Inflammatory responses induced by *Helicobacter pylori* on the carcinogenesis of gastric epithelial GES-1 cells

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Abstract. *Helicobacter pylori* (*HP*) is a pathogenic bacterium associated with chronic gastritis, gastric ulcer and gastric cancer. In the present study, the primary carcinogenesis process of normal gastric epithelial cells (GES-1) infected with *HP* was investigated. It was determined that infected gastric mucosal epithelial GES-1 cells secreted increased interleukin-8 (IL-8) and IL-23, and exhibited enhanced expression of inducible nitric oxide synthase and cyclooxygenase-2, inducing inflammatory reactions and resulting in apoptosis. The bacterial infection significantly increased the expression of carcinogenesis-associated genes, including p16, c-Myc, p53 and p21, as well as the expression of cell surface signaling molecules cluster of differentiation 44 (CD44) and CD54 in GES-1 cells or tissues of patients with gastritis and gastric cancer *in vitro* or *in vivo*. Simultaneously, the migration and invasion abilities of normal gastric epithelial GES-1 cells were increased following *HP* infection. These observations demonstrated that the inflammatory response of *HP* infection could cause normal gastric epithelial cells to undergo significant cancerous reactions, indicating that *HP* is a risk factor for gastric cancer.

Introduction

Helicobacter pylori (*HP*) is etiologically active in the occurrence of gastric cancer, stomach inflammation and peptic ulcer disease (1), infecting ~50% of the whole global population in 2015 (2). In cases of chronic or acute *HP* infection, inflammation is a major cause of peptic ulcer disease and gastric malignancy (3). The pathogenesis of gastric cancer is multi-factorial, with *HP* infection being the probable leading cause (4). The International Agency for Research on Cancer, World Health Organization in 1994 defined *HP* as a class I carcinogen and a cause of human cancer (5). Studies on *HP* infection in rodents have independently provided evidence of the role of the bacterial pathogen in gastric cancer development (6-8). A previous study reported *HP* as the single leading risk factor of gastric cancer development and is estimated to cause ~75% of all cases of gastric cancer globally in 2015 (9).

Long-term inflammation has been demonstrated to intensify gastric barrier penetrability, further injure lamina propria (10) and potentially contribute to numerous extra-gastric dysfunctions (11-13). *HP* utilizes virulence factors in gastric epithelial cells to target the signaling pathways that modulate cell cycle, apoptosis and other special survival processes (14). Among the several reported *HP* factors involved in gastric lining disturbance, the most studied virulence factors include cag pathogenicity island (cagPAI) and vacuolating cytotoxin gene (vacA) (15). CagPAI encodes a type IV secretion system, which is activated in human cancer through the deliverance of the oncoprotein cytotoxin-associated gene A (CagA) into host cells. Chronic inflammation due to the virulence factor CagA is considered to be the mechanism of *HP*-associated gastric cancer occurrence (16). However, the mechanisms of tissue damage caused by the aforementioned factors remain unknown.

The present study focused on experiments on the carcinogenesis of *HP* infection in normal gastric epithelial cells and investigated the specific molecular mechanisms of carcinogenesis in the *HP*-infected GES-1 cells.

Materials and methods

GES-1 cell culture. Normal epithelial GES-1 cells of human gastric mucosa (Cell Resource Center of Shanghai Academy of Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in wells or flasks at 37˚C in

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an atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Little Chalfont, UK). The medium contained 10% (v/v) fetal bovine serum (FBS; GE Healthcare Life Sciences), 0.25 μg/ml amphotericin B, 0.1 mg/ml streptomycin and 100 U/ml penicillin.

HP culture. The wild-type *HP* strain 26695 was obtained from the Chinese Center for Disease Control and Prevention (Beijing, China). *HP* was cultured for 72 h at 37˚C in an atmosphere containing 5% CO_2 on commercial *HP* culture plates.

GES-1 and HP co-culture. Following washing once with PBS, GES-1 cells were placed in 6-well plastic plates at a density of $3x10^5$ cells/well in 1 ml FBS-free DMEM. *HP* was restored by a swab from the agar plates and suspended in DMEM at an optical density of 0.6 at 600 nm, which corresponded to $3x10^6$ colony-forming U/ml. The *HP* was added to cells in DMEM without FBS and antibiotics at a multiplicity of infection (MOI) of 100:1, and co-cultured at 37˚C and 5% CO_2 for 24 or 48 h. The morphology of *HP*-infected GES-1 cells was observed by Olympus CKX53 Inverted Fluorescence Microscope (magnification, x40; Olympus Corporation, Tokyo, Japan).

Apoptosis assay. Apoptosis was measured using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), according to the manufacturer's protocols and analyzed by FlowJo 7.6 software (Tree Star, Inc., Ashland, OR, USA). Specifically, following washing twice with PBS, cells were re-suspended in 100 μl 1X binding buffer (BD Biosciences; Becton, Dickinson and Company), and then incubated for 15 min with fluorescein isothiocyanate Annexin V and propidium iodide (each 5 μl; BD Biosciences; Becton, Dickinson and Company) at 37˚C in the dark. Subsequently, 400 μl 1X binding buffer was added to each tube, followed by detection on a FACS Canto II flow cytometer (BD Biosciences; Becton, Dickinson and Company).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The primers of β-actin, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), p16, c-Myc, p53 and p21 designed by Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) and primers sequences were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Total RNA was isolated from HP-infected GES-1 cells using a TRIzol® reagent (Thermo Fisher Scientific, Inc.) and detected spectrophotometrically. The cDNA was prepared using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). A mixture consisting of RNA (500 ng/μl; 2 μl), 5X M-MLV RT buffer (12 μl), dNTP mixes (2.5 mM; 6 μl), RNase inhibitor (30 U/μl; 1 μl), M-MLV RT (5 U/μl; 4 μl), Oligo dT(18) primer (500 ng/μl; 3 μl) and diethyl pyrocarbonate water (17 μl) was cultured at 37˚C for 1 h. RT-qPCR was performed on an Eppendorf PCR system (Eppendorf, Hamburg, Germany). cDNA (1 μl), forward and reverse primers (each 0.5 μl), SYBR® Premix ExTaq (8 μl; Takara Biotechnology Co., Ltd., Dalian, China) and ddH_2O (9 μl) were added to the mix, followed by 32 cycles of amplification as follows: Denaturation at 94˚C for 2 min; 32 cycles of 94˚C for 20 sec, 55˚C for 35 sec, 72˚C for 25 sec; and extension at 72˚C for 5 min. The following primers were used: iNOS, forward, 5'-GAG-CTTCTACCTCAAGCTATC-3', and reverse, 5'-CCTGATGTTGCCATTGTTGGT-3'; COX-2, forward, 5'-AGATCATCTCTGCCTGAGTATCTT-3', and reverse, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'; p21, forward, 5'-CCATGATGTTGATGCCCTAC-3', and reverse, 5'-TTGCCTGCCTTCCTTTCT-3'; p53, forward, 5'-CAGTCTACCTCCCGCCATAA-3', and reverse, 5'-CTCCCAAACATCCCTCACAG-3'; c-Myc, forward, 5'-GGGCTTTATCTAACTCGCTGTA-3', and reverse, 5'-GCTATGGGCAAAGTTTCGTG-3'; p16, forward, 5'-GAAGAAAGAGGAGGGGCTGG-3', and reverse, 5'-CTGCAGACCCTCTACCCACC-3'; and β-actin, forward, 5'-CCTGGCACCCAGCACAAT-3', and reverse, 5'-GCTGATCCACATCTGCTGGAA-3'. Subsequently, fluorescence was detected. Specific amplification was guaranteed by dissociation curves. Each sample was tested in triplicate, and the mean Cq value was calculated (17).

Western blotting. The GES-1 cells from each group were collected, washed with PBS twice on ice and incubated with a Radioimmunoprecipitation Assay protein lysate (Beyotime Institute of Biotechnology, Beijing, China; cat. no. P0013B) containing 1% benzene sulfonyl fluoride for 30 min at 4˚C. The protein concentration in the supernatant was then measured with a BCA protein concentration kit (Beyotime Institute of Biotechnology; cat. no. P0010). Proteins (30 μg) were treated by 10% SDS-PAGE electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were then immunoblotted with primary antibodies against β-actin (dilution, 1:2,000; cat. no. MAB8929), Cox-2 (dilution, 1:1,000; cat. no. AF4198), iNOS (dilution, 1:1,200; cat. no. MAB9502), p16 (dilution, 1:800; cat. no. AF5779), c-Myc (dilution, 1:1,000; cat. no. MAB3696), p53 (dilution, 1:1,600; cat. no. AF1355) and p21 (dilution, 1:1,000; cat. no. AF1047; R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4˚C, followed by cultivation with goat IgG horseradish peroxidase-conjugated antibody (R&D Systems, Inc.; dilution, 1:1,000; cat. no. HAF019) at 37˚C for 60 min and Enhanced Chemiluminescence Western Blotting Detection reagents (cat. no. RPN2209; GE Healthcare Life Sciences). The bands were visualized using a LAS-4000 system (GE Healthcare Life Sciences) and were analyzed quantitatively using ImageJ v1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Cytokine quantification by ELISA. GES-1 cells cultured in 6-well plates were processed in lipopolysaccharide (LPS; 50 ng/ml; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China; cat. no. L8880) or *HP* (MOI of 100) medium for 24 or 48 h. The concentration of interleukin-8 (IL-8) and IL-23 in supernatants of stimulated cells was detected by IL-8 ELISA kit and IL-23 ELISA kits (Wuhan Boster Biological Technology, Ltd., Wuhan, China; cat. nos. EK0413 and EK1612, respectively), according to the manufacturer's protocols. Cytokine concentrations were determined by a standard curve.

Wound-healing and Matrigel assays in vitro. Following 24 or 48 h of culture at 37˚C, the GES-1 cells were transferred using trypsinization, counted and seeded at $1x10^5$ cells/ml into 6-well plates, followed by overnight culture at 37˚C to form confluent single layers. Wounds were then induced using a 100 μl pipette tip and imaged at 0, 24 or 48 h under Olympus CKX53 Inverted Fluorescence Microscope at magnification, x100. The migration distance of the monolayer from the wounded area during this time period was detected and expressed as a migration index (the migrating distance following a stimulus relative to that of control cells). Each assay was conducted in triplicate and repeated at least five times.

Invasion assays were conducted *in vitro*, using Transwell plates (Costar; Corning, Inc., Corning, NY, USA) with 8-μm pores. After $2x10^4$ GES-1 cells were processed in LPS (50 ng/ml) or *HP* (MOI of 100) medium for 48 h, they were added to the upper chamber of Transwell plates with Matrigel (Costar; Corning, Inc.) and DMEM in the lower chamber of Transwell plate contained with 5% FBS. Following 24 or 48 h incubation, cells on the upper surface were removed using cotton wool and cells attached to the bottom were fixed with 75% methanol at 37˚C for 15 min and stained with 0.5% crystal violet at 37˚C for 30 min. Images were captured and the cells were counted using Olympus CKX53 Inverted Fluorescence Microscope at x200 magnification.

Flow cytometry. GES-1 cultivated in 6-well plates were infected with *HP* for 24 or 48 h, and then harvested and rinsed twice with PBS containing 0.2% bovine serum albumin (cat. no. B7542; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following being stained with phycoerythrin-labeled monoclonal cluster of differentiation 44 (CD44; dilution, 1:200; cat. no. 562818; BD Biosciences; Becton, Dickinson and Company) or allophycocyanin-labeled CD54 (dilution, 1:200; cat. no. 559771; BD Biosciences; Becton, Dickinson and Company) antibodies or the isotype controls (dilution, 1:200; cat. no. 560787; BD Biosciences; Becton, Dickinson and Company) at 37˚C for 30 min, the cells were washed twice with PBS and fixed in 10% (v/v) formaldehyde-PBS at 37˚C. Finally, cells were sorted and analyzed using a BD FACSCalibur with four-color fluorescence detection system (BD Biosciences; Becton, Dickinson and Company). Statistical data were analyzed by FlowJo 7.6 software. Mean fluorescence intensity and the percentage of positive cells were estimated after the values for the isotype controls were subtracted.

Immunohistochemical (IHC) analysis. Tissues from 6 normal male patients, 6 male patients with HP^+ gastritis and 6 male patients with HP^+ gastric cancer (stomach adenocarcinoma) aged 32-65 years (mean ± SD, 44.3±8.9 years) were collected from Chongqing Cancer Institute (Chongqing, China) or Kunshan First People's Hospital Affiliated to Jiangsu University (Suzhou, China) from January 2013 and January 2017. The HP infection status of normal patients, patients with gastritis and patients with gastric cancer was confirmed by a Carbon 13 breathing experiment because this experiment could indicate that individuals were suffering from HP infection. All protocols were approved by the Ethics Committee of the Chinese Hospital Association (Beijing, China) and written informed consent was provided by all participants in all experiments.

CD44 and CD54 in the membrane of gastric tissue cells from different participants were analyzed with an IHC assay. Briefly, all tissues were fixed with 4% formaldehyde (Beyotime Institute of Biotechnology; cat. no. P0099) at 37˚C for 30 min, and then were embedded in paraffin and cut into slices with a histotome. Following xylene-deparaffinizing and rehydration through AR grade absolute ethanol to distilled water at 37˚C, the 5-mm-thick microarray sections were stained with Hydrogen Peroxide Block (cat. no. ab64218; Abcam, Cambridge, UK). Following cultured with rabbit anti-CD44 (dilution, 1:100; cat. no. 550392; BD Biosciences; Becton, Dickinson and Company) and anti-CD54 antibodies (dilution, 1:100; cat. no. BBA17; R&D Systems, Inc.) at 37˚C for 40 min, the sections were washed with PBS three times and cultivated with Biotin goat anti-rabbit IgG (dilution, 1:100; cat. no. 550338; BD Biosciences; Becton, Dickinson and Company) for 30 min at 37˚C. Subsequently, following colorization with 3,3'-diaminobenzidine, the nuclei were counterstained lightly with hematoxylin at 37˚C. Images were captured and the cells were counted using Olympus CKX53 Inverted Fluorescence microscope, at magnification, x200. The immunostained sections were evaluated by two independent pathologists.

Statistical analysis. Results from three independent experiments are presented as the mean ± standard error of the mean (SEM). Data were processed on SPSS 16.0 for Windows (SPSS,Inc., Chicago, IL, USA) and tested using one-way analysis of variance (ANOVA). The one-way ANOVA and post-hoc Tukey's test were conducted on GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) based on an assumption of normal distribution. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Apoptosis of HP-infected GES-1 cells. To investigate the effects of *HP* on cell survival and physiology, the morphology and viability of *HP*-infected GES-1 cells was analyzed by microscopy and flow cytometry, respectively (Fig. 1). *In vitro*, the majority of GES-1 cells were in good condition, and only a small portion underwent *HP* infection-induced apoptosis, indicating that the virulence factor toxicity of *HP* could induce extensive apoptosis.

Inflammation-associated genes and proteins iNOS and COX-2 in GES-1 cells influenced by HP. The host cells employ reactive oxygen species and nitric oxide to eliminate an invading pathogen, but this reaction mechanism could also regulate the inflammatory response of host cells (18). A total of 2 major enzymes (iNOS and COX-2) involved in the metabolism of *HP*-infected GES-1 cells (Fig. 2) were therefore examined using RT-qPCR and western blotting. The gray of bands was scanned on ImageJ, with β-actin as the standard. The gene and protein expression of iNOS and COX-2 were determined to be upregulated following infection.

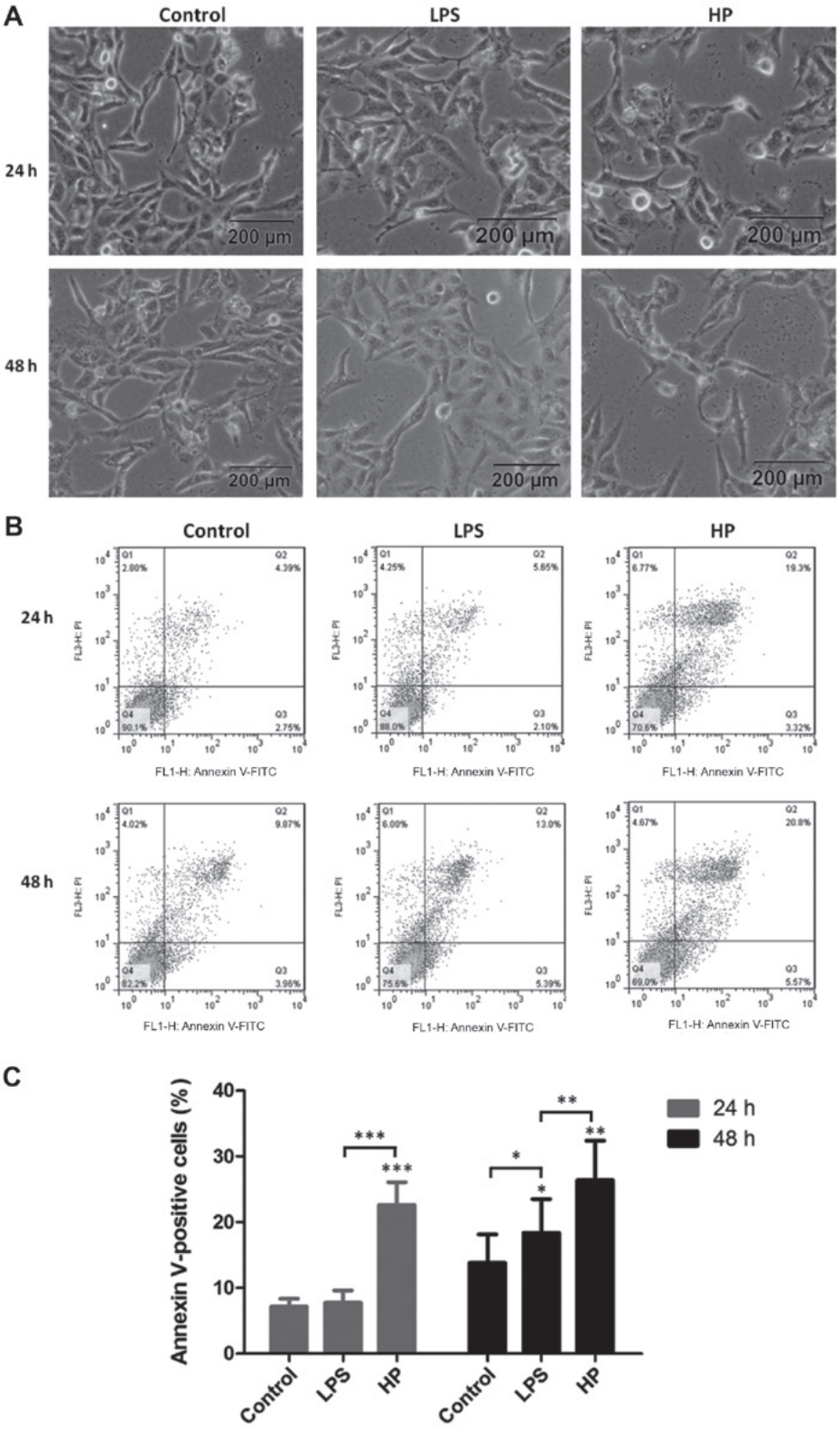

Figure 1. Apoptosis analysis of GES-1 cells infected with *HP* detected by microscope and flow cytometry. (A) Morphological characterization of GES-1 cells stimulated with LPS or *HP* medium for 24 or 48 h. Scale bar, 200 μm. (B) Flow cytometry results of GES-1 cells treated with LPS or *HP* for 24 or 48 h. (C) Quantification of apoptosis. Results presented as the mean ± standard error of the mean. n=3, results are representative of three separate experiments (*P<0.05 vs. control or LPS, **P<0.01 vs. control and ***P<0.001 vs. control or LPS). LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Inflammatory factors IL-8 and IL-23 regulated by HP. Since immune cells are the main secretor of cytokines, the inflammatory response capacity of GES-1 cells following *HP* infection was detected by measuring inflammatory factors (IL-8 and IL-23) in the supernatant using ELISA (Fig. 3). IL-8 and IL-23 levels were significantly increased in infected

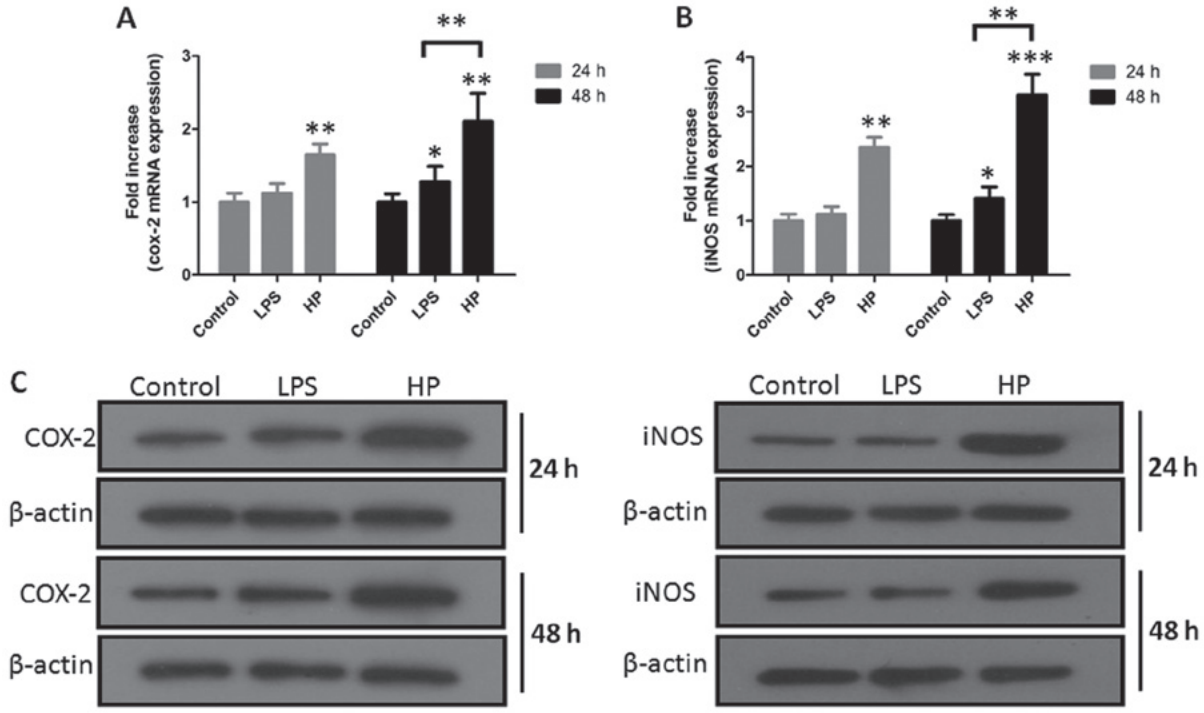

Figure 2. Expression of COX-2 and iNOS in GES-1 cells infected with *HP* for 24 or 48 h from the gene and protein levels. Total mRNAs of (A) COX-2 and (B) iNOS isolated from *HP*-infected GES-1 cells were analyzed using reverse transcription-quantitative polymerase chain reaction. (C) Western blotting results of COX-2 and iNOS proteins in GES-1 cells infected with *HP* for 24 or 48 h. Quantification of COX-2 and iNOS expression were conducted and data are presented as the mean ± standard error of the mean of three independent experiments (*P<0.05 vs. control, **P<0.01 vs. control or LPS and ***P<0.001 vs. control). LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.

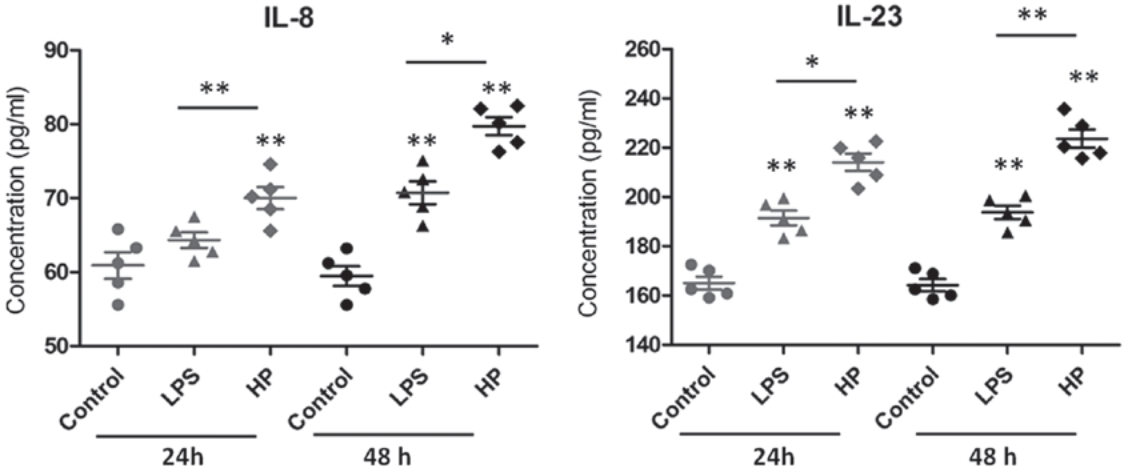

Figure 3. IL-8 and IL-23 in the supernatants of GES-1 cells treated with LPS and *HP* for 24 or 48 h were determined by ELISA. Each symbol per condition represents the data obtained from one test. Horizontal lines represent the median values of 5 experiments (*P<0.05 vs. LPS and **P<0.01 vs. control or LPS). LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*; IL, interleukin.

cells, compared with the control or LPS-treated groups. These results indicated that normal gastric mucosal cells can also secrete a number of cytokines to inflammatorily respond to and resist the bacterial invasion.

Expression of cell carcinoma-associated genes and proteins induced by HP. Since long-term repeated *HP* infection could induce chronic gastritis, gastric ulcer or gastric cancer, the expression of cancer-associated genes and proteins, including p16, c-Myc, p53 and p21, was analyzed in *HP*-infected GES-1 cells using RT-qPCR and western blotting (Fig. 4). It was determined that the gene and protein expression levels of p16, c-Myc, p53 and p21 were all enhanced following *HP* infection, compared with the control or LPS-treated groups, indicating that *HP* may be a risk factor of gastric cancer.

Cell migration and invasion capabilities of HP-infected GES-1. Since *HP* infection could change the expression of cancer-associated genes, causing the host cells to become cancerous, the cell migration and invasion abilities of

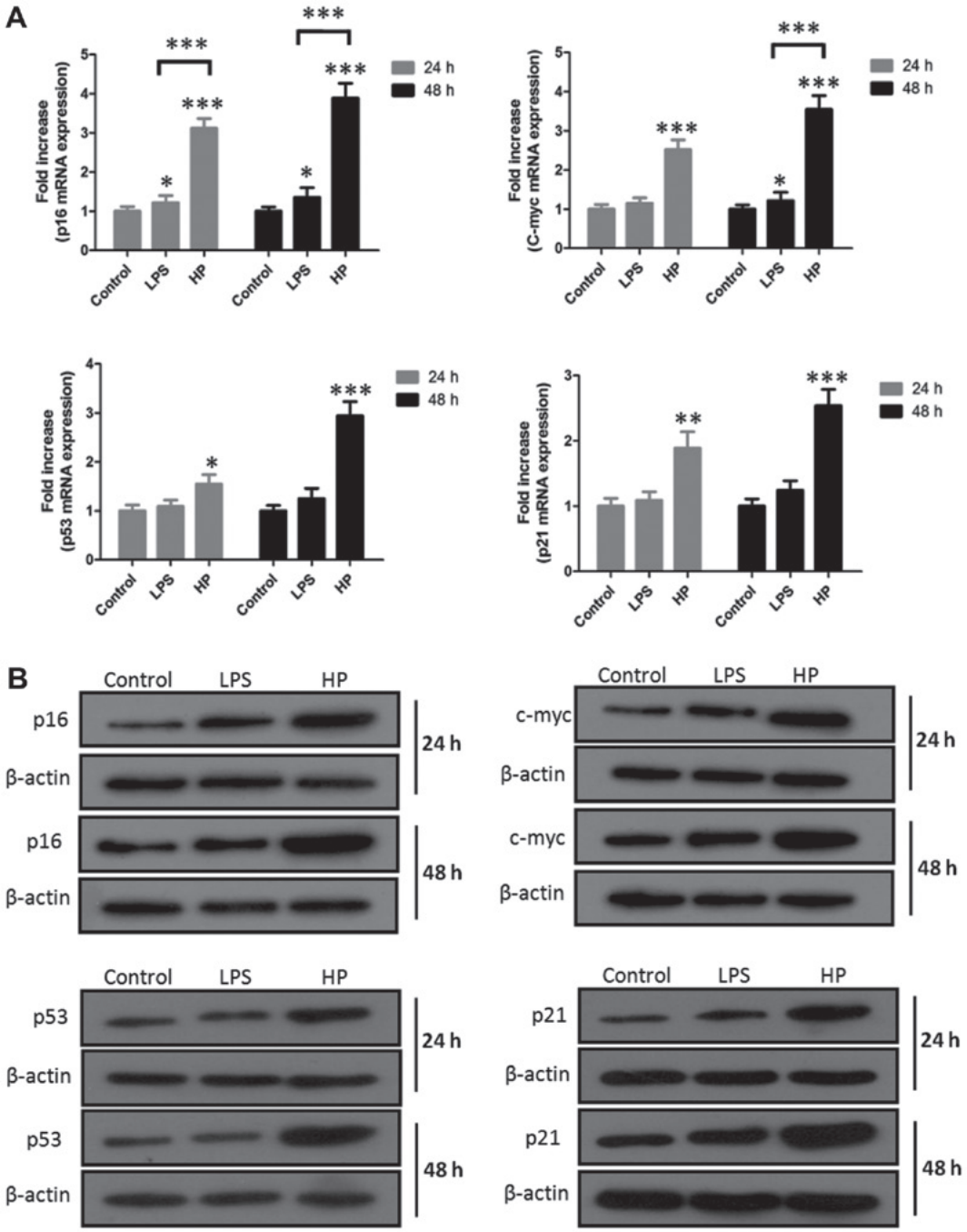

Figure 4. Cancer-associated genes and proteins p16, c-Myc, p53 and p21 in *HP*-infected GES-1 cells were analyzed by RT-qPCR and western blotting. (A) RT-qPCR results of p16, c-Myc, p53 and p21 genes. (B) Western blotting results for p16, c-Myc, p53 and p21 proteins in GES-1 cells. Quantification of p16, c-Myc, p53 and p21 expression was conducted and data are presented as the mean ± standard error of the mean of three independent experiments (*P<0.05 vs. control, **P<0.01 vs. control and ***P<0.001 vs. control or LPS). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*.

HP-infected GES-1 cells were investigated by wound-healing experiments (Fig. 5A and B) and Matrigel assay (Fig. 5C and D). As expected, the migration and invasion abilities of *HP*-infected GES-1 cells were significantly increased, compared with the control or LPS-treated groups.

Expression of CD44 and CD54 in HP-infected GES-1 cells by flow cytometry. CD44 and CD54 are associated with tumor invasion and metastasis, and are highly expressed in various malignant tumor types, including gastric cancer (19). The CD44/54 protein expression levels in *HP*-infected GES-1 cells were quantified by flow cytometry (Fig. 6). Results demonstrated that CD44 and CD54 were upregulated in *HP*-infected cells, and that CD54 protein levels were all overexpressed.

Expression of CD44 and CD54 in HP^{+} gastric mucosa tissues. The expression of CD44 and CD54 proteins in the tissues of different participants was quantified by IHC (Fig. 7) and it was determined that they were increased in *HP*-infected tissues.

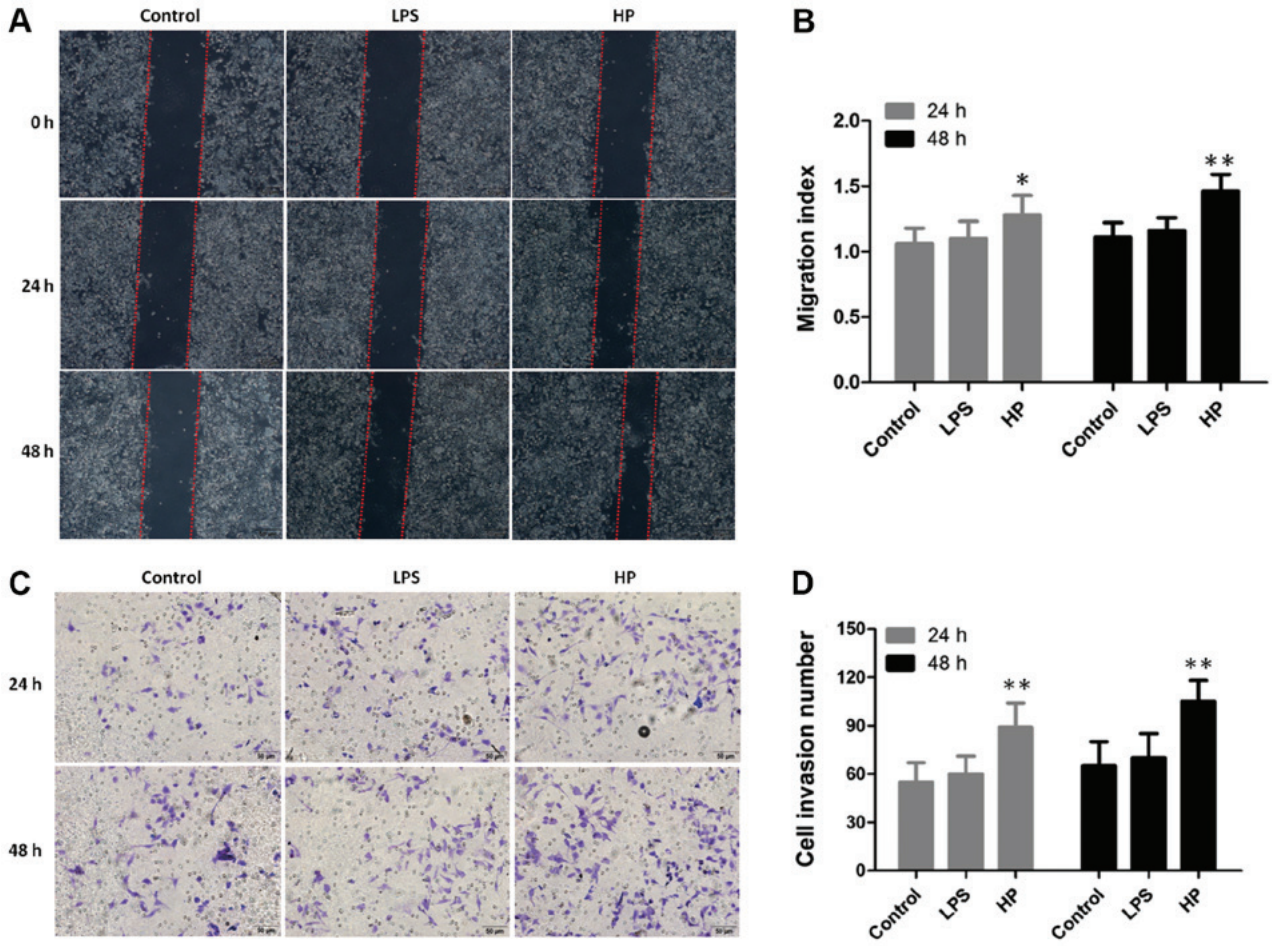

Figure 5. Migration capability analysis of *HP*-infected GES-1 by wound healing and Matrigel assays. (A) Wound healing data are depicted for LPS and *HP* treatment. Scale bar, 50 μm. (B) Bars represent the migration index of each treatment, expressed as a value relative to the distance traveled by the cell monolayer, compared with that of control. (C) The invasion migration of GES-1 cells treated with LPS and *HP*. Scale bar, 50 μm. (D) Quantification analysis of invasion ability of GES-1 cell. Error bars represent the standard errors for three experiments carried out in triplicate (*P<0.05 vs. control and **P<0.01 vs. control). LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*.

The increased CD44 and CD54 were positively associated with the severity of the disease, indicating that *HP* infection may critically affect the expression of CD44/54.

Discussion

HP, a well-known gastric pathogen, can induce the generation of inflammatory factors, including IL-1β, IL-6, IL-8, IL-23 and tumor necrosis factor-α (20-23), resulting in gastroduodenal inflammation, peptic ulcer disease or gastric cancer (24,25). IL-8 in the gastric mucosa following *HP* infection has been associated with the intensity of gastritis and risk of gastric cancer (26,27). IL-23 generated in the gastric mucosa could imply the occurrence of chronic gastritis, and in the existence of *HP*, may be outputted from the inflammatory gastric mucosa following the kinetics of IL-1β (28). VacA-activated p38 signaling pathway could induce (COX-2 expression and thereby increase the formation of prostaglandin E2 (29). It was determined that IL-8 and IL-23 were markedly upregulated in *HP*-infected GES-1 cells, causing an inflammatory response, which in turn induced the apoptosis of GES-1 cells. *HP* infection can increase the expression of iNOS and COX-2, which regulate cellular inflammatory responses, thereby contributing to the inflammatory response (30).

HP possesses various virulence factors, including VacA and CagA, that allow it to chronically survive in the gastric mucosa (31). VacA and CagA, two major virulence factors, have been reported to induce inflammation in the gastric mucosa and be associated with gastric cancer (32). Accumulative evidence supports CagA as a pro-oncogenic factor, and these observations are from mouse transgenic experiments, in which CagA overexpression results in uniform hypertrophy, low frequency and late onset focal tumorigenesis of the gastric epithelium, notably without significant induction of gastritis or atrophy (33,34). VacA has a variety of biological activities, including the induction of apoptosis and gastric inflammation, and contribution to gastric carcinogenesis (35-37).

HP upregulates the expression of p16-INK4 via its promoter in SGC-7901 cells, and activates its promoter with the involvement of specificity protein 1 (38). Telomerase mobility and c-Myc levels in gastric diseases may be affected by *HP* infection, particularly chronic atrophic gastritis (39). Similarly, *in vivo* p53 expression is significantly increased in the *HP*-positive gastritis group, compared with the non-gastritis group, and the expression levels of Ki-67 and p21 were increased significantly in the *HP*-positive gastritis group(40). *HP* increases the proliferation and increases the expression of

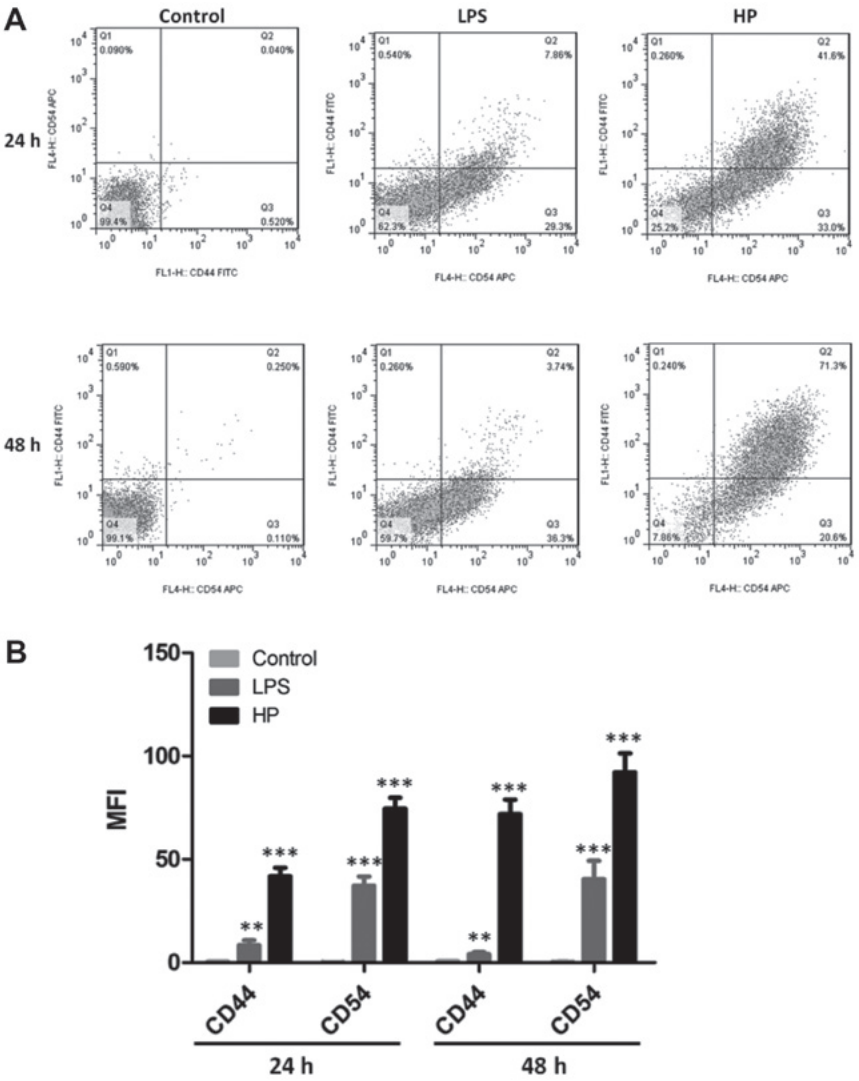

Figure 6. Analysis of CD44/54 molecules by flow cytometry in *HP* infection GES-1. (A) GES-1 cells treated with LPS or *HP* medium were analyzed on FACS and obtained fluorescence intensities of CD44/CD54. (B) Bar graphs represent the mean fluorescence intensities of CD44/54 (mean ± standard error of the mean, 3 independent experiments), and statistical analysis was performed using SPSS 16.0 ($^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control). MFI, mean fluorescence intensity; CD, cluster of differentiation; LPS, lipopolysaccharides; *HP*, *Helicobacter pylori*.

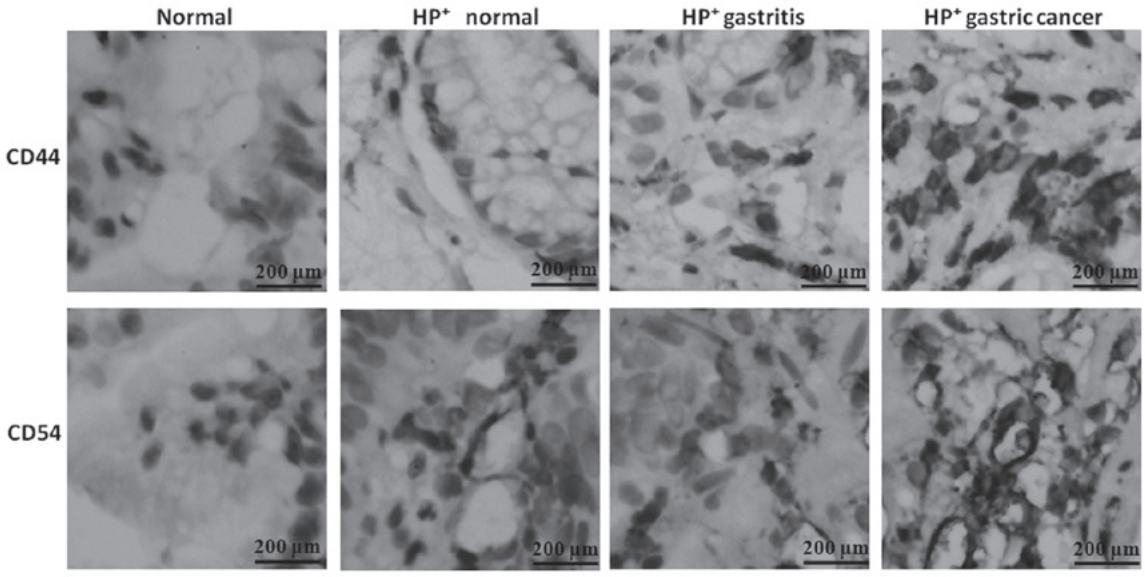

Figure 7. Immunohistochemical staining of CD44/54 in the membrane of gastric tissue cells of normal patients and patients with HP^+ gastritis and HP^+ gastric cancer. All pictures are in high-power fields. Scale bar, 200 μm. CD, cluster of differentiation; *HP*, *Helicobacter pylori*.

p53, but not p21, in gastric mucosa, indicating that the action of p53 may be independent of p21 activity (41). In a previous study, *Mongolian gerbils* were infected with *HP* for a number of hours, and *HP* induced an acute accumulation of p53 in the gastric mucosa, followed by a relatively low plateau for a number of weeks, and then a second peak (42). This dynamic change may depend on the balance between the p53 degradation induced by *HP* and the intracellular self-defense mechanism. The aberrant activation of oncogenes, DNA damage and high level of inflammation induced by *HP* may trigger the intrinsic cellular protection mechanisms, which upregulate the p53 protein (43). Notably, salt and stress synergize *HP*-induced gastric lesions, cell proliferation, and p21 expression without p53 increases in Mongolian gerbils (44). *HP* infection could inhibit the proliferation and induce the apoptosis of endothelial cells through increased phosphorylated p53, p21 and B-cell lymphoma 2-associated X expression, which may contribute to gastric mucosal injury and to delay healing of gastric lesions (45).

Notably, cell carcinogenesis-associated genes and proteins, including p16, c-Myc, p53 and p21, were determined to be upregulated in *HP*-infected gastric epithelial cells, and it was considered that *HP* induced the inflammatory response of GES-1 cells and induced the expression of p16, c-Myc, p53 and p21 in GES-1 cells at the initial stage of infection. However, as the duration of infection continues, *HP* carcinogenic factor proteins CagA and VacA enter into host cells to activate various signaling pathways, including nuclear factor (NF-κB) NF-κB and Janus kinase-signal transducer and activator of transcription, in cells to induce cell carcinogenesis through changing the expression of tumor suppressor genes, including p53 and p21 (46-50). Additionally, *HP* infection significantly promoted the migration and invasion abilities of gastric epithelial cells. These changes may eventually result in inflammation-associated oncogenesis. However, further experiments are required to investigate the process of inflammation-induced carcinogenesis and its specific mechanisms.

The inflammatory events caused by *HP* infection can contribute to gastric cancer occurrence. The circulating immune cells move to the infected mucosa via the interaction between their ligands and receptors in the endothelial zone (51). CD44, a surface marker associated with cancer stem cells, is upregulated in severe gastric lesions (52). Following co-culturing with cytotoxin-associated gene pathogen island-positive *HP*, the steady-state mRNA levels and surface CD54 in epithelial cells were upregulated. Additionally, *HP* stimulated the CD54 promoter via the NF-κB binding site and induced CD54 expression in gastric epithelial cells in an NF-κB-dependent manner that may advocate leukocyte linking upon inflammation (53). CD44 and CD54 were upregulated in epithelial cells following *HP* infection and abnormally expressed in the mucosa tissues of patients with *HP*-infected gastritis or gastric cancer.

Experiments demonstrated that the infection of *HP* subjected normal gastric epithelial cells to significant inflammatory responses and primary cancerous reactions, indicating that *HP* is a risk factor for gastric cancer and the inflammatory responses induced by *HP*-infection were associated with cell carcinogenesis. Nevertheless, the mechanism of carcinogenesis of gastric epithelial cells associated with the inflammation response induced by *HP* should be further investigated.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW and LT conceived and designed the experiments. YY and SL performed the experiments. QZ and YY analyzed the data. JW and LT drafted and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Kunshan First People's Hospital, Affiliated to Jiangsu University.

Patient consent for publication

All patients whose tissues were used for immunohistochemical analysis in the present study consented to publication.

Competing interests

The authors declare that they have no competing interests.

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