Helicobacter pylori induces malignant transformation of gastric epithelial cells in vitro

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Epidemiologic studies have demonstrated that Helicobacter pylori infection is associated with increased risk for the development of gastric cancer. Animal studies have also shown that H. pylori infection leads to gastric carcinogenesis, especially intestinal phenotypes. However, no in vitro study has been carried out for cell transformation induced by H. pylori. The present study aimed to investigate whether ‘chronic’ H. pylori infection induces gastric epithelial cell transformation, and elucidate the underlying mechanisms of transformation induced by H. pylori. The immortalized ‘normal’ gastric epithelial cell line, GES-1, was co-cultured for 45 days with H. pylori strains B975 and L301. The cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Ki-67 antigen, and colony formation assay. The cell transformation was determined by observing cell morphology and measuring the expression of E-cadherin, β-catenin, and transcription factor-4 (TCF-4) at both protein and mRNA levels. H. pylori induced morphologic changes in GES-1 cells and significantly increased the proliferation of GES-1 cells. Moreover, H. pylori up-regulated the expression of β-catenin and TCF-4, and also induced the nuclear accumulation of β-catenin. In addition, the diffusive gastric cancer-related gene, E-cadherin, was up-regulated at the protein level, but down-regulated at the mRNA level. H. pylori infection is capable of inducing GES-1 transformation to present with the characteristics of intestinal-type gastric cancers in vitro, likely through the β-catenin/TCF-4 signaling pathway.

Key words: Helicobacter pylori; GES-1 cell; cell transformation; intestinal phenotypes of gastric cancers.

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Chronic infection of the gastric mucosa by the human pathogen Helicobacter pylori is a leading cause for the development of gastroduodenal diseases such as chronic gastritis, peptic ulcers, and mucosa-associated lymphoid tissue lymphoma (1). More importantly, chronic H. pylori infection has been classified as a definite carcinogen for the development of gastric carcinoma (2), which is the fifth most common malignancy worldwide, and is still the second most common cancer and first leading cause of cancer death in China (3). In addition to H. pylori infection, the environmental and dietary factors and genetic background of the host all contribute to the development of gastric cancer. It has been widely accepted that the development of gastric cancer, especially the intestinal type, is a complicated multi-step process in vivo, in which the malignant transformation is involved. The process usually begins from the normal mucosa to chronic gastritis, gastric glandular atrophy,
intestinal metaplasia, then dysplasia and finally to early adenocarcinoma (4). The previous studies on H. pylori-infected Mongolian gerbils confirmed that H. pylori infection could lead to gastric cancer, especially the intestinal type (5–9). However, to our knowledge, there remains no study that determines whether and how (if any) H. pylori induces the transformation of gastric epithelial cells in vitro.

During the development of gastric cancer, there are malfunctions or alterations of signaling pathways and the abnormal expression of related genes and factors, such as oncogenes, tumor suppressor genes, cellular adhesion molecules (CAM), and telomeres (10, 11). Current studies indicate that the Wnt/β-catenin signaling pathway plays a crucial role in the pathologic process of carcinogenesis and advance (11, 12), and is particularly related to the development of gastrointestinal tumors (13, 14). The activation of the Wnt/β-catenin signaling pathway has been observed in about 30% of gastric cancer patients, often as a result of N-terminal mutations in β-catenin injuring its proper degradation (15). β-catenin and transcription factor-4 (TCF-4), which are the proteins involved in the Wnt/β-catenin signaling pathway, are associated with intestinal phenotypic expression in human gastric cancer, and thus regarded as biomarkers for intestinal-type gastric cancer (16–20).

E-cadherin, a kind of calcium-dependent CAM, normally binds with β-catenin to form the complex at the cell membrane and mediates the cellular adhesion between the same kinds of cells. E-cadherin is also involved in the carcinogenesis of diffusive gastric cancer. Recently, several studies have demonstrated that the expression of E-cadherin is down-regulated in diffuse gastric cancer because of mutations of E-cadherin gene (CDH1) (21–24). In addition, alterations in the E-cadherin/β-catenin cell adhesion complex frequently occur in gastric cancer and are associated with increased nuclear localization of β-catenin (25). Thus, perturbation in the expression or function of E-cadherin/β-catenin genes could result in consequent malignant transformation and tumor progression.

GES-1 is a kind of immortal gastric epithelial cell line, which is derived from fetal gastric epithelial cell after SV40 transfection (26). It has been demonstrated that GES-1 is basically a normal gastric epithelial cell line that can be used for further investigation on the in vitro characteristics of normal gastric mucosa and the development mechanism of gastric cancer (26). Using GES-1, the present study aimed to investigate whether ‘chronic’ H. pylori infection induces gastric epithelial cell transformation, and elucidate the underlying mechanisms of transformation induced by H. pylori by monitoring the morphology of GES-1 cells, and measuring the expression of E-cadherin, β-catenin, and TCF-4 in GES-1 cells after co-culture with H. pylori.

**MATERIALS AND METHODS**

**Culture of human gastric epithelial cells**

We used an immortalized human fetal gastric epithelial cell line, GES-1 (27) (kindly provided by the Department of Cell Genetics at Beijing Institute for Cancer Research, Beijing, China), for the study. GES-1 cells were grown in RPMI 1640 medium (pH 7.2–7.4; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Beijing Zhong Shan-Golden Bridge Biological Technology Co., Beijing, China) and penicillin/streptomycin (both 100 U/mL) at 37 °C in a humidified atmosphere of 5% CO₂. The cells were allowed to reach 80% confluency before passage. The culture medium was replenished with the fresh medium every 2 or 3 days.

**Culture of Helicobacter pylori**

We used two H. pylori strains, B975 and L301 (both provided by the Third Laboratory of Institute of Cancer Research at China Medical University, Shenyang, China). B975 and L301 were isolated from a patient with active chronic gastritis and a patient with gastric cancer, respectively. Both strains share the same virulence factors including cytotoxin-associated gene A protein (CagA), vacuolating cytotoxin A (VacA), and blood group antigen-binding adhesion (BabA).

H. pylori was cultured on brain heart infusion agar plates with 10% sheep blood at 37 °C in a 97% humidified atmosphere of 85% N₂, 5% O₂, and 10% CO₂ under microaerobic conditions. Single colonies were subcultured on the agar plates for 3–4 days, and then further subcultured for 48 h before the bacterial cells were harvested into RPMI 1640 medium with 10% FBS for immediate use.

**Co-culture of GES-1 cells with Helicobacter pylori**

GES-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS without antibiotics in cell culture flasks overnight. Then, bacteria were added in
the ratio (GES-1 cells: bacterial cells) of 1:1 for co-culture. The cells were allowed to reach 80% confluency before passage. The culture was replenished with fresh medium every 2 or 3 days, after three washes with aseptic phosphate-buffered saline (PBS). GES-1 cells cultured under the same conditions but without co-culture with *H. pylori* were used as controls. The culture continued for 45 days, and the cells were used for experiments and analysis during that period of time.

**Cell morphology observation**

The morphology of viable GES-1 cells cultured with or without *H. pylori* was observed under an inverted phase contrast microscope at 72 h and 45 days. In addition, GES-1 cells cultured with or without *H. pylori* for 45 days were placed on chamber slides. The slides were fixed with acetone at 4 °C and stained with hematoxylin and eosin (H&E) for morphologic observation. Moreover, the GES-1 cells harvested from culture flasks after 45 days of co-culture were fixed with 2.5% glutaraldehyde for observation under a transmission electron microscope (TEM, EM208S; Philips, Eindhoven, The Netherlands).

**3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay for cell proliferation**

GES-1 cells cultured with or without *H. pylori* for 45 days (at a concentration of approximately 1 × 10⁴ cells/well) were seeded into wells containing 100 μL of the culture medium of a 96-well plate and incubated overnight at 37 °C. Then, the cells were cultured, replenishing with fresh medium at different time points (i.e., 12, 24, and 48 h). Then, 25 μL of 5 μg/mL MTT (Sigma Chemical Co., St. Louis, MO, USA) labeling reagent was added to the designated wells and cells were further incubated at 37 °C for 4 h. The supernatant was removed, and then 150 μL of dimethyl sulfoxide was added. After the plate was incubated at 37 °C for 10 min, the absorbency was measured using a micro ELISA reader (Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. The experiments were repeated twice, and duplicated wells were used for cells cultured with or without *H. pylori* for 45 days at each time point for each experiment.

**Immunocytochemistry assay for Ki-67 protein expression**

Immunostaining was performed to determine Ki-67 protein expression in GES-1 cells cultured with or without *H. pylori* for 45 days using the labeled streptavidin–biotin technique, according to the manufacturer’s (Zhong Shan-Golden Bridge Biological Technology Co.) instructions. The polyclonal anti-Ki-67 (Biotechnology, Inc., Santa Cruz, CA, USA) was used as the primary antibody. GES-1 cells were placed on the chamber slide in PBS, and then treated with normal goat serum for 30 min to block any non-specific binding, which was followed by incubation overnight at 4 °C with an optimum dilution of the primary antibody (×100 dilution). The negative control was prepared by processing the slides in the same manner, but without the primary antibody. The average optical density was obtained by measuring three randomly selected fields per slide using a micro ELISA reader (Bio-Tek Instruments).

** Colony formation assay for cell viability**

Viable cells cultured with or without *H. pylori* for 45 days (200 cells/well) were seeded into wells of a six-well plate (in triplicate) and cultured in an incubator with 5% CO₂. The medium was replaced 1 week later and the colony count was performed after incubation for another week. Cells in the plate were washed with PBS thrice after removal of the medium, and then fixed with 4% paraformaldehyde for 5 min twice, followed by staining with 0.1% crystal violet for 5 min. The stain was washed off under running tap water, and the plate was allowed to dry. The number of distinctly stained colonies containing at least 50 cells per colony was counted under an inverted microscope. Colony-forming efficiency (CFE) was calculated as the number of colonies generated divided by the seeded cells (i.e., 200) × 100%.

**Immunofluorescence assay for protein expression of β-catenin, E-cadherin, and TCF-4**

GES-1 cells cultured with or without *H. pylori* for 45 days were placed on chamber slides, and were fixed with acetone at 4 °C for 10 min. Then, the cells were washed in 2-ethanesulfonic acid buffer five times (each for 5 min) before addition of 10% goat serum albumin and incubation for 30 min at room temperature. After further three washes (each for 5 min), the cells were permeabilized with 0.5% Triton X-100 for 10 min, washed again for 3–5 min, and then incubated overnight at 4 °C with one of the following primary antibodies: a concentrated murine monoclonal anti-human β-catenin (E5, ×100 dilution; Santa Cruz), a concentrated rabbit polyclonal anti-human E-cadherin (H-108, ×100 dilution; Santa Cruz), or a concentrated rabbit monoclonal anti-human TCF-4 (EP2033Y, ×100 dilution; Epitomics, CA, USA). Then, goat anti-mouse IgG/FITC (×50 dilution; Santa Cruz) or goat anti-rabbit IgG/BRITC (×50 dilution; Santa Cruz) antibodies were added, respectively, and the cells were incubated at 37 °C in the dark for 30 min and washed with PBS. Finally, the cells were mounted on slides with 50% glycerin and
examined under a fluorescence microscope (Olympus CX41-32RFL, Tokyo, Japan).

RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR) for mRNA expression of β-catenin, E-cadherin, and TCF-4

Total RNA was extracted from GES-1 cells cultured with or without H. pylori for 45 days using a total RNA kit (Tiangen Biotech, Beijing, China). RNA (1.5 μg) was reverse transcribed to cDNA using ImProm-II™ Reverse Transcription System Kit (Promega, Southampton, UK) according to the manufacturer’s instructions. RT-PCR was carried out using TaqMan primer sets specific for human E-cadherin, β-catenin, TCF-4, and β-actin, as described previously (Table 1) (28–31). The densitometry analysis was performed using the GDS-8000 System for gel documentation (UVP BioImaging Systems, Upland, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS® version 11.5 (SPSS, Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD) and their differences percentages were compared between groups by the Mann–Whitney test or chi-squared test, where appropriate. A p-value of less than 0.05 (two-sided) was considered statistically significant.

RESULTS

Effect of Helicobacter pylori on the morphology of GES-1 cells

As observed using the inverted phase contrast microscope, GES-1 control cells presented with a polygonal or fusiform shape, a regular appearance, clear edge, and anchorage-dependent growth. Floating cells were rarely observed (Fig. 1, A1). After 72 h of co-culture with H. pylori, irregular appearance emerged, with unclear edges. There were numerous increasing floating cells. After 45 days of co-culture, most GES-1 cells exhibited significant morphologic changes including enlarged cellular size, more irregular appearance, and enlarged cellular nuclei with increased number of nucleoli. Occasionally, giant cells were observed. The number of viable cells declined (Fig. 1, A2 and A3). Similar findings were observed with H&E staining as shown in Fig. 1, B1–B3. The significantly increased pathologic karyokinesis was observed in nuclei. TEM revealed that the cells co-cultured with H. pylori for 45 days of co-culture appeared with an irregular shape, increased cellular microvilli, enlarged nuclei with increased chromatin, and thickened nuclear membrane (Fig. 1, C1–C3). Occasionally, H. pylori was observed surrounding the cells (Fig. 1, C3).

Effect of Helicobacter pylori on proliferation of GES-1 cells

The MTT assay showed that H. pylori significantly inhibited the proliferation of GES-1 cells for up to 48 h after co-culture with the cells; however, after 72 h of co-culture, H. pylori significantly increased the proliferation of the cells (p < 0.001; Table 1). Moreover, L301 had more power than B975 on the proliferation of GES-1 cells (p < 0.001; Table 2).

Table 1. TaqMan primer sets and reverse transcription-polymerase chain reaction procedures for mRNA expression

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Procedures</th>
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<tr>
<td>E-cadherin (29)</td>
<td>5′-TGA AGG TGA CAG AGC CTC TGG AT-3′</td>
<td>5′-TGG GTG AAT TCG GGC TTG TT-3′</td>
<td>94 °C 1 min, 94 °C 39 s, 55 °C 30 sec, 72 °C 30 s, 30 cycles, 72 °C 5 min</td>
</tr>
<tr>
<td>β-catenin (30)</td>
<td>5′-ACA AAC TGT TTT GAA ATT CCA-3′</td>
<td>5′-CGA GTC ATT GCA TAC TGT CC-3′</td>
<td>95 °C 2 min, 95 °C 1 min, 58 °C 30 s, 72 °C 30 s, 35 cycles, 72 °C 10 min</td>
</tr>
<tr>
<td>Transcription factor-4 (31)</td>
<td>5′-TCA CCA ACA GCG AAT GGC-3′</td>
<td>5′-AGG AAG GAT AGC CTG GCG-3′</td>
<td>94 °C 4 min, 94 °C 45 s, 60 °C 30 s, 72 °C 1 min, 33 cycles, 72 °C 5 min</td>
</tr>
<tr>
<td>β-actin (32)</td>
<td>5′-GCA TGG AGT CCT GTG GCA T-3′</td>
<td>5′-CTA GAA GCA TTT GCG GTG G-3′</td>
<td>94 °C 2 min, 94 °C 45 s, 58 °C 45 s, 72 °C 45 s, 30 cycles, 72 °C 7 min</td>
</tr>
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</table>
As shown in Fig. 2, the expression of Ki-67 protein intensity was significantly increased in GES-1 cells co-cultured with the two *H. pylori* strains, B975 (OD: 0.327 ± 0.009) or L301 (OD: 0.358 ± 0.008), compared with that of control cells (OD: 0.308 ± 0.006, p = 0.040 and p = 0.001, respectively). In addition, L301 induced a more significant increase in Ki67 antigen expression than did B975 in GES-1 cells (p = 0.009).

As shown in Fig. 3, *H. pylori* significantly enhanced the colony formation of GES-1 cells. L301 significantly increased the CFE of GES-1 cells, compared with that of control cells (40.6% vs 27.2%, p = 0.042). There was a slight, but not statistically significant, increase...
in the CFE in GES-1 cells co-cultured with B975, compared with control cells (32.3% vs 27.2%, p = 0.229).

**DISCUSSION**

Recently, *H. pylori* infection has been confirmed to be related to the development of gastric cancer by Mongolian gerbil models (6–9). Although mechanisms of *H. pylori*-induced carcinogenesis are only beginning to be understood, inflammation is the most commonly cited mechanism in the carcinogenic process. Inflammation is thought to induce cancer by increasing the production of free radicals, inducing apoptosis and necrosis of epithelial cells and augmenting cell proliferation (32). An important mechanism posited other than inflammation is that *H. pylori* directly interacts with epithelial cells, resulting in protein modulation, gene alterations, and consequently epithelial cell transformation (33). In the present study, we used a non-tumorous gastric epithelial cell line, GES-1, to construct a cell transformation model with *H. pylori* infection *in vitro*, to avoid or minimize the impact of inflammation present *in vivo*. To our knowledge, this is the first time that such a model was used to study the effects of *H. pylori* on the transformation of gastric epithelial cells *in vitro*. We observed that *H. pylori*...
induced GES-1 cells into morphologic changes and significantly increased the proliferation of GES-1 cells. Moreover, *H. pylori* up-regulated the expression of β-catenin and TCF-4, known as intestinal phenotypes of gastric cancer-related genes, and also induced the nuclear accumulation of β-catenin. In addition, the diffusive gastric cancer-related gene, E-cadherin, was up-regulated at the protein level, but down-regulated at the mRNA level.

Although the two *H. pylori* strains we used were isolated from patients with different gastric diseases, both possessed the virulence factor, CagA. It has been demonstrated that CagA activates the anti-apoptotic pathways in gastric epithelial cells to overcome self-renewal of the host cell and help sustain *H. pylori* infection (34). In the present study, after co-culture with the CagA + *H. pylori* strains for 45 days, proliferation and morphology of GES-1 cells presented with the features of transformation cells. Moreover, the colony-forming assay also confirmed that GES-1 proliferation ability was enhanced by *H. pylori* infection. These findings potentially indicate that *H. pylori* is able to induce GES-1 to transform in vitro; however, further extensive investigation based on our preliminary observations is required. It is noted that the strain, L301, isolated from a patient with gastric cancer was more potent than the strain, B975, isolated from a patient with active chronic gastritis, in stimulating the cell proliferation and pathologic karyokinesis of GES-1. This observation indicates that there may be differences in the virulence and pathogenicity between strains isolated from gastric cancer patients and those from active gastritis patients, which should be elucidated in further studies.

A large body of evidence supports a causal role of *H. pylori* in the majority of gastric malignancies. Great strides have been made in understanding the pathogenesis of this bacterium. However, much remains to be studied. Genetic changes can already be detected in intestinal metaplasia, with p16 methylation being significantly associated with *H. pylori* infection in precancerous lesions (35). Studies have also shown decreased E-cadherin expression in the gastric mucosa of *H. pylori*-infected individuals (36), and the interaction of CagA with E-cadherin, which causes cytoplasmic and nuclear accumulation of β-catenin, has been documented and implicated in the development of intestinal metaplasia (37). In the present study, *H. pylori* led to the up-regulated protein and mRNA of intestinal-type gastric cancer-related genes,

Fig. 3. Colony formation of GES-1 cells observed with naked eyes and under a microscope (40×). (A, C) GES-1 control cells; and (B, D) GES-1 cells co-cultured with *Helicobacter pylori* strain, B975. The number of colonies of GES-1 was significantly increased for cells co-cultured with *H. pylori*, compared with control cells.
that is, β-catenin and TCF-4, as shown in Figs 4 and 5. In addition, β-catenin presented with cytoplasmic and nuclear translocation. Similar results were reported in a study using MCF-7 breast cancer cell line, which showed that a predominant cytoplasmic localization of β-catenin after prolonged *H. pylori* infection was associated with deregulation of cell adhesion through disconnection of the E-cadherin–catenin complex from the cytoskeleton (38). Furthermore, we observed that the diffusive gastric cancer-related gene, E-cadherin, was up-regulated at the protein level, but down-regulated at the mRNA level, which further supports that

![Fig. 4](image-url) The expression of E-cadherin, β-catenin, and transcription factor-4 (TCF-4) in GES-1 co-culture and control groups (200×). (A–C and G–I) GES-1 control cells; (D–F and J–L) GES-1 cells exposed to *Helicobacter pylori*. (A) and (D) show the expression of E-cadherin; (B) and (G) show the expression of β-catenin on cell membrane, and (E) and (J) show the expression of β-catenin in the cytoplasm and nucleus; (H) and (K) show the expression of TCF-4; (C) and (F) show the co-localization of E-cadherin and β-catenin; and (I) and (L) show the co-localization of β-catenin and TCF-4.

![Fig. 5](image-url) Effects of *Helicobacter pylori* on the expression of E-cadherin, β-catenin, and transcription factor-4 mRNA in GES-1 cells. Lane 1, GES-1 control cells; lane 2, GES-1 cells co-cultured with B975; and lane 3, GES-1 cells co-cultured with L301.
**Table 3.** The expression of E-cadherin, β-catenin, and transcription factor-4 (TCF-4) mRNA in GES-1 cells with or without co-culture with Helicobacter pylori.

<table>
<thead>
<tr>
<th>Cell group</th>
<th>E-cadherin/β-actin</th>
<th>β-catenin/β-actin</th>
<th>TCF-4/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GES-1 cells</td>
<td>0.568 ± 0.010</td>
<td>0.658 ± 0.027</td>
<td>0.200 ± 0.080</td>
</tr>
<tr>
<td>GES-1 cells co-cultured with B975</td>
<td>0.461 ± 0.016*</td>
<td>1.413 ± 0.124*</td>
<td>0.925 ± 0.062*</td>
</tr>
<tr>
<td>GES-1 cells co-cultured with L301</td>
<td>0.389 ± 0.013*</td>
<td>0.862 ± 0.016*</td>
<td>1.251 ± 0.069*</td>
</tr>
</tbody>
</table>

*p < 0.01 vs GES-1 control cells.

H. pylori interacts with β-catenin/TCF-4 signaling pathway to exert its role in the intestinal-type transformation.

In the *in vitro* model we established that the impact of host inflammation was attenuated (if not eliminated), indicating that the malignant transformation should be mainly attributed to the virulence factors of *H. pylori*. Phosphorylated CagA is known to disrupt tight cell junctions, resulting in cell elongation and the formation of the so-called ‘hummingbird phenotype’ (39). This process may result in the sloughing off of epithelial cells and compensatory cell proliferation. Suzuki et al. (40) reported that the non-phosphorylated CagA activity was involved in interaction with activated Met, a hepatocyte growth factor receptor, which in turn led to the activation of β-catenin and NF-κB signaling, and contributed to the epithelial proliferative and proinflammatory responses associated with the development of chronic gastritis and gastric cancer. Sokolova et al. (41) reported that in Madin-Darby canine kidney cell line, *H. pylori* infection suppressed Ser/Thr phosphorylation and ubiquitin-dependent degradation of β-catenin, resulting in up-regulation of lymphoid enhancer-binding factor/T-cell factor (LEF/TCF)-dependent transcription. Therefore, CagA may play a pivotal role in the malignant transformation of gastric epithelial cells by means of activation of the signaling pathways; however, further extensive investigation is required to reveal how CagA plays the role and whether and how other virulence factors are involved in the process.

In conclusion, *H. pylori* infection is capable of inducing GES-1 transformation to present with the characteristics of intestinal-type gastric cancers *in vitro*, likely through β-catenin/TCF-4 signaling pathway. Further investigation on molecular mechanisms with regard to how *H. pylori* induces malignant transformation is required.

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