48). Indeed, in cases where Western strains are associated with cancer, most have multiple EPIYA-C motifs (8, 39). This increase may be due to elevated morphological transformation as a result of increased CagA phosphorylation and SHP-2 binding (24).

East Asian CagA containing the EPIYA-D motif demonstrates higher affinity for SHP-2 than Western CagA. This leads to greater morphological changes in infected cells (24) as well as greater levels of inflammation and atrophy (9). These findings, along with the fact that East Asian strains predominate in countries with the highest rates of gastric cancer, suggest that East Asian CagA may have the potential to induce more-severe forms of gastric disease (1, 18, 47).

In order to assess the correlation between the *cagA* genotype and *H. pylori*-induced disease severity, we examined a collection of isolates from South Korea, which has one of the highest rates of *H. pylori* colonization (44) and one of the highest rates of gastric cancer in the world (22, 41). Additionally, the majority of South Korean strains carry the East Asian *cagA* allele (16). Here, we present molecular epidemiologic evidence that there is a significant association between the development of gastric cancer and infection with *H. pylori* strains carrying the EPIYA-ABD genotype.

MATERIALS AND METHODS

Bacterial strains. Korean bacterial strains along with G27-MA (2), and its isogenic derivatives G27-MA Δ*cagA* (3) and G27-MA ΔPAI (provided by Manuel Amieva and constructed as described in Galgani et al. [21]), were cultured as previously described (14). Briefly, bacterial stocks preserved at −80°C were grown and expanded on antibiotic-supplemented horse blood agar plates. Overnight liquid cultures, consisting of brucella broth (BB) (Acumedia, Lansing, MI) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 10 μg/ml vancomycin (Amresco, Solon, OH), were subcultured to an optical density at 600 nm of 0.05 in fresh media and were grown for 18 h under microaerophilic conditions created by an Anoxomat evacuation/replacement system (Spiral Biotech, Norwood, MA).

Clinical isolate acquisition. Isolates were obtained from patients presenting with gastric symptoms to the Division of Gastroenterology in the Department of Internal Medicine at the College of Medicine of the Catholic University of Korea in Seoul, Korea. Written informed consent was received from each patient, and the protocol was approved by the Institutional Review Board of Human Research at the Catholic University of Korea. Biopsy samples were collected at the site of visible mucosal disturbance, and histology was performed to provide a diagnosis along with culture for the presence of gram-negative, spiral-shaped bacteria that produced a functional urease enzyme. Subsequently, a single-colony isolate was selected from each biopsy sample for further characterization. An extensive breakdown of the epidemiological characteristics of the patients can be found in Table 1, and a complete list of strains is available in Table S1 in the supplemental material. Strains are named such that the letters following the strain number indicate the disease state as follows: cancer (-CA), duodenal ulcer (-DU), gastritis (-G), and gastric ulcer (-GU).

***cagA* genotyping.** All the primers used in this study are listed in Table 2. Genomic DNA was extracted using the Easy-DNA kit (Invitrogen, Carlsbad, CA), and genotyping of the C terminus of *cagA* was performed by PCR using a modified version of the strategy developed by Argent et al. (7), as schematically depicted in Fig. 1A and B and Fig. S1 in the supplemental material. Briefly, amplification with primers cagA28F or cag2 and cagA-P1C or cagA-pA-1 (R) identifies an EPIYA-A motif. Amplification with primers cagA28F or cag2 and a 1:1 mixture of primers cagA-P2TA and cagA-P2CG indicates an EPIYA-B motif. Amplification using primers cagA28F or cag2 and cagA-P3E identifies the presence of either an EPIYA-C motif or an EPIYA-D motif, and an additional amplicon with cagA28F or cag2 and the unique cagA-pD (R) primer categorizes CagA as having an EPIYA-D motif. In some cases where the PCR amplification was inconclusive and to confirm results of the PCR genotyping, *cagA* was am-

TABLE 1. Epidemiological breakdown of the Korean collection

Epidemiological characteristic	All patients						Patients with EPIYA-ABD *cagA*						Patients with all other genotypes					
	Total		Female		Male		Total		Female		Male		Total		Female		Male	
	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)	No. of isolates (%)	Age range (yr) (mean)
Genotyped isolates	234[a] (90)	14–86 (50)	112 (49)	21–86 (52)	116 (51)	14–82 (49)	200[a] (85)	14–86 (51)	90 (46)	21–86 (52)	104 (54)	14–82 (49)	34 (15)	28–82 (50)	22 (65)	28–82 (51)	12 (35)	33–81 (49)
Disease state of genotyped isolates																		
Gastritis	108[b] (46)	19–82 (49)	69 (64)	21–82 (49)	38 (36)	19–78 (48)	87[b] (81)	19–78 (49)	57 (66)	21–75 (49)	29 (34)	19–78 (48)	21 (19)	28–82 (49)	12 (57)	28–82 (50)	9 (43)	36–61 (46)
Gastric ulcer	42 (18)	34–84 (55)	10 (24)	46–84 (57)	32 (76)	34–82 (54)	38 (90)	34–84 (55)	8 (21)	46–84 (58)	30 (79)	34–82 (53)	4 (10)	48–81 (61)	2 (50)	48–56 (52)	2 (50)	58–81 (70)
Duodenal ulcer	54[c] (23)	14–72 (45)	19 (39)	31–72 (51)	30 (61)	14–70 (41)	45[c] (83)	14–72 (44)	11 (28)	36–72 (52)	29 (72)	14–70 (42)	9 (17)	31–72 (49)	8 (89)	31–72 (51)	1 (11)	33 (N/A)[d]
Gastric cancer	30 (13)	37–86 (58)	14 (47)	37–86 (61)	16 (53)	38–70 (55)	30 (100)	37–86 (58)	14 (47)	37–86 (61)	16 (53)	38–70 (55)	0 (0)	0 (0)	0	0 (0)	0	0 (0)
Total	260[a]	14–86 (51)	126 (50)	21–86 (52)	128 (50)	14–82 (50)												

[a] Six without age or sex information.
[b] One without age information.
[c] Five without age information.
[d] N/A, not applicable.

TABLE 2. Primer sequences

Primer	Sequence (5′–3′)	Reference or source
cag2[a]	GGAACCCTAGTCGGTAATG	37
cagA28F	TTCTCAAAGGAGCAATTGGC	8
cagA-P1C	GTCCTGCTTTCTTTTTATTAACTTKAGC	8
cagA-pA-1 (R)	CTTGTCCTGYTTTCTTTTTATTAAC	This study
cagA-P2TA	TTTAGCAACTTGAGTATAAATGGG	8
cagA-P2CG	TTTAGCAACTTGAGCGTAAATGGG	8
cagA-P3E	ATCAATTGTAGCGTAAATGGG	8
cagA-pD (R)	TTGATTTGCCTCATCAAAATC	This study
cagA seq (R)[a]	TGGTTGAATCCAATTTTATC	This study
grace2	TCATGCGAGCGGCGATGT	This study

[a] This primer was also used for sequencing.

plified with cag2 and either cagA seq (R) or grace2 (which lies within the downstream conserved glutamate racemase gene) and then sequenced using primers cag2 and cagA seq (R). Sanger dideoxy sequencing was performed at the Uniformed Services University of the Health Science Biomedical Instrumentation Center (Bethesda, MD) or at Cosmo Genetech Co., Ltd (Seoul, Korea). The resulting DNA sequences were analyzed using Vector NTI version 9.1 (Invitrogen) and Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI).

CagA protein expression. After 24 hours, lawns of bacteria were harvested from horse blood agar plates, pelleted, resuspended in 1× phosphate-buffered saline (PBS), and mixed with 5× Laemmli sample buffer. Bacterial lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 10% separating gel and a 4% stacking gel, and proteins were transferred to nitrocellulose membranes using a semidry transfer apparatus (Owl; Thermo Scientific, Rochester, NY). Membranes were probed with a 1:5,000 dilution of mouse immunoglobulin G1 (IgG1) anti-CagA monoclonal antibody (Austral Biologicals, San Ramon, CA), followed by a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Alternatively, membranes were probed with a 1:5,000 dilution of rabbit IgG anti-CagA polyclonal antibody b-300 (Santa Cruz Biotechnology), followed by a 1:20,000 dilution of HRP-conjugated bovine anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Proteins were detected using the Pierce ECL Western blotting substrate kit (Thermo Scientific/Pierce, Rockford, IL) and photographic film with a Series XXXV A rapid processor (S&W Imaging, Frederick, MD) or using the SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific/Pierce) and a LAS-3000 Intelligent Dark Box with LAS-3000 Lite capture software (Fujifilm, Stamford, CT).

CagA phosphorylation assays. The CagA phosphorylation assays were essentially conducted as previously described (13). Briefly, six-well tissue culture plates were seeded with 3.5×10^5 AGS cells per well and allowed to grow for 3 days in normal cell culture media, Dulbecco's modified Eagle's media without L-glutamine (Quality Biological, Inc., Gaithersburg, MD), supplemented with 10% fetal bovine serum, 10 μg/ml vancomycin, and 2 nM L-glutamine (Quality Biological, Inc.). Two hours prior to infection, AGS cells were washed with 1× PBS, and 3 ml of fresh media was added to each well. Liquid cultures of *H. pylori* were resuspended in 1 ml of 1× PBS and used to infect the AGS cells at a multiplicity of infection (MOI) of 100. Infections were allowed to proceed for 5 h, at which point the media was removed, and the cells were washed with 1× PBS and lysed with 5× Laemmli sample buffer. Infected cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 6% separating gel and a 4% stacking gel. Proteins were transferred to nitrocellulose membranes by semidry transfer. Membranes were then probed with a 1:5,000 dilution of an anti-phospho-tyrosine monoclonal antibody, pY100 (Cell Signaling Technology, Danvers, MA), followed by a 1:20,000 dilution of HRP-conjugated goat anti-mouse IgG secondary antibody and detection as described above. Membranes were subsequently stripped (using a heated 10-mM dithiothreitol solution) and reprobed with the polyclonal anti-CagA antibody b-300 as described above. Densitometry was performed using MultiGauge software (Fujifilm).

A low level of cross-reactivity for CagA with the anti-phospho-tyrosine antibodies (pY100 and pY99; BD Biosciences, San Jose, CA) was observed. Hence, bacterial lysates were run adjacent to their corresponding infected cell lysates so that any cross-reactivity could be accounted for when comparing the ratio of phosphorylated CagA to that of total CagA for infected cell lysates (Table 3).

Cell elongation studies. The cell elongation studies were essentially conducted as previously described (13). Six-well tissue culture plates were seeded with 2.7×10^5 AGS cells per well and allowed to grow in normal cell culture media for 22 h. After 22 h and approximately 2 h before infection, the media was removed, the cells were washed with 1× PBS, and 1 ml of fresh cell culture media supplemented with 10% BB was added to each well. Eighteen-hour liquid cultures of *H. pylori* that were suspended in 1 ml of the BB-supplemented cell culture media were then used to infect cells at an MOI of 100. Infections were allowed to proceed for 9 hours, at which point cells were fixed with 2% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) and stained with Giemsa (Sigma-Aldrich, Inc., St. Louis, MO) per the manufacturer's directions. Cells were analyzed using an Olympus BX60 (Olympus America Inc., Center Valley, PA) and were digitally photographed using a Spot RT color camera (Diagnostic Instruments, Sterling Heights, MI). One hundred cells were counted to assess the number of cells displaying the hummingbird phenotype, which is characterized by the presence of fingerlike protrusions (40). In each case, the infections and analysis were replicated to verify the reproducibility of the results. Any strain that on average induced more than 60% of the AGS cells to display the hummingbird phenotype in biologically independent experiments was considered positive for the presence of a functional CagA.

IL-8 induction assay. Six-well tissue culture plates were seeded with 4.2×10^5 AGS cells per well and allowed to grow in normal cell culture media for 24 h. At this point, the media was removed and replaced with media lacking serum for a period of 24 h before *H. pylori* infection. Approximately 2 hours prior to infection, the cells were washed with 1× PBS, and 1 ml of fresh media without serum was added to each well. Eighteen-hour liquid cultures of *H. pylori* were pelleted and resuspended in 700 μl of cell culture media without serum and used to infect the semiconfluent AGS cells at an MOI of 100. After 5 hours, the cell culture supernatant was collected, samples were centrifuged at 16,100 relative centrifugal force for 10 min, and the supernatant was transferred to a new tube and stored at −20°C until later use. Human interleukin-8 (IL-8) concentration was measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) by following the manufacturer's directions. The change in IL-8 concentration was calculated in comparison to that of G27-MA ΔPAI. An independent biological repeat of each infection was conducted, and strains were considered to induce IL-8 if the average change was more than 10-fold.

Statistical analysis. The Fisher exact test was used to analyze the association between disease state and EPIYA motif genotype. Log linear modeling was used to assess whether this association was consistent across the age and sex subgroups. We fit a saturated model using categorical variables representing genotype, disease state, sex, and age groups. A backward selection algorithm identified two- and three-way associations among these variables that were statistically significant at the 5% level. Data were analyzed using SPSS version 14 software (SPSS Inc., Chicago, IL).

Nucleotide sequence accession numbers. The sequences for the C-terminal region of CagA from 47 strains have been deposited in GenBank under accession numbers FJ458117 to FJ458163.

RESULTS

Sample acquisition/*cagA* genotyping. A total of 260 *H. pylori* clinical isolates were obtained from patients presenting with gastric maladies (Table 1). Six of these were missing the epidemiological data of age and gender. Of the remaining 254, the mean patient age was 51 years, with an age range of 14 to 86 years. There were 126 females (49.6%), with a mean age of 52 years and an age range of 21 to 86 years, and 128 males (50.4%), with a mean age of 50 years and an age range of 14 to 82 years. Of the 254 samples, 45.3% were from patients with gastritis, 43% from patients with ulcers (21.7% gastric ulcers and 21.3% duodenal ulcers), and 11.8% from patients with cancer.

Four different PCRs were conducted for each strain in order to genotype *cagA* (Fig. 1A and B; see Fig. S1 in the supplemental material). As previously described, three of these PCRs identify different EPIYA motifs; one identifies the EPIYA-A motif, one the EPIYA-B motif, and one an EPIYA-C or EPIYA-D motif (7). To definitively identify the EPIYA-D motif, we also designed and employed primer cagA-pD (R), which is well conserved among strains carrying the EPIYA-D motif and was one of the first primers designed to specifically

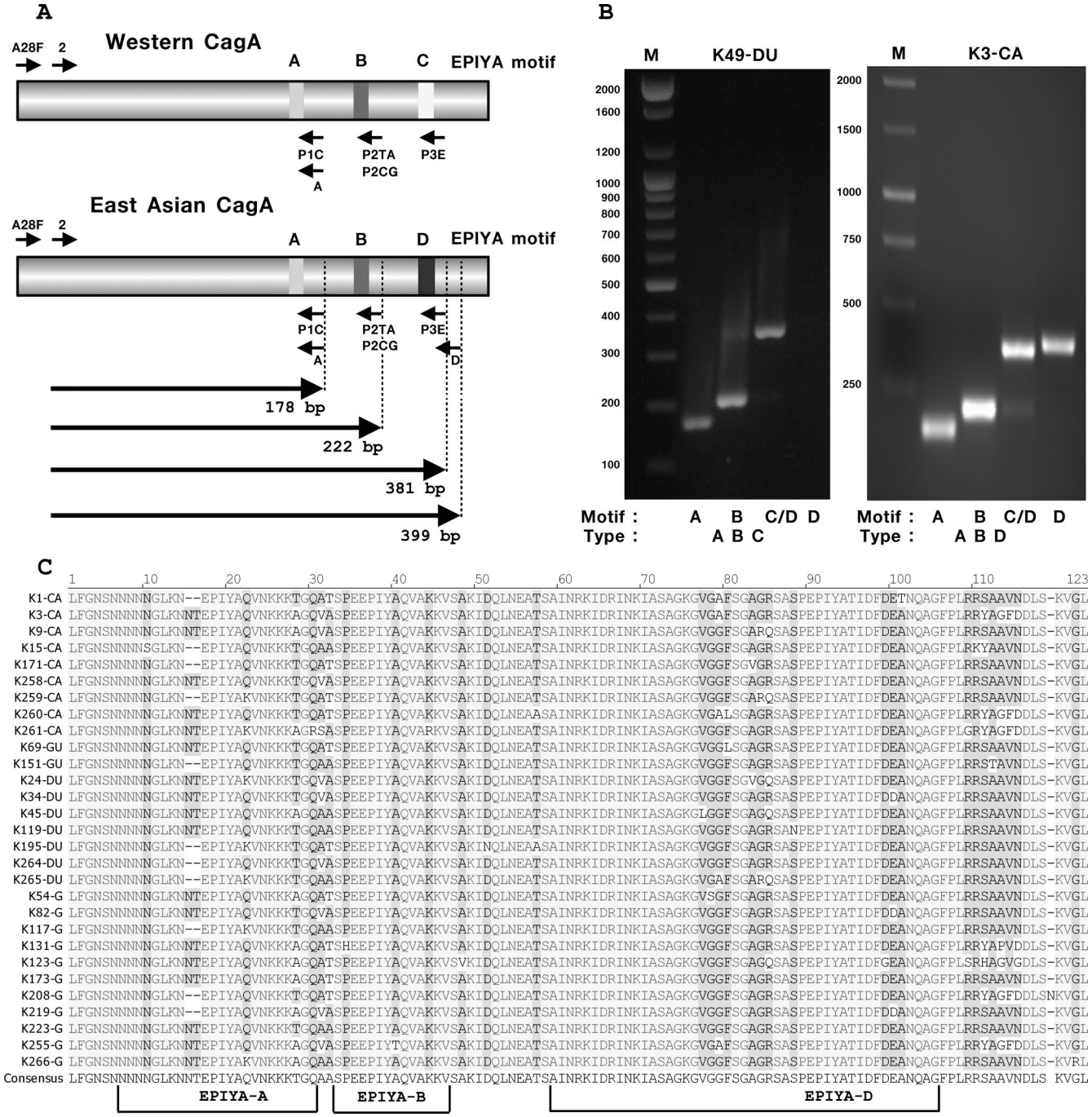

FIG. 1. Genotyping of the *cagA* variable EPIYA motif. (A) Schematic representation of the *cagA* variable region. Western CagA (EPIYA-ABC) is depicted on the top, and East Asian CagA (EPIYA-ABD) is depicted on the bottom. The annealing positions (small arrows), names of the primers used in this study, and expected sizes (large arrows) of the amplified specific EPIYA motif products as based on the strain K3-CA DNA sequence are shown. Primer names are abbreviated as follows: cagA28F, A28F; cag2, 2; cagA-P1C, P1C; cagA-pA-1 (R), A; cagA-P2TA, P2TA; cagA-P2CG, P2CG; cagA-P3E, P3E; and cagA-pD (R), D. (B) PCR amplicons of K49-DU and K3-CA using the forward primer 2 and the reverse primer A, P2TA and P2CG (equimolar mixture), P3E, or D. "M" designates the size markers (in base pairs). Type indicates the resulting EPIYA motif identified. (C) Amino acid alignment of the carboxy terminus of CagA from 29 Korean strains carrying the EPIYA-ABD motif. The EPIYA-A, -B, and -D motifs are indicated below the consensus sequence.

amplify the EPIYA-D motif. Using this technique, we were able to genotype 234 strains (Table 1). These strains displayed the same age ranges as the full collection and a had mean age of 50 years. Again, the proportion of females (112) to males (116) was virtually identical to that of the larger collection, 49.1% to 50.9%, respectively. The remaining 26 strains, which all came from non-cancer patients, failed to yield PCR products or gave incorrectly sized bands and thus were not further analyzed.

TABLE 3. Analysis of CagA expression and function

Strain	EPIYA motif	CagA expression	Phosphorylation of CagA[a]	Induction of hummingbird phenotype[b]	IL-8 induction[c]
K82-G	ABD	−	−	−	−
K255-G	ABD	−	−	−	−
K264-DU	ABD	−	−	−	±
K111-DU	ABD	+	−	+	
K123-G	ABD	+	−	−	−
K17-CA	ABD	+	±	+	+
K26-DU	ABD	+	±	+	+
K208-G	ABD	+	±	+	+
K21-CA	ABD	+	±	+	
K23-DU	ABD	+	±	+	
K25-DU	ABD	+	±	+	
K42-DU	ABD	+	±	+	
K104-CA	ABD	+	±	+	
K182-DU	ABD	+	±	+	
K193-G	ABD	+	±	+	
K238-DU	ABD	+	±	+	
K248-G	ABD	+	±	+	
K259-CA	ABD	+	±	+	
K3-CA	ABD	+	+		
K6-CA	ABD	+	+		
K10-CA	ABD	+	+		
K16-CA	ABD	+	+		
K19-CA	ABD	+	+		
K28-DU	ABD	+	+		
K34-DU	ABD	+	+		
K35-DU	ABD	+	+		
K36-DU	ABD	+	+		
K37-DU	ABD	+	+		
K41-DU	ABD	+	+		
K43-DU	ABD	+	+		
K44-DU	ABD	+	+		
K45-DU	ABD	+	+		
K46-DU	ABD	+	+		
K47-DU	ABD	+	+		
K48-DU	ABD	+	+		
K57-G	ABD	+	+		
K60-G	ABC	+	+		
K64-G	ABCC	+	+		
K74-G	ABD	+	+		
K77-G	ABD	+	+		
K78-G	AABD	+	+		
K80-CA	ABD	+	+		
K85-G	BD	+	+		
K93-DU	ABC	+	+		
K107-DU	ABD	+	+		
K109-G	ABD	+	+		
K112-G	ABD	+	+		
K113-G	ABD	+	+		
K115-G	ABC	+	+		
K117-G	ABD	+	+		
K131-G	ABD	+	+		
K162-G	ABD	+	+		
K172-G	ABCC	+	+		
K175-G	ABD	+	+		
K178-G	ABD	+	+		
K183-G	ABD	+	+		
K185-G	ABD	+	+		
K196-G	ABD	+	+		
K197-G	ABD	+	+		
K209-G	ABD	+	+		
K218-G	ABD	+	+		
K220-DU	ABD	+	+		
K223-G	ABD	+	+		
K235-G	ABD	+	+		
K241-G	ABD	+	+		
K258-CA	ABD	+	+		
K260-CA	ABD	+	+		
K261-CA	ABD	+	+		
K262-G	ABC	+	+		
K263-G	ABABD[d]	+	+		
K265-DU	ABD	+	+		
K266-G	ABD	+	+		
K24-DU	ABD	+	++		
K27-DU	ABD	+	++		
K146-G	ABD[e]	+	++		
K154-G	ABCCCC	+	++		
K165-G	ABD	+	++		

[a] ++, near or above G27-MA (positive control); +, slightly below the level of G27-MA; ±, below the level of G27-MA but above background; −, not detectable.
[b] +, greater than 60% of cells displayed the hummingbird phenotype; −, less than 60% of cells displayed the hummingbird phenotype.
[c] +, induction was greater than 20-fold more than G27-MA ΔPAI; ±, induction was 10- to 20-fold more than G27-MA ΔPAI; −, induction was less than 10-fold more than G27-MA ΔPAI.
[d] Second EPIYA-B motif's proline is replaced with leucine, ELIYA.
[e] EPIYA-B motif's proline is replaced with a serine, ESIYA.

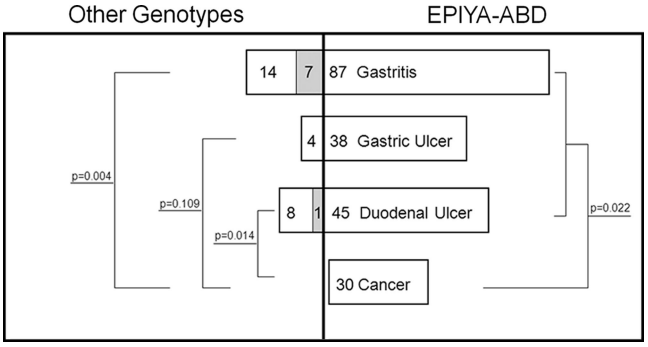

FIG. 2. Schematic depiction of the distribution of the *cagA* genotypes stratified by disease state within this Korean population. Distributions of *cagA* genotypes and EPIYA-ABD versus all other EPIYA motifs within the four different disease states are shown as follows: gastritis, gastric ulcers, duodenal ulcers, and cancer. The shaded portion within the other genotypes subgrouping corresponds to the isolates that contain an alternative EPIYA-ABD motif. Calculated *P* values, using the Fisher exact test, are shown.

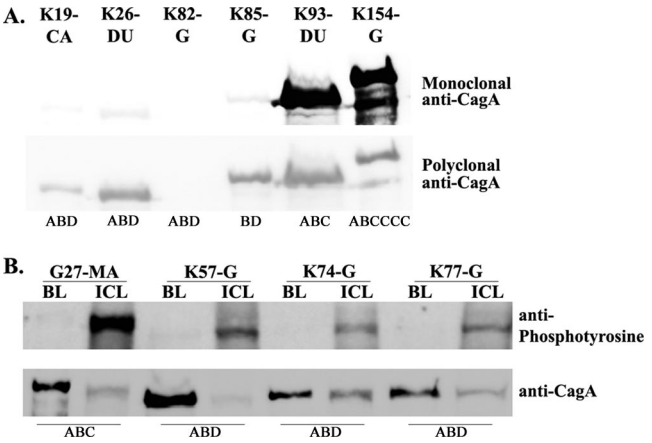

FIG. 3. Expression, delivery, and phosphorylation of CagA. (A) Western blot analysis of bacterial lysates from the six indicated Korean strains was conducted using a monoclonal anti-CagA antibody (top) or the polyclonal anti-CagA antibody b-300 (bottom). (B) Lysates from the bacterial cells alone (BL) and AGS cells infected with the same bacterial strain (ICL) were assessed for delivery and phosphorylation of CagA. Membranes were probed with an anti-phosphotyrosine antibody, pY100 (top), stripped, and subsequently reprobed with the polyclonal anti-CagA antibody b-300 (bottom). Data are shown from the positive control G27-MA and three indicated Korean isolates.

To confirm the *cagA* genotyping results, we sequenced the C-terminal region of the *cagA* gene from 47 of the 234 genotyped strains. These sequences verified that the PCR genotyping method was accurate. Alignments of the predicted amino acid sequences of those strains carrying the EPIYA-ABD motif can be found in Fig. 1C.

Of the 234 genotyped strains, 208 isolates (88.9%) carried an EPIYA-D motif, therefore classifying them as East Asian, and 26 isolates (11.1%) were determined to carry Western CagA (see Table S1 in the supplemental material). Among the East Asian strains, eight carried an incomplete EPIYA-ABD motif or contained additions of one or more motifs (EPIYA-AABD, -BD, -BBD, -ABAB*D, and -AB*D, with a mutation within the EPIYA-B motif designated by an asterisk), and thus, we subdivided the strains based on the presence of CagA containing a complete EPIYA-ABD motif versus all other EPIYA motifs. Given these characteristics, 34 individuals were determined to have "other genotypes," which includes alternative EPIYA-ABD as well as Western motifs.

We next analyzed the distribution of the *cagA* genotype among disease states. There were 108 gastritis patients, 54 duodenal ulcer patients, 42 gastric ulcers patients, and 30 gastric cancer patients. EPIYA-ABD CagA composed 80.6% of gastritis patients, 83.3% of duodenal ulcer patients, 90.5% of gastric ulcer patients, and 100% of gastric cancer patients. Stratification of the patients based on age, sex, and disease categories can be found in Table 1, and a schematic depiction of the distribution of the *cagA* genotypes stratified by disease state within this Korean population can be found in Fig. 2.

Even though this collection was evenly distributed for the factors of age and gender, several trends for each were observed based on statistical analysis. Not surprisingly, age is statistically linked to disease state ($P < 0.001$). Moreover, as has been suggested in other studies (38), males were more likely to have ulcers (odds ratio, 3.89; confidence interval, 1.81 to 8.36), whereas females were more likely to have gastritis (odds ratio, 1.91; confidence interval, 1.91 to 5.67). Conversely, the cancer patients were evenly distributed by gender, with 46.7% being female and 53.3% being male. This differs from what is most often seen in the *H. pylori* literature, which shows that men are anywhere from 1.5 to 2.5 times more likely to be afflicted with gastric cancer than women (reviewed in reference 35). A significant three-way association was also observed using log linear modeling between gender, disease, and *cagA* allele ($P = 0.009$).

Given the fact that 100% of the gastric cancer patients were infected with *H. pylori* encoding CagA with the EPIYA-ABD motif, we conducted statistical analysis to assess the relationship between disease state and genotype. The Fisher exact test showed that the proportion of patients with the EPIYA-ABD genotype varied significantly ($P = 0.022$) by diagnosis (Fig. 2). In fact, the proportion of cancer patients with the EPIYA-ABD genotype (100%) was significantly higher than the proportion with that genotype in gastritis patients (80.6%; $P = 0.004$) or duodenal ulcer patients (83.3%; $P = 0.014$) but not gastric ulcer patients (90.5%; $P = 0.109$) (Fig. 2). Taken together, these data suggest that there is a definitive link between infection with *H. pylori* strains carrying *cagA* which encodes the EPIYA-ABD motif and the development of gastric cancer.

CagA protein expression. Given the fact that we saw a significant statistical link between the presence of the EPIYA-ABD genotype and gastric cancer but that some strains that carry *cagA* do not actually express the CagA protein (25), we next sought to determine if genotypically *cagA*$^+$ strains were phenotypically CagA positive. Bacterial lysates from a subset of 77 randomly chosen strains were assessed for expression of CagA. Of the 77 isolates examined using a monoclonal antibody, four samples (K19-CA, K82-G, K255-G, and K264-DU) showed no appreciable CagA expression (Fig. 3A and data not shown). Given the fact that CagA shows heterogeneity in the carboxy terminus that may affect protein structure and monoclonal antibody recognition, we also utilized a polyclonal anti-

CagA antibody to ensure that CagA was not actually expressed in these strains. As shown in Fig. 3A, the polyclonal antibody was better able to detect CagA in the majority of strains. This included K19-CA, for which CagA was not detected with the monoclonal antibody. Using this assay, three of the 77 strains (K82-G, K255-G, and K264-DU) expressed no detectable levels of CagA (Table 3 and data not shown).

Delivery and phosphorylation of CagA. Once CagA is expressed, it must be delivered to host cells via the type IV secretion apparatus and phosphorylated by host cell kinases to be biologically active (40). Therefore, we next conducted phosphorylation assays to determine if CagA could be delivered to and phosphorylated in host cells. Of the 77 strains tested, 59 of the isolates efficiently delivered CagA to the host cells, as detected by the appearance of a strongly phosphorylated CagA band (Fig. 3B and Table 3). Of the remaining 18 isolates, an intermediate level of phosphorylated CagA was detected for 13 strains, and no detectable phosphorylated CagA was found for 5 strains (K82-G, K111-DU, K123-G, K255-G, and K264-DU) (Table 3). Importantly, the three strains shown to be negative for CagA expression were included among these five, and K19-CA, which was only detected with the polyclonal CagA antibody, was positive for phosphorylation.

Cell elongation assay. Upon injection of CagA into host cells, it becomes phosphorylated and causes striking host cell elongation, which is known as the hummingbird phenotype (40). Thus, to reassess the presence of functional CagA in the 18 isolates that produced either an intermediate phenotype or no detectable level of phosphorylated CagA, the ability to induce the hummingbird phenotype was assessed in cultured AGS cells. A wild-type strain, G27-MA, and its isogenic Δ*cagA* mutant were used as positive and negative controls, respectively. The percentage of cells displaying the hummingbird phenotype for the G27-MA Δ*cagA*-infected cells was 23% and for the G27-MA-infected cells was 80% (Fig. 4A and B). The range for the 18 Korean isolates was between 31.5% (K82-G) and 79% (K25-DU) (Fig. 4C and D; Table 3). Given the large range of changes, we conservatively required that a strain must induce at least 60% of AGS cells to display the hummingbird phenotype to be considered positive for delivery of functional CagA. Four of the 18 samples tested did not meet this threshold (K82-G, K123-G, K255-G, and K264-DU). These samples also showed no detectable levels of phosphorylated CagA via the phosphorylation assay.

Induction of IL-8. Since several isolates were identified that did not express any detectable level of functional CagA as measured by the phosphorylation assay and induction of the hummingbird phenotype, we finally assessed the assembly of the type IV secretion system on the bacterial surface for this subset of strains. Proper assembly of the type IV secretion system has been shown to result in the induction of IL-8 in cultured AGS cells (10). Therefore, we assessed IL-8 induction with the four strains that failed to produce phosphorylated CagA and failed to induce the hummingbird phenotype. Additionally, as a positive control, we analyzed several strains that did induce the hummingbird phenotype. One (K264-DU) out of the four hummingbird phenotype-negative samples induced IL-8 (at least 10-fold above the level induced by G27-MA ΔPAI), indicating that the failure to detect CagA and phosphorylated CagA or to induce the hummingbird phenotype was not impacted by a lack of a functional type IV secretion system (Fig. 4E and Table 3).

DISCUSSION

Herein, we show a significant statistical link between the presence of the CagA EPIYA-ABD motif and the development of gastric cancer. In fact, 100% of gastric cancer patients analyzed in this South Korean population were infected with *H. pylori* strains encoding CagA containing the EPIYA-ABD motif. Statistical analysis with the Fisher exact test showed that the proportion of EPIYA-ABD genotype varied significantly by diagnosis ($P = 0.022$) and that this distribution was statistically different than that of gastritis patients ($P = 0.004$) or duodenal ulcer patients ($P = 0.014$) (Fig. 2). These data suggest that the distribution of alleles is not random and is important in the case of gastric cancer. While on the whole, the presence of the *cagA* gene did not strictly correlate to the expression and delivery of CagA to host cells, all of the analyzed cancer strains did express a functional CagA that could be delivered to and phosphorylated in host cells. This is the first time that a specific *cagA* allele has been statistically linked to gastric cancer. However, it should be noted that the EPIYA-ABD allele is not necessarily a predictor of cancer since there was a high percentage of peptic ulcer patients, both gastric (90%) and duodenal (83%), infected with isolates containing EPIYA-ABD CagA. Alternatively, these data could indicate that patients infected with *H. pylori* containing non-EPIYA-ABD motifs are more likely not to develop cancer.

This association between the East Asian *cag*A genotype and gastric cancer may be due to higher affinity for SHP-2 (25). Binding of CagA with SHP-2 occurs via interaction of the phosphorylated EPIYA motifs and the SH2 domains from the host cell protein. This interaction changes the conformation of SHP-2 to its active form. Thus, the stronger affinity of East Asian CagA for SHP-2 results in longer periods of SHP-2 activity. This likely explains why East Asian strains cause greater morphological damage and greater levels of induction for multiple cellular pathways, resulting in increased proliferation, morphogenesis, and cell motility compared to those of Western strains (23–25, 29, 36, 46).

While we did isolate eight EPIYA-AABD, -BD, -BBD, -ABAB*D, and -AB*D motifs, the vast majority of East Asian strains that we examined showed strong conservation and a lack of duplication in the -D region. This suggests that variation in East Asian *cagA* is not as favorable as in Western isolates, where the EPIYA-C motif is found to vary widely among isolates (4, 6). Similar results have been seen by Argent et al., who showed by sequence analysis of >500 East Asian strains available in GenBank that the percentage of other East Asian alleles compared to those coding for EPIYA-ABD CagA was fairly small: 88.3% of East Asian strains contained an EPIYA-ABD CagA (4). Also, it is interesting that all of the cancer strains were specifically EPIYA-ABD, which suggests that among East Asian isolates this combination of EPIYA motifs is most favored for cancer development. It should be noted that in a study of Japanese cancer patients, all *H. pylori* isolates contained the EPIYA-D motif, and the majority of those isolates (84%) contained an EPIYA-ABD motif (9). Alternatively, the other 16% were made up of isolates carrying

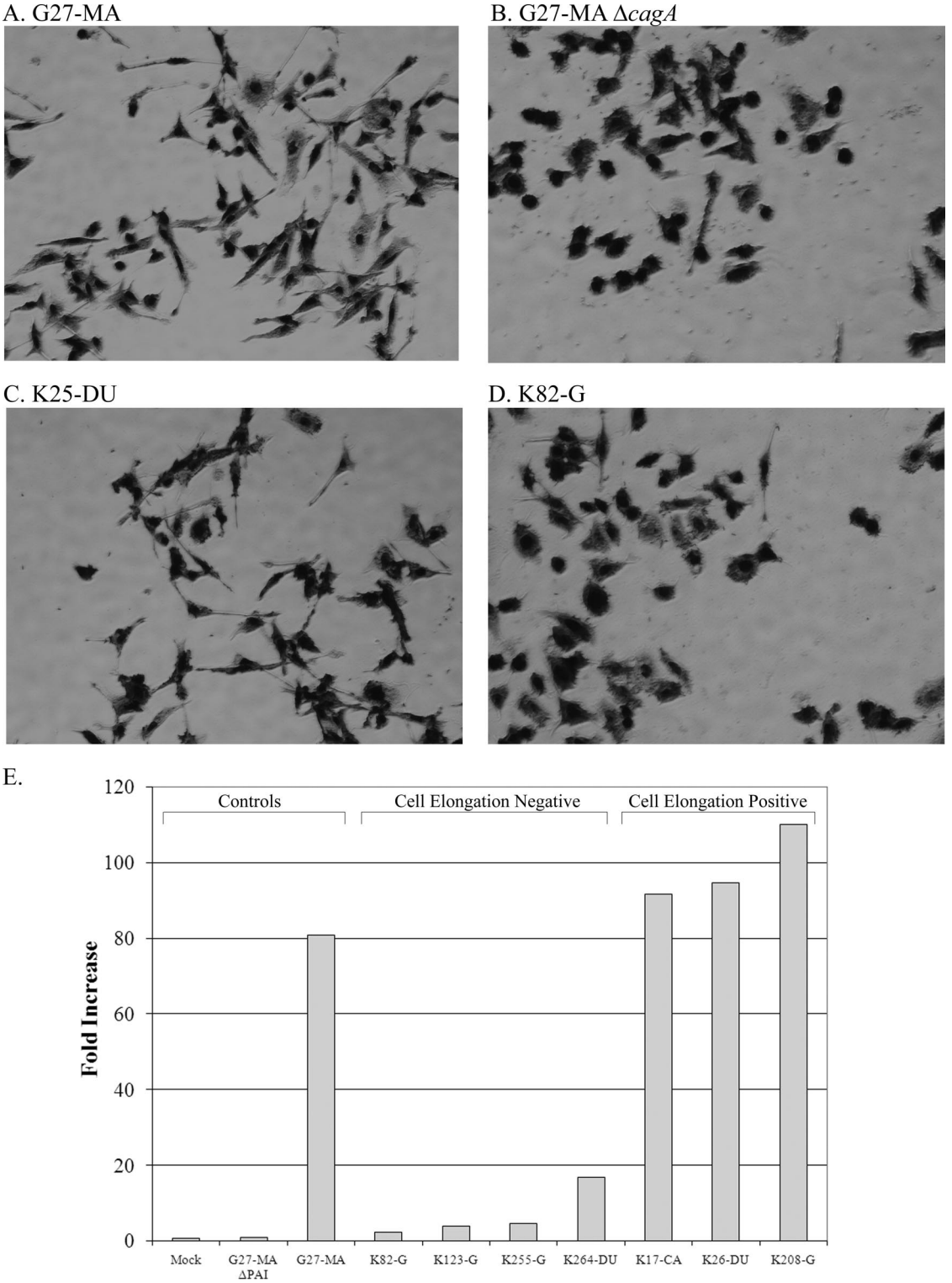

FIG. 4. Morphological changes and induction of IL-8. (A to D) Induction of morphological changes in AGS cells when measured after 9 h of infection with the following strains: G27-MA (positive control) (A), G27-MA Δ*cagA* (negative control) (B), K25-DU (C), and K82-G (D). (E) Induction of IL-8 from the indicated strains expressed as an increase above the induction elicited by G27-MA ΔPAI (negative control) after a 5-hour infection.

the EPIYA-AABD, -ABBD, -ABABD, and -ABDBD motifs (9).

The reason for the EPIYA-ABD conservation is unknown, but perhaps a single EPIYA-D motif allows for optimal SHP-2 binding. The presence of extra motifs may contort CagA's conformation and destabilize binding to SHP-2. Additionally, it is known that CagA phosphorylated at EPIYA-A and -B motifs binds to Csk, activates a negative feedback loop that

inactivates the Src family kinases, and ultimately reduces the level of phosphorylated CagA in the cell (45). Thus, it is reasonable to suggest that the presence of additional EPIYA-A or -B motifs in association with an EPIYA-D motif would more strongly activate this negative feedback loop (4). In support of the importance of conservation of the EPIYA-ABD motif in the disease state, seven out of the eight isolates containing an EPIYA-D motif but not a complete standard EPIYA-ABD motif caused only gastritis.

Multiple host, dietary, environmental, and bacterial virulence factors have been shown to play a role in *H. pylori*-induced disease. In this molecular epidemiologic study, we have shown a definitive statistical difference in the distribution of the *cagA* allele coding for the EPIYA-ABD motif in cancer versus other disease states; 100% of the cancer patients were infected with *H. pylori* strains carrying the EPIYA-ABD genotype. Currently, the reason for this correlation is unclear, and further study is required to elucidate the molecular role that the EPIYA motif plays in cancer development.

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