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ORIGINAL ARTICLE

# *Helicobacter pylori* Infection Activates the Akt–Mdm2–p53 Signaling Pathway in Gastric Epithelial Cells

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**Abstract**
*Backgrounds and Aims* Although *Helicobacter pylori* is widely accepted as a causative factor of many gastric diseases, the signaling pathways affected by *H. pylori* and subsequent effects on cell apoptosis and proliferation remain unclear. Here, we investigated the molecular mechanisms mediating *H. pylori* infection in gastric epithelial cells.
*Methods* Tissues from 160 patients with various gastric diseases with or without *H. pylori* infection were obtained and analyzed by immunohistochemistry for Akt, pAkt, Mdm2, p53, and Bax expression. In vitro, human gastric epithelial cells, GES-1, were incubated with *H. pylori* culture filtrates. Cell viability was measured by MTT assay. Apoptosis was evaluated by Annexin V/PI double staining followed by flow cytometry, DNA electrophoresis, and comet assay. mRNA and protein expression was assessed by RT-PCR and Western blot analysis.
*Results* In patient tissues, *H. pylori* infection was associated with significantly elevated levels of pAkt in chronic nonatrophic gastritis (CNAG), Mdm2 in dysplasia, p53 in metaplastic atrophy (MA), and Bax in CNAG and MA. In vitro, *H. pylori* culture filtrates reduced GES-1 cell viability in a time- and dose-dependent manner, induced G0/G1 arrest, triggered apoptosis, and increased DNA fragmentation. Mdm2 and Bax mRNA expression and pAkt, Mdm2, p53, and Bax protein expression were significantly upregulated when treated with *H. pylori* culture filtrates. Akt inhibition by LY294002 decreased Mdm2 expression, upregulated p53, and enhanced *H. pylori*-induced growth inhibition of GES-1 cells.
*Conclusions* These findings suggest that Akt–Mdm2–p53 signaling is involved in the molecular response of GES-1 cells to *H. pylori* infection.

**Keywords** *Helicobacter pylori* · Akt · Mdm2 · p53 · Apoptosis · GES-1

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## Introduction

*Helicobacter pylori*, a Gram-negative bacterium, is a dominant species of the human gastric microbiome. *H. pylori* infection is one of the greatest risk factors for the development of many upper gastrointestinal diseases, including gastric inflammation (gastritis), gastric and duodenal ulcers, gastric cancer (GC), and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1, 2]. As many of these diseases result in the imbalance of cell proliferation and apoptosis of gastric epithelial cells, the

effect of *H. pylori* infection on these processes warrants further investigation. Previous reports have shown that *H. pylori* infection increases the proliferation of gastric epithelial cells [3, 4]. However, other studies have shown that *H. pylori* infection may lead to the disorganization of gastric epithelial architecture [5] and induce cell apoptosis [6, 7]. Thus, the true molecular impact of *H. pylori* on cellular homeostasis remains to be elucidated.

The Akt–murine double minute 2 (Mdm2)–p53 signaling pathway has been implicated in the regulation of cell proliferation, survival, and apoptosis [8]. Akt phosphorylates substrate proteins, including Mdm2, and triggers the translocation of Mdm2 from the cytoplasm into the nucleus, where nuclear Mdm2 interacts with the p53 tumor suppressor protein [8]. Binding of Mdm2 with p53 suppresses the transcriptional activity of target genes and leads to the degradation of p53 [9]. Moreover, the Akt–Mdm2–p53 pathway creates a negative feedback loop by the inhibition of p53-mediated Akt activation through PTEN-dependent [10, 11] and PTEN-independent [12] mechanisms.

The importance of p53 in the regulation of gastric epithelial cell survival and death has been widely addressed [13–15]. In a previous study, we determined that Mdm2 and p53 expression was closely associated with different gastric pathologies that were dependent on *H. pylori* infection [16]. However, whether *H. pylori* infection mediates the activation of the Akt–Mdm2–p53 signal transduction pathway and subsequently affects the proliferation, and apoptosis of human gastric epithelial cells still needs clarification. In this present study, we examined the expression of Akt, pAkt, p53, Mdm2, and Bax in clinical patient tissue samples and investigated the functional role of the Akt–Mdm2–p53 signaling pathway in the human gastric epithelial cell line, GES-1, after *H. pylori* infection. Our findings provide valuable insight into understanding the pathogenesis of gastric diseases related to *H. pylori* infection.

## Materials and Methods

### Patients and Sample Collection

A total of 160 patients receiving upper gastroduodenoscopy at the First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, from January 2007 to September 2009, were recruited after signing an informed consent. Samples were collected from the gastric antrum or gastric lesions during gastroduodenoscopy. *H. pylori* infection was examined by rapid urease test and pathological examination using Giemsa staining. Among the collected samples, 80 cases were diagnosed as *H. pylori*-positive when positive results were obtained from both tests (42 males and 38 females; average age 53.1 ± 13.5 years), and 80 cases were diagnosed as *H. pylori*-negative when negative results were obtained from the two tests (51 males and 29 females; average age 53.7 ± 12.3 years). In addition, pathological examination of tissues diagnosed 40 patients with chronic nonatrophic gastritis (CNAG), 40 patients with metaplastic atrophy (MA), 40 patients with dysplasia (Dys), and 40 patients with GC according to the WHO classification and the updated Sydney system [17, 18]. Within each disease group, 20 patients were *H. pylori*-positive and 20 patients were *H. pylori*-negative. The Ethics Committee of the First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, granted ethical approval for this study.

### Reagents

High-glucose Dulbecco's modified Eagle medium (DMEM) culture medium and LY294002 were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Gibco/Life Technologies (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from Hangzhou Sijiqing Corp. (Hangzhou, Zhejiang, China). Brucella broth was bought from Shanghai Municipal Center for Disease Control and Prevention (Shanghai, China). Trypsin, PV-9000 kit, and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were obtained from Beijing Zhong Shan Golden Bridge Biological Technology Co., Ltd. (Beijing, China). The cell genomic DNA extraction kit, oligo(dt), PCR mix, and RNase inhibitor were purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China). The DNA damage detection kit for the comet assay and Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit were purchased from KeyGEN Biotech (Nanjing, Jiangsu, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Beijing Qihuasheng Biotechnology Co., Ltd. (Beijing, China). Agarose and dimethylsulfoxide (DMSO) were obtained from Promega (Madison, WI, USA). TRIzol solution was bought from Invitrogen/Life Technologies (Grand Island, NY, USA). dNTPs were obtained from MBI (Davis, CA, USA). PCR primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Primary antibodies, including anti-pAkt (Ser473), anti-pAkt (Thr308), and anti-Bax, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-Akt, anti-Mdm2, and anti-p53 antibodies were bought from Abcam (Cambridge, MA, USA).

### Immunohistochemical Analysis

Paraffin-embedded tissue sections were used for immunohistochemical analysis. Immunostaining was carried out

with a PV-9000 kit according to the manufacturer's instructions. The samples were incubated with anti-Akt (1:1,000 dilution), anti-p-Akt (1:100 dilution), anti-p53 (1:100 dilution), anti-Mdm2 (1:100 dilution), or anti-Bax (1:100 dilution) followed by incubation with the appropriate secondary antibody. Antibody binding was visualized with DAB according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. All immunostained sections were examined in a blind fashion by two investigators who had no knowledge of patient clinical status.

For evaluation, five fields of view at 200× magnification were randomly selected from each sample, and 100 tumor cells were evaluated in each field. The degree of immunohistochemical staining was recorded using a semiquantitative grading system that considered both the intensity of staining and the proportion of tumor cells that had an unequivocal positive reaction. Cells with cell membrane and/or cytoplasm showing yellow or brown-yellow staining were identified as immuno-positive cells. Staining was rated using the following grading system: 0, positive staining in <5.0 % of tumor cells; 1, positive staining in 5 % $\geq$ 25 % of tumor cells; 2, positive staining in 25 % $\geq$ 50 % of tumor cells; 3, positive staining in 50 % $\geq$ 75 % of tumor cells; 4, positive staining in >75 % of tumor cells. Grades for stain intensity were as follows: 0, no staining; 1, light yellow staining; 2, brown-yellow staining; and 3, dark brown staining. The staining index was calculated as the staining intensity was multiplied by the positive area: negative (−), 0–1; weakly positive (+), 2–3; moderately positive (++), 4–5; and strongly positive (+++), 6–7.

## In Vitro Culture of GES-1 Cells

Human SV40-transformed and immortalized gastric epithelial cells, GES-1, were provided by the Beijing Institute for Cancer Research (Beijing, China). Cells were maintained in DMEM culture medium supplemented with 10 % heat-inactivated FCS and were incubated in a 5 % $CO_2$-humidified incubator at 37 °C. When cells reached 90 % confluency, they were digested with 0.25 % trypsin and subcultured into new flasks.

## Preparation of Culture Filtrates from *H. pylori* and Treatment of GES-1 Cells

The *H. pylori* strain, NCTC 11637, CagA+ VacA+, was provided by the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China). Bacteria were grown on agar plates and incubated in a multigas incubator (Sanyo MCO-175 M, Japan) at 37 °C in a humidified 85 % $N_2$, 10 % $CO_2$, and 5 % $O_2$ atmosphere. For large-scale liquid culture, *H. pylori* was inoculated from a fresh agar plate into an Erlenmeyer flask containing brucella broth. Cultures were incubated for 48–72 h with shaking (200 rpm) at 37 °C under microaerophilic conditions. Bacterial growth was estimated directly by spectrophotometric measurement of the OD600 using a ultraviolet–visible (UV–Vis) spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). Cultures with an OD600 value ranging between 0.6 and 0.7 were pelleted by centrifuging at 10,000 rpm at 4 °C for 20 min. The supernatants were carefully collected and filtered through a 0.22-μm pore filter. The concentration of total protein in the culture filtrates was 11.1 mg/mL as determined by the Biuret method. The culture filtrates were aliquoted and stored at −80 °C until use. GES-1 cells were incubated with culture filtrates at dilutions of 1:2, 1:4, or 1:8. The liquid culture medium alone was used as a control.

## Determination of Cell Viability

To evaluate the viability of GES-1 cells following *H. pylori* culture filtrate treatment, MTT assay was performed. Briefly, a GES-1 cell suspension was added to each well of a 96-well plate. The cell density was adjusted to approximately $1 \times 10^5$ cells/mL. Twenty-four hours after seeding, culture medium was replaced with *H. pylori* culture filtrates or liquid culture medium alone as a control. After 3, 6, 24, 48, or 72 h of treatment, MTT reagent (5 mg/mL) was added to each well. After an additional 4 h of incubation at 37 °C, the medium was removed and replaced with DMSO to resuspend the MTT metabolic product. The absorbance of the dissolved formazan was measured at 490 nm (A490) using a scanning microplate spectrophotometer (Model 680, Bio-Rad, USA). Cell survival rate was calculated as a percentage using the following formula:

$$(A_{\text{Sample}}/A_{\text{Control}}) \times 100.$$

## Evaluation of DNA Damage in Individual Cells

The comet assay was used to measure DNA damage in individual cells as previously described [19]. Briefly, glass slides were pre-coated with 0.5 % normal-gelling-temperature agarose (NMA). Approximately $1 \times 10^6$ cells were gently mixed with 0.7 % low-gelling-temperature agarose (LMA) and added onto the liquid NMA. The agarose cell layer was allowed to air-dry to form a thin film before the addition of another LMA layer. After the agarose was solidified, the slides were submerged in pre-coated lysis buffer for 1–2 h at 4 °C followed by incubation in an alkaline electrophoresis buffer (1 mmol/L EDTA and 300 mmol/L NaOH) for 20–60 min at room temperature. Electrophoresis was conducted for 20–30 min at

25 V. Slides were neutralized in 0.4 mmol/L Tris–HCl (pH 7.5) and stained with propidium iodide (PI) or ethidium bromide (EB) for 10 min in the dark. DNA fluorescence was observed using a fluorescent microscope. A total of 50 cells were randomly selected for analysis from each sample. The nuclei diameter and tail length were measured by Image-Pro Plus 6.0 software. The percentage of DNA damage was calculated by dividing the amount of DNA in the tail over the total DNA intensity (DNA content).

### Assessment of Cell Apoptosis by DNA Electrophoresis

GES-1 cells were exposed to culture filtrates derived from *H. pylori* (1:4 dilution) for 48 h, and genomic DNA was extracted using the genomic DNA extraction kit per the manufacturer's instructions [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. In brief, cells were collected and prepared as a single cell suspension. After centrifugation at 10,000 rpm for 1 min, the supernatant was removed, and cells were resuspended with GA lysis buffer. Proteinase K (20 mg/mL) was added, and the cell suspension was incubated with GB buffer for 10 min at 70 °C. Samples were mixed with 100 % ethanol and were loaded onto a CB3 column. After centrifuging at 12,000 rpm for 30 s, the upper solution was removed, and the samples in CB3 column were washed sequentially with GD buffer, PW solution, and PW solution. The samples were air-dried and eluted with Tris–EDTA buffer (TE). DNA was analyzed using 1 % w/v agarose gel electrophoresis.

### Assessment of Cell Cycle and Cell Apoptosis by Flow Cytometric Analysis

GES-1 cells were treated with *H. pylori* culture filtrates (1:4 dilution) for 48 h. For cell cycle analysis, cells were collected and washed with PBS. The cell density was adjusted to $1 \times 10^6$ cells/mL. Cells were fixed with 70 % acetic acid for 18 h at 4 °C. After fixation, cells were washed with PBS and stained with PI solution for 30 min in the dark. The fluorescence was analyzed by a flow cytometer. To identify cell apoptosis, $1 \times 10^5$–$5 \times 10^5$ cells were suspended in binding buffer (50 mM HEPES, 700 mM NaCl, and 12.5 mM $CaCl_2$, pH 7.4) and mixed with Annexin V–FITC (fluorescein isothiocyanate) and PI for 5–15 min at room temperature in the dark. Cell apoptosis was analyzed using flow cytometric analysis within 1 h after staining.

### Isolation of Total RNA and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from $1 \times 10^6$ cells using TRIzol/chloroform extraction and isopropyl alcohol precipitation. The RNA concentration and purity were determined by an ultraviolet spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). Total RNA (10 μL) was reverse transcribed using oligo(dt) primers and M-MLV reverse transcriptase. PCR was performed using a Gene Amp PCR system 9600 (Perkin-Elmer, Waltham, MA, USA). PCR amplification was carried out with the following specific primers: p53, forward, 5′-ACCMAGTCMA ATGA-3′, reverse, 5′-GCAAGAAGCCCAGACG-3′, product size 174 bp; Mdm2, forward, 5′-GCTGAAGAGGGCTTTG AT-3′, reverse, 5′-TGGTGTAAAGGATGAGCT-3′, product size 623 bp; Bax, forward, 5′-GTGCACCAAGGTGCCGGA AC-3′, reverse, 5′-TCAGCCCATCTT CTTCCAGA-3′, product size 205 bp; and β-actin, forward, 5′-AAGGAAGGCTG GAAGAGTGC-3′, reverse, 5′-CTACAATGAGCTGCGTGT GG-3′, product size 528 bp. The PCRs were heated to 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C (53 °C for p53; 58 °C for Bax) for 30 s, and 72 °C for 60 s. PCR products were separated by agarose gel electrophoresis, and mRNA levels were determined by a gel image analysis system (GSG-2000, Hema Medical Instrument Co. Ltd., Zhuhai, Guangdong, China). The densitometric values were used for the statistical analyses. The housekeeping gene, β-actin, was used as an internal control, and the relative mRNA expression was calculated for each target gene.

### Inhibitor Treatment Using LY294002

To assess the impact of Akt signaling, LY294002, a PI3-kinase inhibitor, was used. Cells were randomly divided into four treatment groups: Control, Hp, Hp + LY294002, and LY294002. In the LY294002 alone treatment group, cells were pre-treated with 40 μM LY294002 for 1 h, followed by 40 μM LY294002 plus the culture medium alone. In the Hp + LY294002 treatment group, cells were pre-treated with 40 μM LY294002 for 1 h, followed by incubation with 40 μM LY294002 plus *H. pylori* culture filtrate. In the Hp treatment group, 40 μM DMSO was used as a control for LY294002, followed by incubation with *H. pylori* culture filtrate. After 24 h of incubation, cells were harvested for analysis.

### Protein Extraction and Western Blot

Total protein was extracted from cells using lysis buffer. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Shanghai Generay Biotech. Co., Ltd., Shanghai, China). Equal amounts of protein extract (30 μg) were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane (Whatman, Erdmannhausen, Germany). Membranes were blocked with

Tris-buffered saline with 0.1 % Tween-20 (TBS-T) with 5 % w/v nonfat dry milk and then incubated with primary antibodies overnight at 4 °C. After washing with TBS-T, membranes were incubated with secondary antibodies for 4 h at room temperature. After washing, bands were visualized using an enhanced chemiluminescence (ECL) kit (Pierce/Thermo Fisher Scientific, Rockford, IL, USA). To quantify protein levels, bands of target proteins were analyzed using the GSG-2000 gel image analysis system (Hema Medical Instrument Co. Ltd., Zhuhai, Guangdong, China).

### Statistical Analysis

Data were analyzed by SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Grade-level comparisons were made using the Mann–Whitney *U* test. Statistical significance among groups was determined using the Kruskal–Wallis *H* test. Correlation analysis was made by Spearman's rank correlation. Count data are presented as mean ± standard deviation (SD). Differences between two groups were evaluated with *t* test. Comparisons between multiple groups were performed with one-way analysis of variance. $P < 0.05$ was recognized as significantly different.

## Results

### IHC Expression Analysis of Akt, pAkt, Mdm2, p53, and Bax Proteins in Gastric Mucosa Tissues Obtained from *H. pylori*-Infected or Non-infected Subjects

To understand the effects of *H. pylori* infection on patients with different gastric diseases, we recruited 160 subjects with or without *H. pylori* infection to establish clinical relevance. Patient tissue samples were analyzed using immunohistochemistry, and staining intensities of Akt, phosphorylated Akt (pAkt), Mdm2, p53, and Bax proteins were determined using a semiquantitative grading system. Although no statistical difference was detected in the expression level of total Akt protein between patients with or without *H. pylori* infection in all groups ($P > 0.05$), pAkt expression was elevated in CNAG patients infected with *H. pylori* ($P < 0.05$, Table 1). Mdm2 and p53 protein levels were upregulated in *H. pylori*-infected Dys and MA patients, respectively ($P < 0.05$). Additionally, *H. pylori* infection was associated with increased Bax expression in CNAG and MA patients ($P < 0.05$). Representative immunohistochemical images of staining are presented in Fig. 1. Spearman's correlation analysis determined that pAkt levels were positively associated with the expression of Mdm2 in the gastric mucosa of *H. pylori*-infected patients with CNAG, MA, and Dys ($r = 0.263$; $P < 0.05$). This pathological analysis suggests that the Akt–Mdm2–p53 signaling pathway might be involved in the physiological changes that occur in the gastric mucosa of subjects infected with *H. pylori*.

### Culture Filtrates from *H. pylori* Reduced Cell Viability and Growth of GES-1 Cells

In order to understand the influence of *H. pylori* infection on gastric mucosal tissue in vitro, we investigated the effect of *H. pylori* culture filtrates on cultured human GES-1 cells, an SV40-transformed and immortalized gastric epithelial cell line. As demonstrated in Fig. 2a, *H. pylori* culture filtrates reduced cell viability of GES-1 cells in a time-dependent manner starting after 24 h as compared with control ($P < 0.05$). In addition, attenuation of cell viability using culture filtrates from *H. pylori* appeared to be dependent on concentration, as the high dose (1:2 dilution) suppressed GES-1 cell viability more dramatically than the moderate (1:4 dilution) or low doses (1:8 dilution, $P < 0.05$). Note that a dilution of 1:8 of culture filtrates initially decreased cell viability after 24 h, but then gradually increased cell viability after a prolonged incubation of 48 or 72 h.

Based on those results, experimental conditions using *H. pylori* culture filtrates at the moderate 1:4 dilution and 48-h incubation time were used in the following experiments. As seen in Fig. 2b, flow cytometric analysis demonstrated that treatment with culture filtrates for 48 h greatly elevated the percentage of GES-1 cells in the G0/G1 stage relative to control cells (Control 45.4 ± 4.89 %; HP 98.7 ± 1.12 %; $P < 0.05$) (Fig. 2b). These results suggest that culture filtrates from *H. pylori* may induce G0/G1 arrest in GES-1 cells.

### *H. pylori* Culture Filtrates Induced Apoptosis

To characterize cell death induced by *H. pylori* culture filtrates, we utilized Annexin V–PI double staining followed by flow cytometric analysis to detect the percentage of apoptotic cells after treatment. As seen in Fig. 3a, b, the percentage of Annexin V–PI double-positive cells increased significantly after exposure to *H. pylori* culture filtrates (Control 1.65 ± 0.440 %; HP 26.9 ± 5.34 %; $P < 0.05$). Using electrophoresis, DNA fragmentation, an indicator of apoptosis, was observed in cells treated with *H. pylori* culture filtrates, whereas no fragmented DNA was observed in control cells (Fig. 3c).

DNA damage in individual cells was further measured by comet assay. As shown in Fig. 3d, e, comet tail length as well as the percentage of DNA in the tail was significantly increased in cells treated with *H. pylori* culture

**Table 1** Immunohistochemical analysis of Akt, pAkt, Mdm2, mutant p53, and Bax protein in patients with various gastric disorders with or without *H. pylori* infection

| Disease subtype | HP infection | *N* | Akt | | | | | pAkt | | | | | Mdm2 | | | | | p53 | | | | | Bax | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | − | + | ++ | +++ | *P* value | − | + | ++ | +++ | *P* value | − | + | ++ | +++ | *P* value | − | + | ++ | +++ | *P* value | − | + | ++ | +++ | *P* value |
| CNAG | Negative | 20 | 2 | 3 | 3 | 12 | 0.834 | 9 | 8 | 2 | 1 | **0.023** | 2 | 10 | 3 | 5 | 0.618 | 17 | 3 | 0 | 0 | 0.435 | 4 | 16 | 0 | 0 | **0.023** |
| | Positive | 20 | 0 | 4 | 6 | 10 | | 2 | 12 | 3 | 3 | | 0 | 10 | 7 | 3 | | 15 | 5 | 0 | 0 | | 2 | 12 | 4 | 2 | |
| MA | Negative | 20 | 0 | 3 | 4 | 13 | 0.379 | 4 | 11 | 2 | 3 | 0.456 | 1 | 8 | 7 | 4 | 0.729 | 19 | 1 | 0 | 0 | **0.019** | 2 | 10 | 3 | 5 | **0.016** |
| | Positive | 20 | 0 | 3 | 1 | 16 | | 8 | 6 | 4 | 2 | | 0 | 8 | 8 | 4 | | 13 | 7 | 0 | 0 | | 0 | 4 | 6 | 10 | |
| Dys | Negative | 20 | 1 | 4 | 6 | 9 | 0.884 | 4 | 11 | 4 | 1 | 0.609 | 0 | 12 | 4 | 4 | **0.000** | 14 | 1 | 1 | 4 | 0.789 | 1 | 2 | 6 | 11 | 0.072 |
| | Positive | 20 | 0 | 3 | 9 | 8 | | 4 | 9 | 5 | 2 | | 0 | 2 | 1 | 17 | | 14 | 4 | 0 | 2 | | 1 | 7 | 6 | 6 | |
| GC | Negative | 20 | 0 | 5 | 1 | 14 | 0.909 | 2 | 8 | 5 | 5 | 0.742 | 0 | 3 | 1 | 16 | 0.458 | 12 | 2 | 1 | 5 | 0.356 | 2 | 6 | 2 | 10 | 0.301 |
| | Positive | 20 | 2 | 0 | 5 | 13 | | 2 | 9 | 5 | 4 | | 0 | 1 | 6 | 13 | | 13 | 6 | 1 | 0 | | 0 | 10 | 6 | 4 | |

Protein expression was evaluated using a semiquantitative analysis of staining intensity and percentage of positively stained cells

*CNAG* chronic nonatrophic gastritis, *MA* metaplastic atrophy, *Dys* dysplasia, *GC* gastric cancer

$P < 0.05$ are indicated in bold

filtrates compared to control cells (Control 5.88 ± 10.5; HP 44.0 ± 17.5; $P < 0.05$). These data indicate that *H. pylori* culture filtrates may induce apoptotic cell death in cultured GES-1 cells.

### *H. pylori* Culture Filtrate Altered the Expression of Akt, pAkt, Mdm2, p53, and Bax After Treatment

In order to understand the mechanism of *H. pylori*-mediated inhibition of cell viability and induction of apoptosis, the Akt–Mdm2–p53 signaling pathway was investigated. RT-PCR analysis revealed that Mdm2 and Bax mRNA expression was significantly upregulated in cells treated with *H. pylori* culture filtrates (Mdm2: Control 0.36 ± 0.06 vs. HP 0.79 ± 0.04, $P < 0.05$; Bax: Control 0.64 ± 0.10 vs. HP 0.90 ± 0.03, $P < 0.05$). No statistical difference was found in the mRNA levels of p53 between *H. pylori* culture filtrate-treated and control cells (Control 0.76 ± 0.10 vs. HP 0.79 ± 0.08, $P > 0.05$; Fig. 4a, b).

Western blot analysis demonstrated that phosphorylated Akt at residues S473 and T308 was upregulated 1.8- and 2.8-fold, respectively, in GES-1 cells after treatment with *H. pylori* culture filtrate ($P < 0.05$), while total Akt protein expression was not altered (Fig. 4c, d). Moreover, Mdm2, p53, and Bax protein expression was also significantly increased by 1.9-, 1.8-, and 2.2-fold, respectively, with *H. pylori* culture filtrate treatment as compared with controls ($P < 0.05$, Fig. 4c, d). These data suggest the Akt–Mdm2–p53 signaling pathway is involved in the *H. pylori*-mediated biological response in GES-1 cells.

### Akt Inhibition Modulated the Expression of Mdm2 and p53

The PI3 kinase inhibitor, LY294002, was employed to evaluate the impact of Akt–Mdm2–p53 signaling in the *H. pylori*-mediated cell response. As seen in Fig. 5, treatment with LY294002 in the presence of *H. pylori* culture filtrate significantly decreased S473 and T308 phosphorylation and Mdm2 expression, but upregulated p53 expression ($P < 0.05$). These results indicate that Akt inhibition by LY294002 was sufficient to overcome the *H. pylori*-mediated effect on the Akt–Mdm2–p53 signaling pathway.

### Blockage of Akt Signaling Enhanced *H. pylori*-Mediated Growth Inhibition of GES-1 Cells

Lastly, we examined the effects of Akt inhibition on the viability of cells treated with *H. pylori* culture filtrate. As Fig. 6 demonstrates, inhibition of Akt signaling by LY294002 further reduced cell growth in GES-1 cells that were incubated with *H. pylori* culture filtrate (12 h: Control 100 ± 0 % vs. HP 74 ± 5 %, $P < 0.05$; 24 h: Control

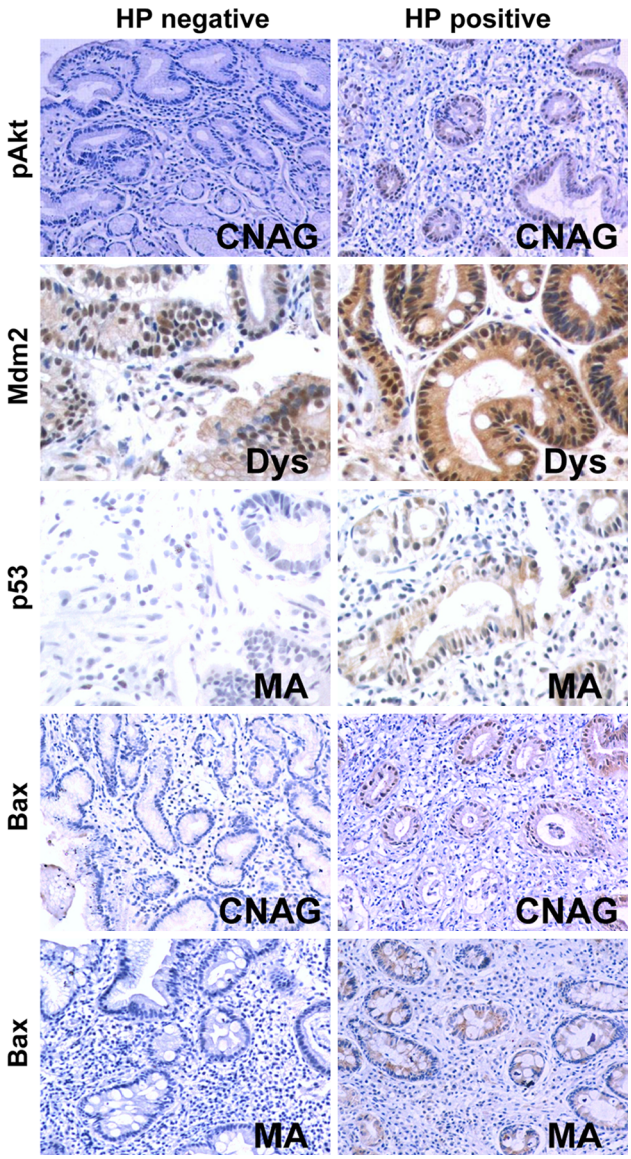


**Fig. 1** Immunohistochemical analysis of pAkt, Mdm2, p53, and Bax protein expression in gastric mucosa tissue specimens derived from *H. pylori*-infected or non-infected subjects. Representative images are shown with positive immunostaining seen in *brown*. Magnification: ×200

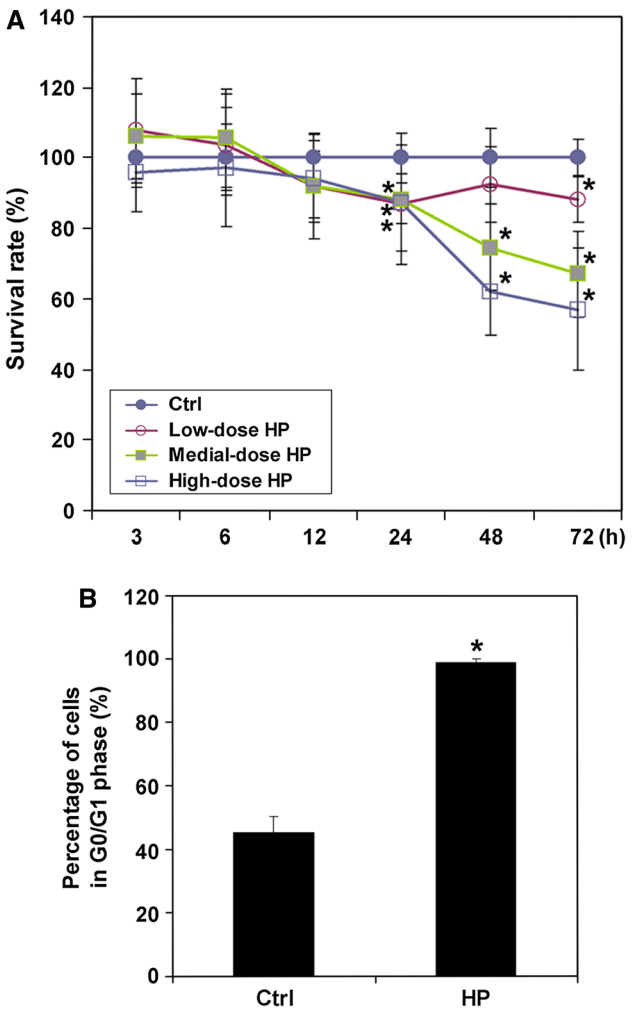


**Fig. 2** Culture filtrates from *H. pylori* reduced viability and induced G0/G1 arrest in GES-1 cells. **a** GES-1 cells were exposed to various dilutions of *H. pylori* culture filtrates as follows: high-dose HP, 1:2 dilution; medial-dose HP, 1:4 dilution; and low-dose HP, 1:8 dilution for the indicated time periods. Control cells (Ctrl) were incubated with *H. pylori* culture medium alone for the same time period. Cell survival rate was determined by MTT assay. Data were quantified from 12 independent experiments. $*P < 0.05$ compared with control. **b** Cell cycle analysis was conducted using flow cytometry. The percentage of cells in the G0/G1 stage was calculated from three independent experiments. $*P < 0.05$ compared with control

100 ± 0 %; HP 79 ± 4 %; $P < 0.05$). These findings confirm the functional involvement of the Akt signal transduction pathway in the *H. pylori*-induced molecular response in gastric epithelial cells.

## Discussion

In a previous study, we determined that Mdm2 and Bax expression levels progressively increased in tissues from normal gastric mucosa (NGM) to GC and that *H. pylori* infection was associated with increased mutant p53 and Bax expression [16]. In this study, we wanted to elucidate the mechanism of *H. pylori* action by studying the Akt–Mdm2–p53 signaling axis in human gastric epithelial cells infected with *H. pylori*. Our findings demonstrate that pAkt, Mdm2, p53, and Bax proteins were upregulated in tissues of *H. pylori*-infected patients with various types of gastric diseases. In vitro analysis using GES-1 cells revealed that culture filtrates from *H. pylori* suppressed cell viability, induced G0/G1 arrest, and triggered apoptosis. Furthermore, the Akt–Mdm2–p53 signaling pathway was activated in GES-1 cells upon *H. pylori* infection.

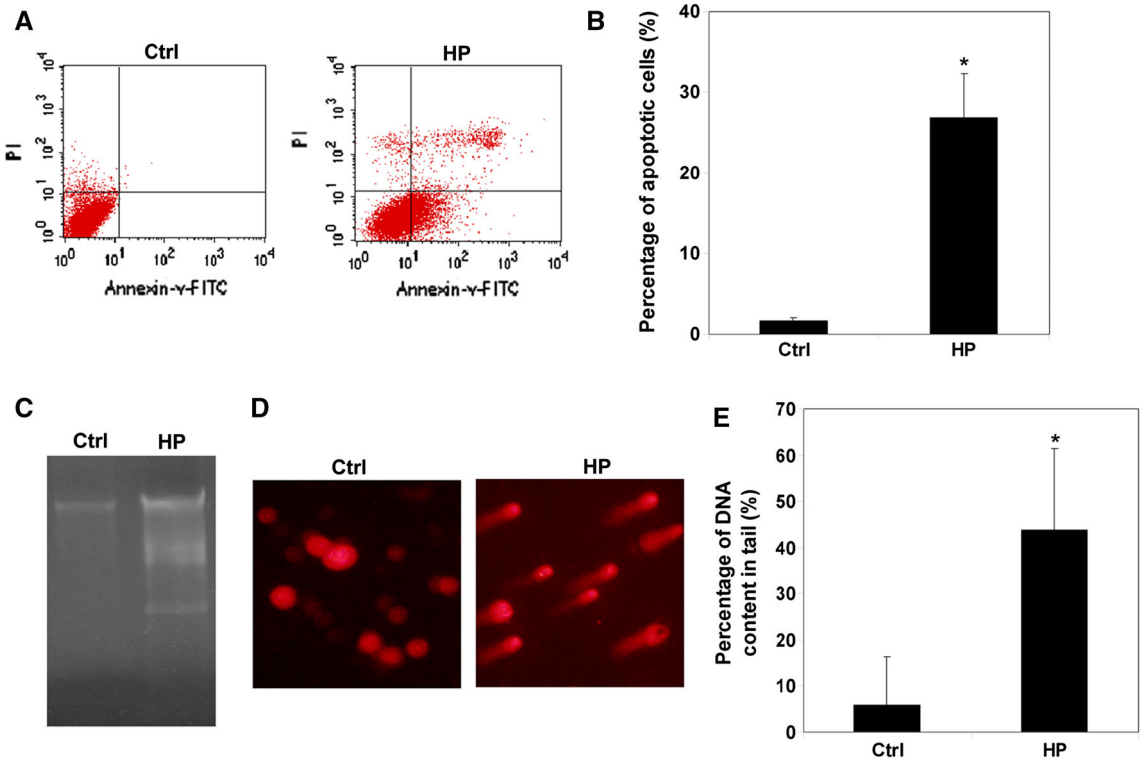


**Fig. 3** Apoptosis of GES-1 cells after treatment with *H. pylori* culture filtrates. GES-1 cells were exposed to *H. pylori* culture filtrates (HP, 1:4 dilution) for 48 h. Control cells (Ctrl) were incubated with culture medium alone for the same time period. **a** The percentage of apoptotic cells was evaluated using Annexin V–PI double staining followed by flow cytometric analysis. **b** Data were quantified from three independent experiments. $*P < 0.05$ compared with control. **c** Cell apoptosis was also assessed by agarose gel electrophoresis. DNA fragmentation was only observed in cells of the HP group. **d** DNA damage in individual cells was measured by comet assay. **e** The average percentage of DNA content in tails was calculated from three independent experiments with each evaluating at least 50 cells. $*P < 0.05$ compared with control

Increased phosphorylation of Akt has been linked with high-risk gastric mucosa disease [20]. Here, we also observed increased pAkt in CNAG patients infected with *H. pylori*. Others have shown that p53 and Mdm2 expression was significantly elevated in *H. pylori*-infected gastric mucosa as compared to normal gastric mucosa [21]. In a study by Nakajima et al. [22], no significant differences were detected in the expression of Mdm2 in patients with and without *H. pylori*-infected chronic gastritis, although Mdm2 expression was higher in intestinal metaplasia and GC tissues than in chronic gastritis with *H. pylori* infection. Consistent with these findings, we also found that Mdm2 expression gradually increased with persistent *H. pylori* infection and was associated with gastric mucosa damage. Specifically, *H. pylori* infection enhanced Mdm2 expression in patients with gastric Dys.

As Mdm2 binds to and leads to the degradation of p53 [9], we also analyzed mutant p53 levels, which are more stable than wild-type p53, in gastric mucosa. In our pathological analysis, those patients with *H. pylori* infection and metaplastic atrophy exhibited elevated p53 expression. In accordance with this result, previous studies have demonstrated that mutant p53 levels were upregulated in *H. pylori*-infected patients with intestinal metaplasia as compared with non-infected patients [23, 24]. It is possible that *H. pylori* infection may induce DNA damage [25] and lead to p53 mutations that ultimately contribute to gastric mucosa carcinogenesis at a later stage.

The pro-apoptotic gene Bax is a target downstream factor for p53 [26]. Here, we found that *H. pylori* infection upregulated Bax expression in CNAG and MA patients, while no significant difference was found in patients with Dys and GC. *H. pylori* infection has been found to upregulate the level of Bax in patients with chronic gastritis. However, in patients with GC, upregulation of the anti-apoptotic gene, Bcl-2, counteracts the pro-apoptotic effects of Bax [27] providing one explanation for the lack of difference in Bax expression observed in this study. Thus, *H. pylori* infection might be critical for the regulation of the Bax/Bcl-2 ratio and its subsequent impact on gastric carcinogenesis. Moreover, a p53 homolog, p73, is strongly induced by *H. pylori* in vitro and in vivo [28]. Importantly, p73 has also been shown to induce cell apoptosis by activating all p53 targets, including the Bax protein [29].

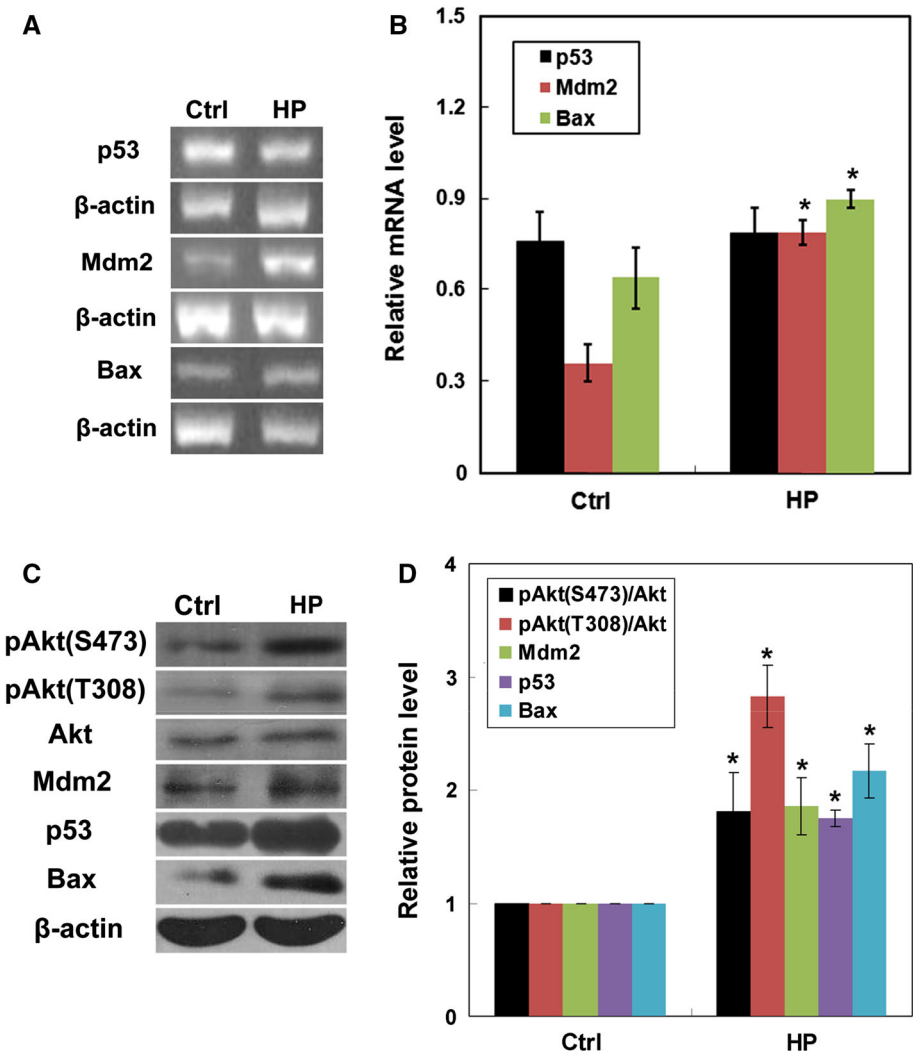


**Fig. 4** Altered expression of Akt, pAkt, Mdm2, p53, and Bax after *H. pylori* culture filtrate treatment. GES-1 cells were exposed to culture filtrates derived from *H. pylori* (HP, 1:4 dilution) for 48 h. Control cells (Ctrl) were incubated with culture medium alone for the same time period. The mRNA levels of p53, Mdm2, and Bax were determined by RT-PCR. β-Actin was used as internal control. **a** Representative results of amplified products. **b** Data were quantified from three independent experiments. $^*P < 0.05$ compared with control. The protein expressions of total Akt, pAkt at residues S473 and T308, Mdm2, p53, and Bax were evaluated by Western blotting. β-Actin was used as internal control. **c** A representative immunoblot. **d** Banding intensities were quantified from three independent experiments. The expression of S473 and T308 was normalized to total Akt, and the expression of Mdm2, p53, and Bax was normalized to β-actin. $^*P < 0.05$ compared with control

Therefore, we could not rule out the possibility that Bax might be induced by p73. Future studies will focus on exploring the precise role of p53 in inducing Bax and apoptosis.

We found that *H. pylori* culture filtrates were capable of suppressing cell viability and inducing apoptosis in vitro. Moreover, medial- or high-dose *H. pylori* culture filtrates greatly increased DNA damage. Consistent with these findings, CagA-positive *H. pylori* culture filtrates have also been shown to induce DNA damage in human gastric epithelial tumor cells in vitro [30]. *H. pylori* infection also induces oxidative stress and triggers cell apoptosis in gastric epithelial cells [6, 7]. The literature supports that *H. pylori* regulates cellular migration and apoptosis by stimulating the Akt signaling pathway in gastric epithelial cells [31]. In this study, the *H. pylori* culture filtrates used were positive for CagA. It should be noted that CagA itself can induce ROS production; excessive ROS production has been linked to gastric carcinogenesis [32]. Thus, *H. pylori* infection as well as its metabolic products and secreted proteins may be catalysts for gastric epithelial cell damage.

Importantly, activation of the Akt–Mdm2–p53 signaling axis appears to be the downstream molecular mechanism in gastric epithelial cells infected with *H. pylori*. Increased Akt phosphorylation, Mdm2, p53, and Bax levels were detected in cells infected with *H. pylori*, consistent with the IHC analysis of tissue sections. Activation of Akt upregulates Mdm2 expression [33] and subsequently regulates cell survival through the regulation of p53 [34]. In this study, inhibition of Akt signaling by the specific inhibitor, LY294002, upregulated p53, but downregulated Mdm2 levels in cells infected with *H. pylori*, suggesting that Mdm2 might be partially regulated by the Akt signal, and the activation of Akt and MDM2 during filtrate infection might moderately suppress p53 action. However, the resulting DNA damage may have significantly upregulated p53, resulting in the elevated p53 levels observed with filtrate infection.

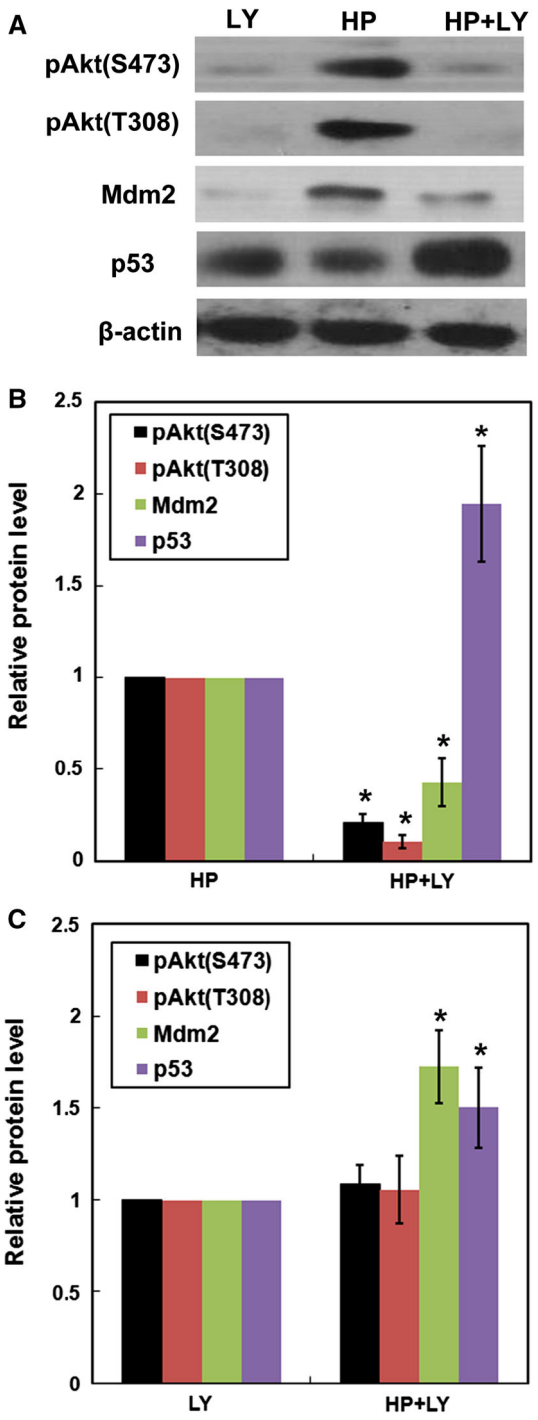


**Fig. 5** Inhibition of the Akt signaling pathway by LY294002 suppressed Mdm2, but elevated p53 protein expression. GES-1 cells were exposed to culture filtrates derived from *H. pylori* (HP, 1:4 dilution) for 48 h. To inhibit the Akt pathway, cells were pre-treated with the Akt inhibitor, LY294002, followed by LY294002 and HP culture filtrate co-incubation (HP + LY). Cells treated with LY294002 alone were used as a control. The protein expression of pAkt at residues S473 and T308, Mdm2, and p53 was evaluated by Western blotting. β-Actin was used as an internal control. **a** Representative immunoblot. **b**, **c** Data were quantified from three independent experiments. Protein expression was normalized to β-actin. $*P < 0.05$ compared with HP or LY alone

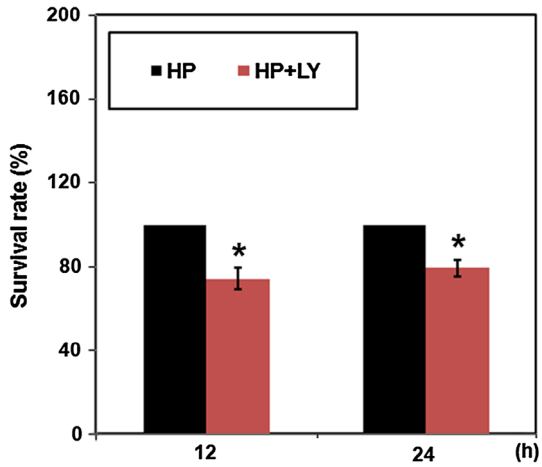


**Fig. 6** *H. pylori*-mediated growth inhibition is further attenuated by LY294002 in GES-1 cells. GES-1 cells were exposed to culture filtrates derived from *H. pylori* (HP, 1:4 dilution) for 12 or 24 h. To inhibit the Akt signaling pathway, cells were pre-treated with the Akt inhibitor, LY294002, followed by the co-incubation of LY294002 and HP culture filtrate (HP + LY). Cell survival rate was determined by MTT assay. Data were quantified from four independent experiments. $*P < 0.05$ compared with control

Blockage of Akt signaling by LY294002 inhibited cell growth associated with *H. pylori* infection, indicating that Akt activation may serve as an anti-apoptotic signal in *H. pylori*-mediated cell apoptosis. Lee et al. have reported that revaprazan, a novel acid pump antagonist, exerted anti-inflammatory action against *H. pylori* infection by inactivating Akt signaling in human gastric adenocarcinoma cells [35]. This work suggests that Akt signaling might be involved in the regulation of pathophysiologic responses to *H. pylori* infection. Early activation of the Akt signaling pathway may act against apoptosis induced by *H. pylori* infection in gastric epithelial cells. However, Akt signal activation did not inhibit ROS-mediated cell apoptosis although elevated ROS is associated with apoptosis from *H. pylori* infection [36].

In summary, our present study shows the functional involvement of the Akt–Mdm2–p53 signaling pathway in gastric epithelial cells infected with *H. pylori* and its impact on cell viability and apoptosis. These findings may provide valuable insight into the understanding of gastric disease pathology in the presence of *H. pylori* infection. Future studies will continue to explore the underlying associations between Akt, Mdm2, p53 and Bax in gastric diseases.

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**Conflict of interest** None.

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