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ORIGINAL ARTICLE

Comparative genomic study of gastric epithelial cells co-cultured with *Helicobacter pylori*

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Abstract

AIM: To identify genes potentially involved in *Helicobacter pylori* (*H. pylori*)-induced gastric carcinogenesis.

METHODS: GES-1 cells were co-cultured with *H. pylori* strains isolated from patients with gastric carcinoma (GC, n = 10) or chronic gastritis (CG, n = 10) for *in vitro* proliferation and apoptosis assays to identify the most and least virulent strains. These two strains were *cagA*-genotyped and used for further *in vivo* carcinogenic virulence assays by infecting Mongolian gerbils for 52 wk, respectively; a broth free of *H. pylori* was lavaged as control. Genomic profiles of GES-1 cells co-cultured with the most and least virulent strains were determined by microarray analysis. The most differentially expressed genes were further verified using quantitative real-time polymerase chain reaction in GES-1 cells infected with the most and least virulent strains, and by immunohistochemistry in *H. pylori* positive CG, precancerous diseases, and GC biopsy specimens in an independent experiment.

RESULTS: GC-derived *H. pylori* strains induced a potent proliferative effect in GES-1 cells in co-culture, whereas CG-derived strains did not. The most (from a GC patient) and least (from a CG patient) virulent strains were *cagA*-positive and negative, respectively. At week 52, CG, atrophy, metaplasia, dysplasia, and GC were observed in 90.0%, 80.0%, 80.0%, 90%, and 60.0%, respectively, of the animals lavaged with the most virulent strain. However, only mild CG was observed in 90% of the animals lavaged with the least virulent strain. On microarray analysis, 800 differentially expressed genes (49 up- and 751 down-regulated), involving those associated with cell cycle regulation, cell apoptosis, cytoskeleton, immune response, and substance and energy metabolisms, were identified in cells co-cultured with the most virulent strain as compared with those co-cultured with the least virulent strain. The six most differentially expressed genes (with a betweenness centrality of 0.1-0.2) were identified among the significant differential gene profile network, including *JUN*, *KRAS*, *BRCA1*, *SMAD2*, *TRAF1*, and *HDAC6*. Quantitative real-time polymerase chain reaction analyses verified that HDAC6 and TRFA1 mRNA expressions were significantly more up-regulated in GES-1 cells co-cultured with the most virulent strain than in those co-cultured with the least virulent strain. Immunohistochemistry of gastric mucosal specimens from *H. pylori*-positive patients with CG, intestinal metaplasia (IM), dysplasia, and GC showed that moderately positive and strongly positive HDAC6 expression was detected in 21.7% of CG patients, 30.0% of IM patients, 54.5% of dysplasia patients, and 77.8% of GC patients (P < 0.001). The up-regulation of TRAF1 expressions was detected in 34.8%, 53.3%, 72.7%, and 88.9% specimens of CG, IM, dysplasia, and GC, respectively (P < 0.001).

CONCLUSION: The overexpression of *HDAC6* and *TRAF1* in GES-1 cells co-cultured with the GC-derived strain and in *H. pylori*-positive dysplasia and GC suggests that HDAC6 and TRAF1 may be involved in *H. pylori*-induced gastric carcinogenesis.

Key words: *Helicobacter pylori*; Gastric carcinoma; Proliferation; Genomic profiles

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a pathogenic bacterium colonizing gastric mucosae, especially in the antrum. It has been accepted to be the primary cause of upper gastrointestinal disorders, such as acute and chronic gastritis, peptic ulcer disease, and gastric cancer[1]. *H. pylori* infection is common worldwide with a prevalence of approximately 50%, especially in Eastern Asian populations, and the infection is usually a life-long event[2]. However, over 80% of *H. pylori*-infected individuals remain asymptomatic for their whole lifetime despite the presence of chronic gastric inflammation or chronic gastritis (CG), caused by *H. pylori* infection[3]. The subsequent outcomes of persistent *H. pylori* infection are highly variable. Approximately, 10%-20% of *H. pylori*-infected individuals are subject to peptic ulcer disease, including gastric and duodenal ulcers[4]. Of note, individuals infected by *H. pylori* are at a higher risk of gastric carcinoma (GC) (1%-2%) and mucosa-associated lymphoid tissue (MALT) lymphoma (< 1%)[5]. Therefore, gastric cancer may well be an infectious disease[6]. Additionally, it has been reported that *H. pylori* infection is associated with esophageal diseases, such as gastroesophageal reflux disease, Barrett's esophagus, and esophageal carcinoma, as well as extra-gastrointestinal diseases, such as cardiovascular diseases, although controversies exist[7,8]. Such variation in clinicopathological outcomes of *H. pylori* infection is believed to result from the variations in the virulence of different strains, genetic background of the host, and more importantly, the host-to-pathogen interactions.

It has been widely accepted that *H. pylori* is the major cause of GC in most (65%-80%) patients[9]. Dietary risks, such as nitrate- and nitrite-enriched smoked or salted foods, are attributed to the *in vivo* biochemical activities of *H. pylori*[10]. Genetic susceptibility has also been identified in 10% of GC patients[11]. The canonical paradigm of gastric carcinogenesis has been established for more than two decades as a consecutive but dynamic progression of *H. pylori* infection, namely, CG, gastric atrophy (GA), intestinal metaplasia (IM), dysplasia, and finally GC[12]. Thus, *H. pylori* infection plays a leading role in the pathogenesis of GC[13].

It is likely that *H. pylori* strains themselves are highly variable in virulence to gastric mucosal epithelia, especially in terms of the carcinogenic effect. Genomic profiling analyses have identified a wide range of genetic variations among *H. pylori* strains isolated from patients with different gastric pathologies. Global gene expression profiles also vary greatly in human gastric epithelial immortalized cells infected with spiral *vs* coccoid *H. pylori*[14]. These findings suggest that gastric epithelial cells tune in the expression of their genes, especially those associated with tumorigenesis, in response to specific *H. pylori* strains or a specific virulent factor of the strain. In addition to the core genes, strain-specific genes are thought to play an essential role in *H. pylori* propagation and pathogenesis.

How gastric epithelial cells respond to *H. pylori* clinical isolates derived from patients with different pathologies, such as GC and CG specimens, at the genome-wide level remains unknown. Therefore, this study was carried out to identify genes potentially involved in *H. pylori*-induced gastric carcinogenesis, by comparing the genomic profiles between gastric epithelial cells co-cultured with *H. pylori* strains isolated from patients with GC and those co-cultured with strains from patients with CG.

MATERIALS AND METHODS

Patients and specimens

The study protocol was approved by the Institutional Review Boards at the Third Xiangya Hospital of Central South University and Hunan Provincial Hospital, respectively. All patients gave written informed consent prior to the enrollment. A total of 350 outpatients who underwent upper gastrointestinal endoscopy at the Department of Gastroenterology, the Third Xiangya Hospital of Central South University, and the Department of Gastroenterology of Hunan Provincial Hospital were consecutively enrolled. Gastric mucosal biopsy specimens were obtained from 182 patients. Three gastric biopsy specimens, 3-5 cm to the pylorus, were collected for the rapid urease test and the histological examination. The rapid urease test was performed using a rapid urease test kit (Sanqiang Biotechnology, Sanming, China). Of the 182 patients, 113 patients were found positive for *H. pylori* infection as detected by the rapid urease test. The histological classification followed the updated Sydney system[15]; the most serious pathology was documented as the histological diagnosis of each patient with concomitant mucosal pathologies. Thus, 23 patients were histologically diagnosed with CG, 30 with intestinal metaplasia (IM), 33 with dysplasia, and 27 with GC. In the present study, gastric specimens from CG and GC patients were used

for *H. pylori* isolation and subculture.

H. pylori isolation and subculture

Gastric mucosal specimens from patients with CG and GC were ground into homogenates and inoculated onto a Columbia agar plate (Sangong Biotech, Shanghai, China) supplemented with 6% sheep blood. Plates were incubated at a mixed atmosphere of 10% CO_2, 5% O_2, and 85% N_2, at 37 ℃ for 72 h. *H. pylori* colonies were validated by using colony identification, Gram staining, light microscopy, and urease test (Fujian Sanqiang Biochemical Co. Ltd., Sanming, China) prior to further use. Subculture of *H. pylori* was performed as described above. Twenty *H. pylori* strains were isolated from 10 CG and 10 GC patients, and used for further experiments.

Identification of the most and least virulent strains isolated from CG and GC specimens

GES-1 cell culture and co-culture with *H. pylori*: GES-1 cell line, a human gastric epithelium immortalized cell line, was purchased from Ai-yan Biotechnology Co., Ltd., Shanghai, China. GES-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), at 37 ℃ and in a humidified atmosphere of 5% CO_2. GES-1 cells at the exponential growth phase were harvested and seeded onto 96-well plates, at a density of 5×10^3 cells per well. *H. pylori* strains were resuspended in phosphate buffer solution (PBS) at a density of 3.0×10^8 cfu/mL using a spectrophotometer (Eppendorf, Hamburg, Germany). Following the cell cycle synchronization with 2% serum starvation, GES-1 cells were co-cultured with *H. pylori* strains in GES-1 cell culture media at a cell/bacterium ratio of 5:1, 1:1, 1:50, and 1:200, respectively. The number of bacteria was examined using a spectrometer, whilst that of cells was determined using TC10 automated cell counter (Bio-Rad Laboratories, Philadelphia, PA). A mono-culture of GES-1 cells in the absence of *H. pylori* was used as a control. The number of GES-1 cells was fixed among co-cultures as well as mono-culture. The experiments were performed in duplicate and repeated in triplicate independently.

3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide cell proliferation assay: Following 12 h, 24 h, and 48 h of co-culture, 20 μL 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) solution (Sangong Biotech, Shanghai, China) was added into the culture medium for a further 4-h incubation at 37 ℃ prior to the supplementation of 150 μL dimethyl sulfoxide (Invitrogen, Carlsbad, CA). The optical density at the wavelength of 490 nm ($A_{490\ nm}$) was determined using an ELISA microplate reader (Bio-Tek, Winooski, VT). The experiments were performed in duplicate and repeated in triplicate independently.

Identification of cagA gene in *H. pylori* strains with real-time polymerase chain reaction: The DNA samples were isolated using a bacterial DNA extraction kit (Boehringer Ingelheim GmbH, Ingelheim, Germany). Briefly, the most and least virulent *H. pylori* strains were resuspended in GTEL buffer and pre-incubated in TESK buffer at 55 ℃ for 2 h. The RNA contained in the *H. pylori* strains was eliminated using 20-μg/mL RNase at 55 ℃ for 10 min. The DNA samples were isolated using the phenol-chloroform-isopentanol (25:24:1) extraction technique. Genotyping for *cagA* was performed using the primers of the following sequences (Sangon Biotech Co., Ltd., Shanghai, China): sense, 5'-ATGGAAAATATCATACAACCCC-3', and antisense, 5'-CATCTTCTAAATGGGAAACGCC-3'; length, 268 bp. The thermal cycling condition was as follows: pre-denatured at 96 ℃ for 1 min; denatured at 94 ℃ for 1 min and annealed at 60 ℃ for 1 min, 35 cycles; and extended at 94 ℃ for 1 min. Polymerase chain reaction (PCR) products were separated on 1% agarose gels containing 0.5 g/mL ethidium bromide and visualized by ultraviolet transillumination. *H. pylori* strain NCTC 11639 (Institute of Digestive Disease, Shanghai, China) was used as a positive control and *H. pylori* strain NCTC 12908 (Institute of Digestive Disease, Shanghai, China) as a negative control.

Cell apoptosis analysis with flow cytometry: Following 12, 24, and 48 h of co-culture, GES-1 cells were collected, washed in chilled PBS, and fixed in 70% pre-chilled (-20 ℃) ethanol at 4 ℃ for 18 h, and resuspended at a density of 1×10^6/mL. Fixed GES-1 cells were washed in PBS three times and stained with propidium iodide (Sigma, St Louis, MO) for 30 min prior to the analysis using an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc., Brea, CA). The experiments were performed in duplicate and repeated in triplicate independently.

The *H. pylori* strain that exhibited the most significant cell proliferative effect on MTT assay over 24 h of co-culture (36.8% increase) was harvested from a GC specimen and deemed as the most virulent strain, while a strain from a CG specimen that exhibited the least significant cell proliferative effect on MTT assay over 24 h of co-culture (15.0% increase) was deemed the least virulent.

In vivo carcinogenicity assay in Mongolian gerbils

The two representative strains, the most and the least virulent, were subsequently used to establish a *H. pylori* infection animal model[16]. The animal care and use complied with the regulations established and approved by the Animal Research Committee at Central South University. Seven-week-old specific-pathogen-free male Mongolian gerbils (n = 30) were purchased from the Laboratory Animal Center, Zhejiang Provincial Institute of Medical Sciences, Hangzhou, China. Gerbils were housed in an environment constantly maintained at a temperature of 25 ℃, a relative humidity of 55%, and a 12 h/12 h light/dark cycle. Animals had no access to rodent chow for 12 h and

Table 1 Primer pairs for quantitative real-time polymerase chain reaction

Gene	Primer Sequence (5′ to 3′)		Size of PCR product (bp)
	Sense	Anti-sense	
HDAC6	ACCGCTACGAGCAGGGTA	CGAGACGTGCAGGAAAGC	155
TARF1	TCCCGTAACACCTGATTAA	ACAACTCCCAAACCATACAC	146
GAPDH	AACGGATTTGGTCGTATTGGG	TCGCTCCTGGAAGATGGTGAT	216

PCR: Polymerase chain reaction.

drinking water for 4 h prior to the pretreatment with 0.3 mL 50% ethanol lavage per animal. *H. pylori* strains were cultured for three days using the aforementioned protocol, and resuspended in 7.5% (w/v) heat-inactivated brain heart infusion broth (Sangong Biotech, Shanghai, China) at a density of 1.0×10^9 cfu/mL using a spectrophotometer (Eppendorf). Fasted animals were lavaged with 0.5-mL suspension of the most or least virulent *H. pylori* strain (n = 10 for each strain) per animal three times, at a 12 h interval. Ten animals that were lavaged with the broth alone using the same protocol served as the control. Animals were allowed to resume oral intake two hours following the last lavage. Animals were sacrificed by cervical dislocation 4, 16, 28, 40 and 52 wk (n = 2 at each time point) following the lavage with *H. pylori* suspensions or broth. Fresh gastric mucosal specimens were collected from the gastric antrum and body for the rapid urease test, and the duplicate specimens were fixed in 4% paraformaldehyde for histological examination using hematoxylin and eosin staining.

Determination of the differentially expressed genes between GES-1 cells co-cultured with the most and least virulent strains

Total RNA extraction: The two representative *H. pylori* strains were co-cultured with GES-1 cells for 24 h, respectively, at a cell/bacterium ratio of 1:50, the optimal ratio for *H. pylori*-induced cell proliferation as determined in MTT assay. Mono-cultured GES-1 cells were used as a control. Total cellular RNA was extracted from infected and non-infected cells using Qiagen RNeasy Mini kit (Invitrogen, Carlsbard, CA) for further microarray analysis and quantitative real-time (qRT)-PCR verification. RNA concentration and purity were determined using an ultraviolet spectrometer (Eppendorf). Denaturing agarose gel electrophoresis was performed to validate the integrity of RNA samples. The experiments were performed in duplicate and repeated in triplicate independently.

Oligonucleotide microarray: The GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, United States), containing 48 000 transcript probes, was used to assess the global gene expression of GES cells in response to *H. pylori* infection. Microarray analysis was performed as instructed by the manufacturer. Chip scanning and data analysis were processed using Affymetrix Microarray Suit Software 5.0 to identify significant differential gene expression profiles. A gene with a signal ratio of more than 2.0 (up-regulated) or less than 0.5 (down-regulated) was defined to be a differentially expressed gene when co-cultured GES-1 cells were compared with control cells, or when cells co-cultured with the most virulent strain were compared with those co-cultured with the least virulent strain.

Validation of potential carcinogenesis-associated genes among the most differentially expressed genes

Quantitative real-time polymerase chain reaction in GES cells: Two carcinogenesis-associated genes that encode histone deacetylase 6 (HDAC6) and tumor necrosis factor receptor-associated factor 1 (TRAF1)[17,18] were among the most differentially expressed genes. Due to their unknown roles in the pathogenesis of GC, the transcriptions of HDAC6 and TRAF1 were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Primer pairs for qRT-PCR were listed in Table 1. GAPDH, as an internal control, was co-amplified with the specific genes. Briefly, total cellular RNA was extracted from GES cells infected with the most and least virulent strains as well as uninfected GES cells, and reversely transcribed into cDNA by M-MLV reverse transcriptase and random hexamer primer (Invitrogen, Carlsbard, CA). The cDNAs were amplified by 30 PCR cycles of denaturation at 94 ℃ for 30 s, annealing at 53 ℃ for 45 s, and extension at 72 ℃ for 30 s. As a final step, the extension was at 72 ℃ for 1 min. PCR products were separated on 1% agarose gels containing 0.5 g/mL ethidium bromide and visualized by ultraviolet transillumination. SYBR qPCR Mix (Invitrogen, Carlsbard, CA) was used to monitor DNA synthesis. The experiments were performed in duplicate and repeated in triplicate independently.

Immunohistochemistry in gastric biopsy specimens: In an independent experiment, *H. pylori*-positive gastric mucosal specimens from the 113 patients were used for immunohistochemistry (IHC) to verify the expression of HDAC6 and TRAF1 in different gastric pathologies. Briefly, goat-anti-HDAC6 (1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and rabbit-anti-TRAF1 (1:100; BIOSS, Beijing, China) were used as primary antibodies. Conjugated biotin was identified by using horseradish peroxide-labeled streptavidin (1:200; Santa Cruz Biotechnology, Inc.) and visualized by using 3,3'-diaminobenzidine (1:200; Santa Cruz Biotechnology, Inc.). Overall, at least 200 cells in ten randomly selected fields were counted, and the percentages of positive cells against the total counted cells were calculated for each specimen. The IHC staining intensity was semiquantitatively

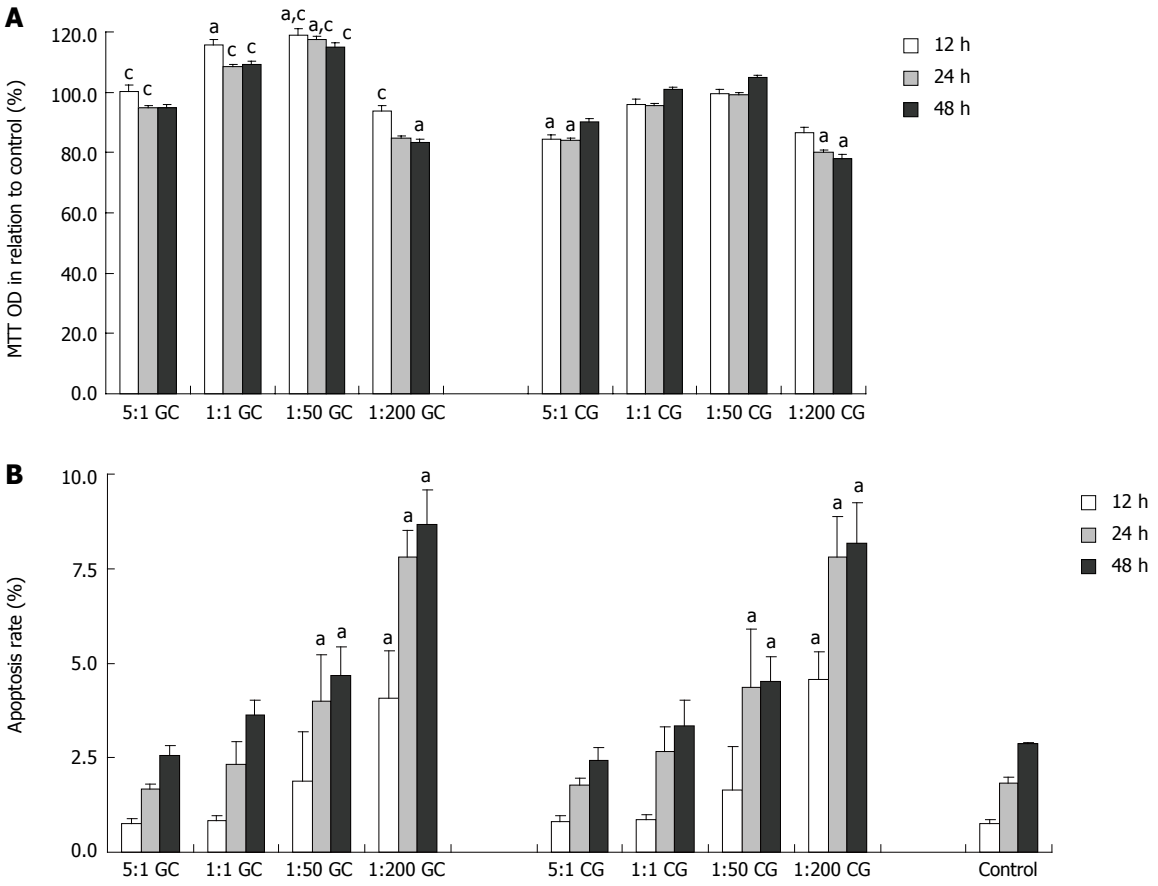

Figure 1 GES-1 cell proliferation as determined by 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide assay (A) and cell apoptosis as determined by flow cytometry (B) after co-culture with *helicobacter pylori* isolated from patients with gastric carcinoma (GC, *n* = 10) or chronic gastritis (CG, *n* = 10) for 48 h in relation to control cells. The ratio denotes the ratio of GES-1 cells *vs Helicobacter pylori* cells. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* CG group. OD: Optical density.

determined as negative (positive cells < 10%), moderately positive (10%-50%), and strongly positive (> 50%). The experiments were performed in duplicate and repeated in triplicate independently.

Statistical analysis

SPSS ver. 13.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis. All numerical data were expressed as mean ± SD, and compared by using the Student's *t*-test or analysis of variance, when appropriate. All categorical data were expressed as percentage and compared by using the χ^2-test or Fisher's exact probability test, when appropriate. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

GC-derived H. pylori strains induced potent proliferation in GES-1 cells

As shown in Figure 1A, GES-1 cells exhibited an overall cell proliferative response over time to the co-culture with *H. pylori* isolated from GC. A low-concentration (5:1) GC-derived *H. pylori* infection *in vitro* had minimal effect on GES-1 cell proliferation. At a cell/*H. pylori* ratio of 1:1 or 1:50, GES-1 cells co-cultured with GC-derived *H. pylori* strains had a significantly higher proliferation rate relative to those with CG-derived *H. pylori* strains and control cells. In contrast, CG-derived *H. pylori* co-culture at a ratio of 5:1, 1:1, and 1:50 had no significant impact on cell proliferation over time. Of note, the co-culture at a higher concentration (1:200) *H. pylori* isolated from either GC or CG compromised the proliferation capability of GES-1 cells.

GC- and CG-derived H. pylori strains induced comparable apoptosis in GES-1 cells

Flow cytometry analysis showed that the apoptosis rate increased over time in cells co-cultured with *H. pylori* and non-infected cells (Figure 1B). In comparison with the control cells, GES-1 cells co-cultured with GC- or CG-derived *H. pylori* strains had a significantly increased apoptotic rate at 24 and 48 h at the ratios of 1:50, and at all time points at the ratio of 1:200. However, there was no significant difference in the increase of apoptotic rate between GES-1 cells co-cultured with GC-derived *H. pylori* strains and those co-cultured with CG-derived *H. pylori* strains.

The most virulent GC-derived H. pylori strain induced gastric mucosal carcinogenesis in the animal model

The most virulent strain, which was derived from a GC patient, and the least virulent strain, which was derived

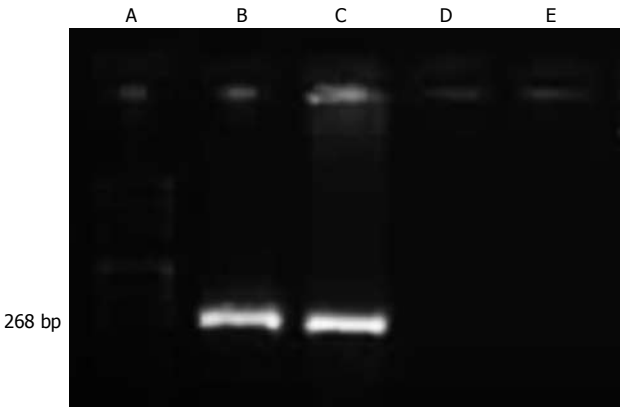

Figure 2 Identification of status of *cagA* gene in *Helicobacter pylori* strains using real-time polymerase chain reaction. A: DNA ladder; B: *Helicobacter pylori* (*H. pylori*) stain NCTC 11639 (positive control); C: The most virulent gastric carcinoma-derived strain; D: The least virulent chronic gastritis-derived strain; E: *H. pylori* stain NCTC 12908 (negative control).

from a CG patient, were genotyped to be *cagA* positive and cagA negative, respectively (Figure 2). *H. pylori* rapid urease testing was found positive in 60.0% (6/10), 70.0% (7/10), and 0.0% (0/10) of the animals lavaged with the most virulent strain, least virulent strain, and control lixivium, respectively. No animals, except one in the group infected with most virulent strain that died at week 3, accidentally died during the experiment. *H. pylori* infection was present in all animals lavaged with *H. pylori*, but in none of the control animals, as shown by histological examination. The animals lavaged with the most virulent strain were more prone to precancerous diseases and gastric carcinoma than those lavaged with the least virulent strain and control lixivium. At week 52, CG, atrophy, metaplasia, dysplasia, and GC were observed in 90.0% (9/10), 80.0% (8/10), 80.0% (8/10), 90% (9/10), and 60.0% (6/10), respectively, of the animals lavaged with the most virulent strain (Table 2). Precancerous lesions including gastric atrophy, intestinal metaplasia, and dysplasia were observed from the 4th week, and gastric cancer occurred as early as at week 28 (Figure 3A, Table 2). However, only mild CG was observed in 90% (9/10) of the animals lavaged with the least virulent strain (Figure 3B, Table 2). In addition, gastric ulceration was observed in 50.0% (5/10) and 40.0% (4/10), respectively, in the two groups. The gastric mucosa was within normal limits in all control animals at any time points (Figure 3C, Table 2).

Microarray analysis identified significant differential gene expression profiles in GES-1 cells co-cultured with the most versus least virulent H. pylori strains

Microarray analysis identified 2834 and 314 differentially expressed genes in GES-1 cells co-cultured with most or least virulent *H. pylori* strain, respectively, compared with non-infected cells (Figure 4). Furthermore, 800 differentially expressed genes (49 up- and 751 down-regulated), involving those associated with cell cycle regulation, cell apoptosis, cytoskeleton, immune response, and substance and energy metabolisms, were identified in cells co-cultured with the most virulent strain compared with those co-cultured with the least virulent strain (Table 3). Six most differentially expressed genes (with a betweenness centrality of 0.1-0.2) were identified among the significant differential gene profile network, including JUN, KRAS, BRCA1, SMAD2, TRAF1, and HDAC6 (Table 4).

Up-regulated expressions of HDAC6 and TRAF1 in GES-1 cells co-cultured with the most vs least virulent H. pylori strains

qRT-PCR analyses verified significant up-regulations of HDAC6 and TRFA1 mRNA expression in GES-1 cells co-cultured with the most virulent strain or least virulent *H. pylori* strain, compared with the control cells (Figure 5A). Furthermore, HDAC6 and TRFA1 mRNA expressions were more significantly up-regulated in GES-1 cells co-cultured with the most virulent strain than in those co-cultured with the least virulent strain (Figure 5B).

Progressive over-expression of HDAC6 and TRAF1 in CG, precancerous disease, and GC specimens on immunohistochemistry

The histology and immunohistochemistry of gastric mucosal specimens from *H. pylori*-positive patients with CG (Figure 6A, E and I), IM (Figure 6B, F and J), dysplasia (Figure 6C, G and K), and GC (Figure 6D, H and L) showed that moderately positive and strongly positive HDAC6 expression were detected in 5 (21.7%) of 23 CG patients, 10 (30.0%) of 33 IM patients, 18 (54.5%) of 33 dysplasia patients, and 21 (77.8%) of 27 GC patients ($P < 0.001$). Furthermore, the positive rate of HDAC6 expression was significantly higher in dysplasia and GC, compared to that in CG ($P = 0.014$; $P < 0.001$) and in IM ($P = 0.049$; $P < 0.001$). However, there was no difference in the positive rate between CG and IM, and between dysplasia and GC. Similarly, the up-regulation of TRAF1 expressions was detected in 8/23 (34.8%), 16/33 (53.3%), 24/33 (72.7%), and 24/27 (88.9%) specimens of CG, IM, dysplasia, and GC, respectively ($P < 0.001$). The positive rate of TRAF1 expression was significantly greater in dysplasia and GC *vs* CG ($P = 0.005$; $P < 0.001$) and in GC *vs* IM ($P = 0.003$), whereas there was no difference between CG and IM and between dysplasia and GC.

DISCUSSION

Gastric carcinogenesis is a pathological process of cell cycle disorder and uncontrolled growth resulting from multiple aberrant gene alterations in response to extrinsic stimulus like *H. pylori* infection. The dysregulation of the balance between cell proliferation and apoptosis plays a pivotal role in this pathogenesis[19]. The proliferation rate of epithelial cells that are co-cultured with *H. pylori* isolated from gastric mucosal biopsy specimens is shown to be two-fold higher than that of the normal control. *H. pylori* eradication can reverse the proliferative effect on gastric mucosal epithelia though[20]. Moreover, both *H. pylori*

Table 2 Pathological outcomes of gastric mucosae from Mongolian gerbils lavaged with the most virulent gastric carcinoma-derived strain, the least virulent chronic gastritis-derived strain, and the control broth over 52 wk

	Pathological outcomes				
	Chronic gastritis	Gastric atrophy	Intestinal metaplasia	Gastric dysplasia	Gastric carcinoma
GC (n = 10)	9 (90.0%)[b]	8 (80.0%)[b,d]	8 (80.0%)[b,d]	9 (90.0%)[b,d]	6 (60.0%)[b,d]
CG (n = 10)	9 (90.0%)[b]	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Control (n = 10)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* CG group. GC: Gastric carcinoma; CG: Chronic gastritis.

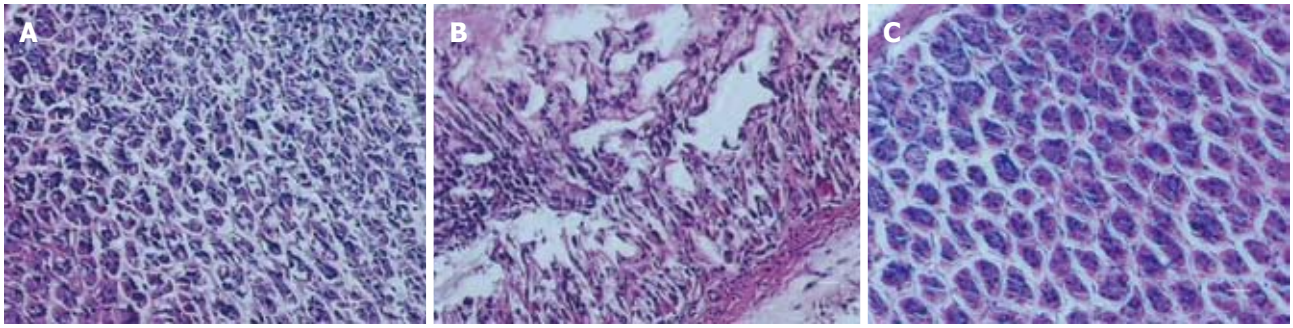

Figure 3 Representative histological microphotographs (hematoxylin and eosin staining, 200 ×, scale bar = 100 μm) of gastric mucosal specimens collected from Mongolian gerbils lavaged with the GC-derived *Helicobacter pylori* strain (A: gastric carcinoma), the CG-derived strain (B: chronic gastritis), and the control lixivium (C) at the 28th week.

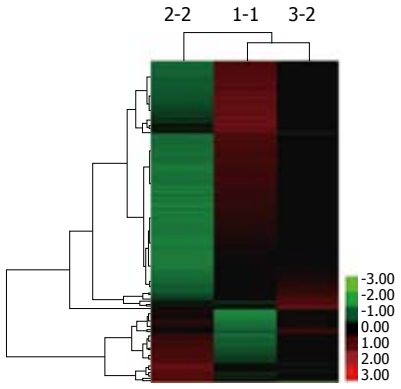

Figure 4 Cluster analysis of gene expression profiles in GES-1 cells in response to the most and least virulent *Helicobacter pylori* strains. The red, green, and black colors indicate up-regulated, down-regulated, and equivalent expression. 1-1: Uninfected GES-1 cells (control); 2-2: GES-1 cells co-cultured with the GC-derived strain; 3-2: GES-1 cells co-cultured with the CG-derived strain. GC: Gastric carcinoma; CG: Chronic gastritis.

intracellular extracts and secretions are found to directly stimulate epithelial growth. Our results indicate that the effects of *H. pylori* co-culture on GES-1 cell proliferation are *H. pylori* concentration dependent, preferably at a cell/*H. pylori* ratio of 1:50; however, the presence of high-concentration *H. pylori* suppresses GES-1 growth instead. The antiproliferative effect of high-concentration *H. pylori* has been reported in multiple cell lines. This inhibitive effect is associated with the production of massive cytotoxic factors, which cause DNA damage, induce cell apoptosis, increase the synthesis of induced nitric oxide synthase, and upregulate p53 expression[21-23]. Of note, GC-derived *H. pylori* strains produced a more potent proliferative effect on GES-1 cells than CG-derived *H. pylori* strains, which is consistent with the findings of Yu *et al*[24]. Moreover, both GC- and CG- *H. pylori* strains had a mild and comparable effect on apoptosis of GES-1 cells in a concentration-dependent but not strain-specific manner as shown by flow cytometry. These findings suggest that *H. pylori* strain-specific virulence may not be associated with gastric epithelial apoptosis, at least *in vitro*.

H. pylori strains isolated from humans are capable of colonizing the gastric mucosa of Mongolian gerbils[16,25]. Additionally, Hirayama *et al*[26] reported that *H. pylori*-infected Mongolian gerbils exhibited a gastric mucosal pathology similar to human *H. pylori* infection. In the present study, infection with the most virulent GC-derived *H. pylori* strain in Mongolian gerbils resulted in a series of mucosal pathologies that manifest as chronic inflammation, atrophy, intestinal metaplasia, or dysplasia more frequently than did the infection with the CG-derived strain over 52 wk. Moreover, GC developed as early as 28 wk following the infection with the GC-derived strain. This observation may be of significant clinical implications. First, previous studies have reported that the time to develop GC is approximately 62 wk[24] or even 72 wk after infection[27]. We assume that less virulent or less carcinogenic strains may have been used in the previous studies, and we propose that more carcinogenic strains should be used in animal experiments related to *H. pylori*-induced carcinogenesis and prevention of *H. pylori*-induced GC. Second and more importantly, it is suggested that the *H. pylori* strains that exhibit a potent epithelial prolifera-

Table 3 Differentially expressed genes with a fold change of > 2.0 or < 0.5 in GES-1 cells co-cultured with the most virulent gastric carcinoma-derived strain *vs* the least virulent chronic gastritis-derived strain

Gene name (n = 185)	Fold change GC/CG	Accession number
Cell cycle related genes		
AMN1	4.748	BG031897
HDAC6	2.025	NM_006044
CCNE2	0.464	AF112857
KIF20B	0.462	NM_016195
DOCK8	0.456	AL161725
ASPM	0.452	AK001380
CENPF	0.450	U30872
NIPBL	0.432	NM_015384
SYCP2	0.424	NM_014258
ASPM	0.414	NM_018123
SGOL2	0.401	N31731
CENPE	0.398	NM_001813
CEP70	0.382	NM_024491
SGOL2	0.375	AW965339
DOCK11	0.363	AI742838
SERPINB3	0.290	BC005224
Apoptosis-related genes		
TRAF1	2.254	NM_005658
TIA1	0.496	AL567227
OPA1	0.474	AB011139
PIK3CA	0.450	NM_006218
NUDT12	0.408	AL136592
PEG10	0.367	BE858180
Cytoskeleton and sports		
ADD3	0.450	AI818488
KIAA0774	0.429	AI818409
KIF14	0.379	AW183154
KIF14	0.370	NM_014875
ADD3	0.344	BE545756
ADD3	0.316	NM_019903
Intracellular transport		
VPS13A	0.435	AW629014
VPS13A	0.438	AW629014
IFT74	0.431	NM_025103
GOLGA4	0.425	NM_002078
ANKRD10	0.419	BE670056
IFT80	0.414	AB037795
FAM8A1	0.412	NM_016255
SNX2	0.406	NM_003100
ANKRD32	0.355	AL136560
DNA synthesis, repair, recombination		
RAD50	0.472	NM_005732
SFPQ	0.457	AV705803
FAM8A1	0.412	NM_016255
LIN9	0.338	BF697734
DNA-binding ,transcription, transcription factor		
ZNF253	5.254	NM_021047
KLF11	3.598	AA149594
SMARCA1	0.496	NM_003069
GOLGB1	0.492	NM_004487
KLF9	0.462	NM_001206
CHD1	0.459	NM_001270
PBX1	0.455	AL049381
TRIP11	0.434	AF007217
SMARCA1	0.433	NM_003069
C8orf83	0.433	BE962119
EPM2AIP1	0.430	BF432224
ELL2	0.420	AI745624
GLCCI1	0.400	AA058770
ARID5B	0.395	BG285011
ZNF644	0.390	NM_016620
BAZ2B	0.365	NM_013450
SAMD9	0.352	AA741307
SAMD9	0.345	NM_017654
TPR	0.346	BF110993
TPR	0.344	AK023111
LCORL	0.339	AI807408
TPR	0.300	AW235355
Cell signal and transduction		
PRKCB	0.498	M13975
DST	0.493	NM_001723
FARP1	0.462	BF725250
GMFB	0.461	NM_004124
PPM1A	0.454	AA886888
IFT81	0.444	NM_014055
PDE10A	0.442	AI143879
ICK	0.428	NM_014920
ANKRD10	0.419	BE670056
IFT80	0.414	AB037795
SNX2	0.406	NM_003100
CNTLN	0.386	AA280904
MAP2K6	0.384	NM_002758
ANKRD32	0.355	AL136560
ARHGAP18	0.307	BE644830
Protein translation, synthesis, decomposition		
RHOBTB3	0.500	NM_014899
ST13	0.490	U17714
EEF2K	0.488	W68180
TSHZ2	0.476	AW953679
TMF1	0.417	AI767750
HMMR	0.410	U29343
CCDC88A	0.387	AB033038
NSBP1	0.321	BC005342
Protein-coding gene		
CXorf39	0.499	AI590719
LOC286052	0.478	AA278233
FLJ40113/LOC440295	0.411	AI632181
LOC100133781	0.374	AA973100
LOC100130360	0.346	BG231554
LOC643401	0.300	BC039509
Ion channel and transport		
IFT74	0.496	AI610355
SLC5A3	0.493	AK024896
SLC2A13	0.489	AL565362
STEAP4	0.488	NM_024636
TMEM56	0.469	AI004375
EXOC5	0.466	BF509391
CACNB2	0.465	AI040163
SLC2A13	0.455	NM_052885
TMEM133	0.435	AF247167
DMXL2	0.405	AB020663
SNX13	0.401	R75838
SEC62	0.397	NM_153039
ATP11C	0.382	BF475862
TMEM106B	0.380	BF513060
PEG10	0.367	BE858180
SORBS2	0.222	AI659533
Cell proliferation, angiogenesis		
ANGPTL4	2.460	NM_016109
TPR	0.492	NM_003292
TPR	0.492	NM_003292
PNN	0.487	U59479
PNN	0.428	U59479
ROCK2	0.404	AL049383
TTK	0.347	NM_003318
Immune-related genes		
CFI	0.477	NM_000204
SERPINB4	0.431	AB046400
PIBF1	0.423	NM_006346
Metabolize-related genes		
AK7	0.477	NM_152327
TTC3	0.471	AI652848
HS2ST1	0.438	NM_012262
MANEA	0.427	AI587307

PLA2G12A	0.416	AV714268
TTC3	0.413	D83077
PPP1CB	0.407	W67887
NUDT12	0.408	AL136592
TTC3	0.403	NM_003316
TTC3	0.403	AI885338
RNF150	0.398	AA722069
SEPP1	0.396	NM_005410
CRYZ	0.387	NM_001889
PDK4	0.374	AV707102
ABAT	0.359	AF237813
PLA2G12A	0.331	AI767751
AGXT2L1	0.326	NM_031279
CYP1B1t	0.314	NM_000104
METTL7A	0.274	NM_014033
Cell structure-related genes		
ARMCX3	0.385	AL121883
ANK3	0.225	NM_020987
Cell Adhesion-related genes		
KITLG	0.447	AI446414
PIK3CA	0.450	NM_006218
ANKRD10	0.419	BE670056
ANKRD32	0.355	AL136560
Stress-related genes		
DNAJB4	0.400	BG252490
LXN	0.294	NM_020169
DNAJB4	0.471	NM_007034
Protein regulation		
LOC727770	0.426	AI359676
TBC1D8B	0.368	AW172431
Cell differentiation		
LIFR	0.374	AA701657
RNA processing		
SR140	0.413	AU152088
Protein receptor		
LANCL1	0.412	NM_006055
Oxidation		
DIO2	0.434	AI038059
Cell growth-related genes		
ITCH	0.441	AA868238
TGFBR3	0.450	AW193698
TGFBR3	0.422	NM_003243
Cytokine receptor		
LTB	2.001	NM_002341

Table 4 Most differentially expressed genes in GES-1 cells co-cultured with the most virulent gastric carcinoma-derived strain vs the least virulent chronic gastritis-derived strain

Gene name	Betweenness centrality	Description	Identified or proposed function
JUN	0.201780	Jun oncogene	Cell growth and/or maintenance, signal transduction molecules, and transcription factors
KRAS	0.185944	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Regulation of cell cycle, cell growth and/or maintenance
BRCA1	0.162070	Breast cancer 1, early onset	DNA damage checkpoint; p53/ATM signaling, and induction of apoptosis
SMAD2	0.121460	SMAD family member 2	Transcription factors
TRAF1	0.092496	Tumor necrosis factor receptor-associated factor 1	DNA damage checkpoint, p53/NF-KB signaling, and cell cycle control
HDAC6	0.092239	Histone deacetylase 6	Cell proliferation and tumor angiogenesis

tive effect are highly virulent and carcinogenic, and that individuals infected with these strains are at high risk for the development of GC and should receive appropriate *H. pylori* eradication therapy.

Human whole-genome microarray analysis identifies a large number of significant differentially expressed genes in GES-1 cells co-cultured with *H. pylori* strains *vs* non-infected control cells, which clearly indicates that *H. pylori* activates and inactivates a series of gene transcription of GES-1 cells *in vitro*. Our transcriptional profiling results were generally consistent with the previous reports. Liu *et al*[14] reported that *H. pylori* infection induced the up-regulated expression of multiple chemokines and chemokine receptors, such as IL-8 and CCL5, as well as of apoptosis-related genes, such as GADD45A. Eftang *et al*[28] reported that *interleukin-8* was the single most up-regulated gene in whole genome profiling of *H. pylori* exposed gastric epithelial cells. MAPK and NF-κB cellular pathways were also powerfully activated; the marked up-regulation of TP53BP2 corresponding to ASPP2 protein may interact with *H. pylori* CagA, and cause marked p53 suppression of apoptosis. Sohn *et al*[29] suggested that the intracellularly translocated CagA may be involved in epithelial mesenchymal transition. However, the present study, for the first time, reports that the expression of HDAC6 and TRAF1 is up-regulated in gastric mucosal cells upon co-culture with GC-derived, *cagA*-positive *H. pylori* strain, and the expressions of these two proteins are progressively up-regulated in CG, intestinal metaplasia, dysplasia, and GC. Furthermore, such response seems to be strain-specific for many genes as the number of differentially expressed genes in cells co-cultivated with the GC-derived strain is 9-fold that in those co-cultured with the CG-derived strain. These significant differentially expressed genes involve genes that are known to be associated with tumorigenesis, among which HDAC6 and TRAF1 are the most prominent ones. The upregulation of HDAC6 and TRAF1 expressions in response to the GC-derived *H. pylori*-strain and in relation to *H. pylori* infection in patients with GC was verified by qRT-PCR in GES-1 cells *in vitro* and by immunohistochemistry in gastric specimens taken from patients with different gastric pathologies. To our best knowledge, this is the first study to identify the overexpression of HDAC6 and TRAF1 in *H. pylori*-associated GC, and to suggest a potential role of these genes in *H. pylori*-induced gastric carcinogenesis.

Histone is an important component of eukaryotic chromatin. Acetylation and deacetylation of histone are essential for the regulation and modification of gene expression[30]. A newly-discovered mechanism of carcinogenesis is that HDAC family proteins aberrantly binding to a specific promoter region may cause cryptic transcription and inhibit normal gene transcription, initiating the malignant transformation[31]. HDAC6 functions to modulate gene expression by removing the acetyl group from histones, which contributes to oncogenic cell trans-

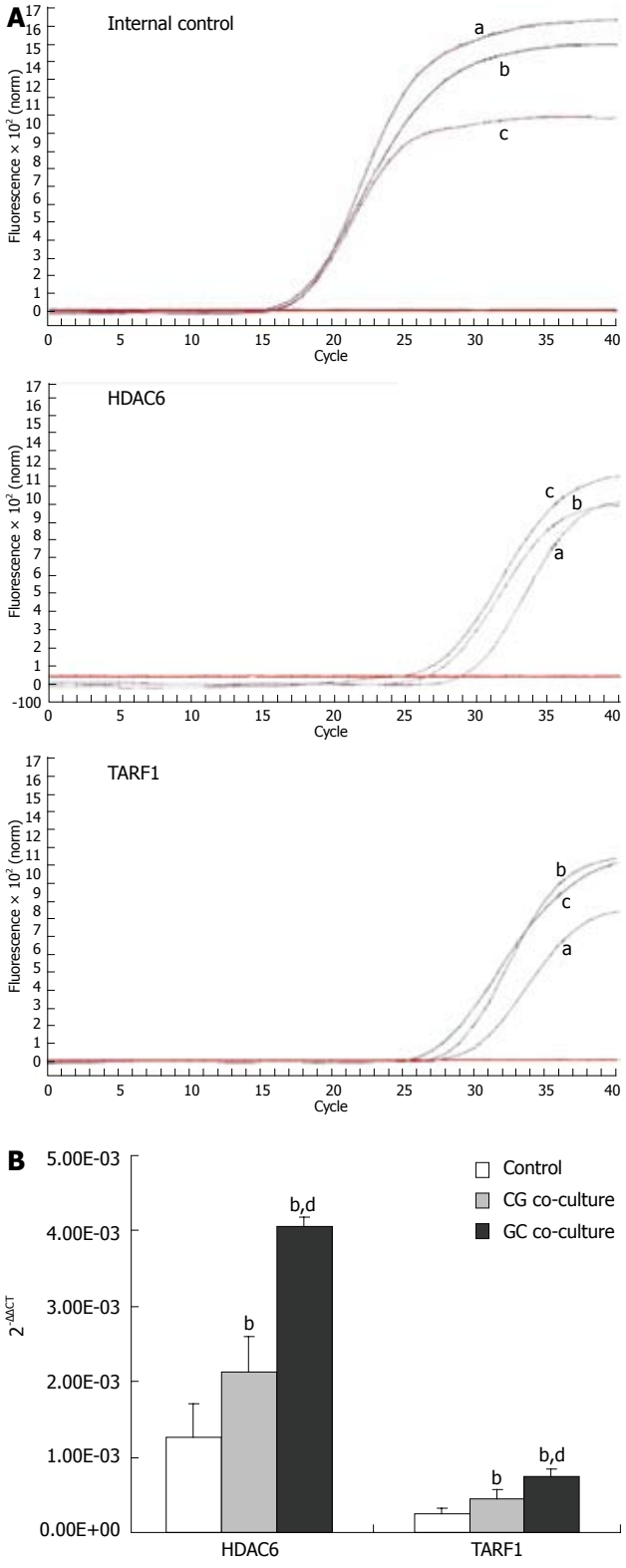

Figure 5 Verification of HDAC6 and TRAF1 up-regulation in GES-1 cells in response to the most and least virulent *Helicobacter pylori* strains as determined by quantitative real-time polymerase chain reaction analysis. A: Representative quantitation of mRNA expression of GAPDH, HDAC6 and TRAF1 in GES-1 cells (a), GES-1 cells co-cultured with the CG-derived *Helicobacter pylori* (*H. pylori*) strain (b) and GES-1 cells co-cultured with the GC-derived *H. pylori* strain (c); B: Graphic analysis showing mRNA expression of *HDAC6* and *TRAF1* expressions in GES-1 cells in GES-1 cells, GES-1 cells co-cultured with the CG-derived *H. pylori* strain and GES-1 cells co-cultured with the GC-derived *H. pylori* strain. [b]$P < 0.01$ *vs* the control; [d]$P < 0.01$ *vs* cells co-cultured with the CG-derived *H. pylori* strain.

formation[32]. Aoyagi *et al*[33] reported that HDAC6 could directly regulate HSP90 expression via deacetylation. The targeted inhibition of HDAC6 reduces the deacetylation of HSP90 but increases the acetylation simultaneously, destroying HSP90 chaperones and resulting in molecular function failure. HDAC6 synergizes with HDAC10 to regulate vascular endothelial growth factor receptors through heat shock protein mediation as well[34]. HDAC6 has been found to be implicated in multiple malignancies, such as esophageal cancer, lung cancer, breast cancer, and oral squamous cell carcinoma[35,36]. Zhang *et al*[35] reported that high-HDAC6-expressing premenopausal breast cancer patients exhibited a favorable tumor-free survival and a sensitive response to endocrine therapy. HDAC is also thought to be associated with breast cancer metastasis as it de-acylates microtubules, whereas the combined use of estrogen antagonist and paclitaxel significantly suppresses the de-acylation of microtubules[37,38]. In the present study, the expression of HDAC6 mRNA was highly up-regulated in GES-1 cells co-cultured with the GC-derived *H. pylori* strain compared with those co-cultured with the CG-derived strain and uninfected control cells. It is likely that *H. pylori* infection activates HDAC6 to dysregulate the synthesis of histones in gastric epithelial cells[39]. However, further investigation is required to elucidate the exact mechanisms of *H. pylori* infection and the functional roles of *H. pylori*-activated HDAC6 overexpression in gastric carcinoma.

TRAF is a new member of the tumor necrosis factor (TNF) family[40]. Seven TRAF isoforms have been reported to interact directly with cell-surface receptors and regulate cell survival/death balance[41]. TRAF1 activates NF-κB to gradually initiate immortalization and tumorigenesis in GC[42]. Sughra *et al*[43] reported that TRAF1 functions primarily to up-regulate the transcription of IKKβ, an inhibitor of NF-κB and to enhance the activity of IKKβ as well. Therefore, TRAF1 activates and interacts with NF-κB simultaneously. The overexpression of TRAF1 has been identified in nasopharyngeal carcinoma and lymphoma[44,45]. The variation in TRAF1 expression is associated with the occurrence, metastasis, and induction of chemotherapy resistance of malignant tumors[46]. Similar to HDAC6, TRAF1 is verified by qRT-PCR to be up-regulated in GES-1 cells co-cultured with *H. pylori* strains, especially the GC-derived strain in the present study. Again, how *H. pylori* infection up-regulates TRAF1 expression and what downstream genes are activated and/or deactivated by *H. pylori*-induced TRAF1 overexpression require further investigation.

The roles of HDAC6 and TRAF1 in *H. pylori*-associated GC remain to be elucidated although their expression is significantly up-regulated in epithelial cells stimulated with GC-derived *cagA* positive *H. pylori* strain. Our GC animal model experiment showed that the proliferative effect *in vitro* of GC-derived *cagA* positive *H. pylori* strain can contribute to the carcinogenesis of gastric mucosa *in vivo*. However, the up-regulated expression of HDAC6 and TRAF1 may not be causative of proliferative or carcinogenic effect of the GC-derived *H. pylori* strain as no

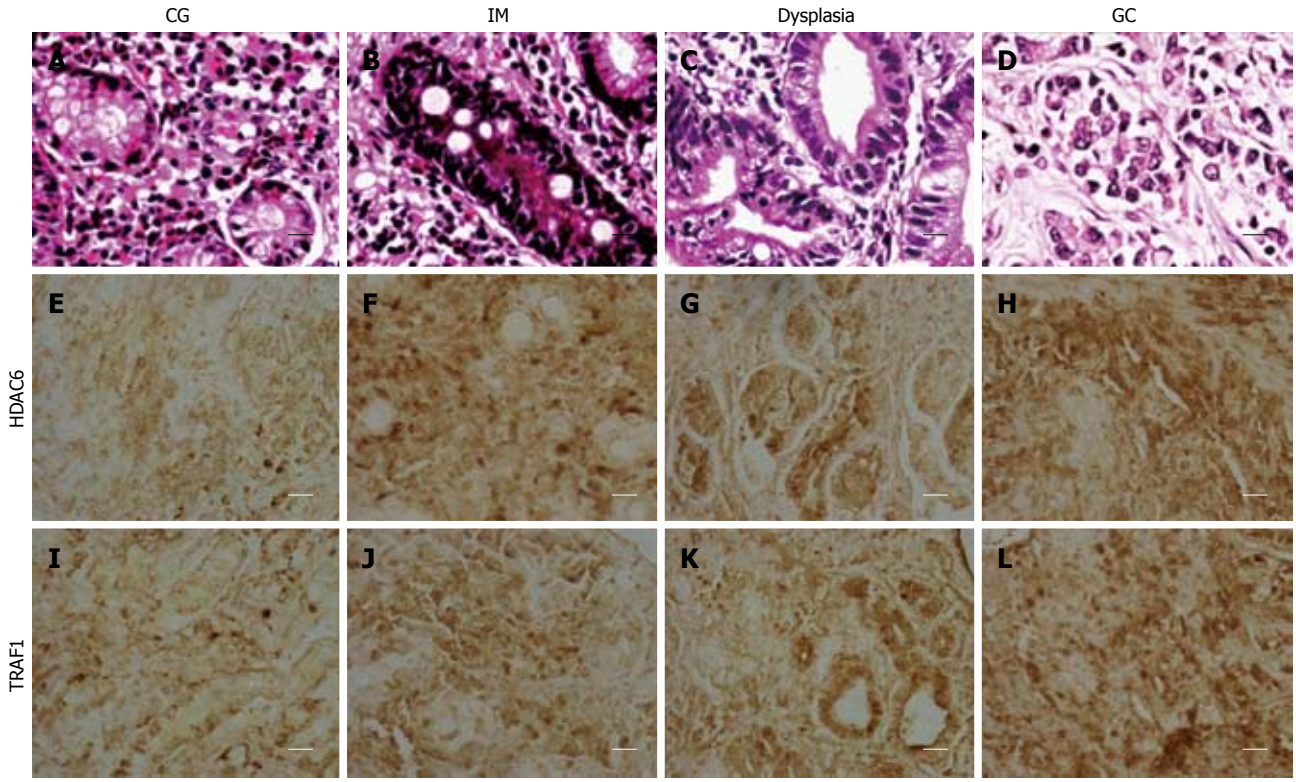

Figure 6 Histology and immunohistochemistry (400 ×, scale bar = 50 μm) of HDAC6 and TRAF1 in gastric specimens of patients with chronic gastritis (CG, *n* = 23), intestinal metaplasia (IM, *n* = 30), dysplasia (*n* = 33), and gastric carcinoma (GC, *n* = 27).

"loss-of-function" or "gain-of-function" experiment has been performed to elaborate the pathogenetic effect of HDAC6 and TRAF1 in GC. Moreover, we were unable to follow up GC patients in subsequent treatment period as they were referred to various general surgeons among multiple institutions at the patients' own will. Therefore, the clinicopathological values of HDAC6 and TRAF1 are yet to be investigated, which are expected to be clinically useful for the prediction of prognosis and treatment resistance.

In conclusion, GC-derived *H. pylori* strains induce a more potent proliferative but comparable apoptotic effect in GES-1 cells as compared to CG-derived strains. HDAC6 and TRAF1 are identified to be up-regulated in GES-1 cells co-cultured with the GC-derived strain, which are further verified *in vivo*. These findings indicate that these two genes may be involved in *H. pylori* induced gastric carcinogenesis, although their exact roles require further investigation.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a pathogenic bacterium colonizing gastric mucosae. It has been accepted to be the primary cause of upper gastrointestinal disorders, such as acute and chronic gastritis, peptic ulcer disease, and gastric cancer. However, the subsequent outcomes of persistent *H. pylori* infection are highly variable. Such variation in clinicopathological outcomes of *H. pylori* infection is believed to result from the variations in the virulence of different strains, genetic background of the host, and more importantly, the host-to-pathogen interactions. How gastric epithelial cells respond to *H. pylori* clinical isolates derived from patients with different pathologies, such as chronic gastritis (CG) and gastric cancer (GC) specimens, at the genome-wide level remains unknown. Therefore, this study was carried out to identify genes potentially involved in *H. pylori*-induced gastric carcinogenesis, by comparing the genomic profiles between gastric epithelial cells co-cultured with *H. pylori* strains isolated from patients with GC and those co-cultured with strains from patients with CG.

Research frontiers

It is likely that *H. pylori* strains themselves are highly variable in virulence to gastric mucosal epithelia, especially in terms of the carcinogenic effect. Genomic profiling analyses have identified a wide range of genetic variations among *H. pylori* strains isolated from patients with different gastric pathologies. Global gene expression profiles also vary greatly in human gastric epithelial immortalized cells infected with spiral *versus* coccoid *H. pylori*. These findings suggest that gastric epithelial cells tune in the expression of their genes, especially those associated with tumorigenesis, in response to specific *H. pylori* strains or a specific virulent factor of the strain. In addition to the core genes, strain-specific genes are thought to play an essential role in *H. pylori* propagation and pathogenesis.

Innovations and breakthroughs

This observation may be of significant clinical implications. First, previous studies have reported that the time to develop GC is approximately 62 wk or even 72 wk after infection. The authors assume that less virulent or less carcinogenic strains may have been used in the previous studies, and the authors propose that more carcinogenic strains should be used in animal experiments related to *H. pylori*-induced carcinogenesis and prevention of *H. pylori*-induced GC. Second and more importantly, it is suggested that the *H. pylori* strains that exhibit a potent epithelial proliferative effect are highly virulent and carcinogenic, and that individuals infected with these strains are at high risk for the development of GC and should receive appropriate *H. pylori* eradication therapy.

Applications

The roles of HDAC6 and TRAF1 in *H. pylori*-associated GC remain to be elucidated although their expression is significantly up-regulated in epithelial cells stimulated with GC-derived *cagA* positive *H. pylori* strain. These findings indicate that these two genes may be involved in *H. pylori* induced gastric carcinogenesis, although their exact roles require further investigation.

Peer review

The authors performed DNA microarray analysis comparing gene expression profiles between GES-1 cell lines co-cultured with highly virulent (cagA+) and low virulent (cagA-) *H. pylori* strains isolated from human gastric mucosa tissue with CG and GC tissue, respectively. These two strains showed different effect on the GES-1 cell line in cell proliferation activity but similar effect on the cell lines in apoptotic property. They identified 800 differentially expressed genes, and from six most differentially expressed genes, they selected TRAF1 and HDAC6. The authors examined HDAC1 and TRAF1 expression at the mRNA level in GES-1 cells co-cultured with the *H. pylori* strains above, and confirmed that these expressions were up-regulated in these cell lines. They also examined the expressions of these molecules immunohistochemically in surgically resected or biopsied specimens. These works are laborious and are considered to be scientifically of significant value.

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