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**Original Paper**

# A Decrease of Histone Deacetylase 6 Expression Caused by Helicobacter Pylori Infection is Associated with Oncogenic Transformation in Gastric Cancer

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**Key Words**
HDAC6 • Precancerous lesion • HP infection • Gastric cancer • Prognosis

**Abstract**
***Background:*** Histone deacetylase 6 (HDAC6) plays a role in the progression of many tumors. However, the relationship between the level of HDAC6 expression and gastric tumorigenesis is still unclear. Here, we illustrate the potential correlation between *Helicobacter pylori* (HP) infection and the variation of HDAC6 expression in different gastric lesions, as well as the clinical significance of HDAC6 expression in gastric cancer (GC) patients. ***Materials and Methods:*** Between 2011 and 2016, 364 patients with different types of gastric lesions were enrolled in Baotou City Central Hospital. Immunostaining of HDAC6 expression and HP infection were performed in the following cohort including 21 normal tissues (Normal); 40 samples with chronic superficial gastritis (CSG); 106 with chronic atrophic gastritis (CAG); 94 with intestinal metaplasia (IM); 64 with dysplasia (DYS) and 39 with gastric cancer (GC). Survival analysis was performed in another 80 GC patients using the Kaplan-Meier method and multivariate Cox regression analyses. The level of HDAC6 expression was determined by Real-time PCR, Western blotting and IHC staining in gastric cell lines and tissues. Furthermore, the correlation between HDAC6 expression and clinicopathological features and prognosis was analyzed in the GC cohort. HP strains were lavaged into Kunming mice to investigate the effects of HP infection on the expression of different HDAC members in this mouse model. ***Results:*** Higher levels of HDAC6 expression were detected in normal and premalignant lesions than in the GC tissues ($p<0.01$), and decreased HDAC6 expression was associated with HP infection and TNM stage ($p<0.01$ and $p=0.048$, respectively). Multivariate analysis revealed that HDAC6 expression was an independent predictor of the outcome of GC patients ($p=0.04$). HP mediated HDAC6 expression in the cell lines and KM mice. HP infection could promote

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HDAC1 and HDAC4 expression as determined by Western blotting. ***Conclusions:*** HDAC6 is a promising biomarker for early diagnosis and prognosis during the oncogenic transformation of gastric cancer.

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## Introduction

Gastric cancer (GC) is one of the most severe malignancies of the digestive tract diagnosed worldwide [1]. Most of the GC incidence and mortality occurs in China [2, 3]. Patients with advanced GC often have poor survival because of the lack of early detection and precise diagnosis [4]. Early detection and prognostic assessment are necessary for GC patients. Particularly, the discovery of novel candidate biomarkers for clinical use is urgent in GC.

HDAC families are associated with tumorigenesis and progression through the epigenetic regulation of tumor behavior [5]. Most evidence indicates that histone deacetylases (HDACs) are overexpressed in cancer. However, among the HDACs involved in liver cancer, HDAC6 has been shown to uniquely function as a tumor suppressor, but the involvement of HDAC6 in GC has not been clarified yet. HDAC6 is a member of the class IIb HDAC family [6-9]. However, whether HDAC6 functions as an oncogene or tumor suppressor in GC is still controversial [9, 10]. Moreover, the mechanism of HDAC6 expression in GC is still unclear. Some data have shown that low HDAC6 expression was associated with poor prognosis in breast cancer and hepatocellular carcinoma [9, 11]. This indicated HDAC6 as a tumor suppressor gene. However, Wang et al. reported the presence of high HDAC6 expression in GC compared with precancerous lesions [12]. In human pancreatic cancer tissues, elevated HDAC6 expression was also detected at both the protein and mRNA levels [13]. Moreover, cytoplasmic HDAC6 expression was found to be induced upon oncogenic Ras transformation [14]. It has been suggested that HDAC6 is required for efficient oncogenic transformation. Hence, the changes in the levels of HDAC6 and the mechanisms of HDAC6 in the development of GC are controversial and remain poorly understood.

It is accepted that HP is an important biological agent responsible for gastric tumorigenesis [15]. It has been linked to genetic modifications including acetylation and methylation of several stomach-specific housekeeping genes [16]. However, the potential correlation between HP infection and the status of HDAC6 expression has not been studied yet. In this study, we will illustrate the potential clinical significance of HDAC6 expression and its relationship with HP infection *in vitro* and *in vivo*.

## Materials and Methods

### *Patients and tissue specimens*

Three hundred sixty-four paraffin-embedded gastric samples were collected from the outpatient service of Baotou City Central Hospital. The patient diagnoses were confirmed by pathology. Clinicopathological factors including HP detection, age, sex, differentiation, and TNM stage were collected. Informed consent was obtained from each patient. The cohort study was approved by the institutional research medical ethics committee of Baotou City Central Hospital.

### *Immunohistochemistry staining*

Routine techniques were used to cut 4 μm sections from paraffin blocks. Polyclonal rabbit HP antibody (1:100 dilution, Cat: PA5-32442, Waltham, USA) and polyclonal rabbit HDAC6 antibody (1:500 dilution, Cat: PA1-31473, Waltham, USA) were purchased from Thermo Fisher Scientific. Antigen was retrieved by microwaving in citrate buffer for 10 min. The slides were incubated with the primary HP antibody at room temperature for 1 h and the primary HDAC6 antibody at 4°C overnight, then incubated with the secondary antibody at room temperature for 1 h. Immunohistochemistry compilation of these slides were developed with DAB (3, 3 –diaminobenzidine, brown) and counterstained with hematoxylin to stain nuclei (blue).



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*Immunostaining evaluation*

Positive expression of HDAC6 was determined as brown staining in the cytoplasm or nuclei. The immunostaining score was dependent on the percentage of positive cells and the staining intensity. The scoring was done according to the following pathology standards: 0, less than 25% of tumor cells were positive; 1, 25%-50%; 2, 50%-75%; 3, 75%-100%. The staining intensity was evaluated as 0, no stain; 1, weak; 2, moderate; 3, strong. The samples that scored more than or equal to 2 were considered as "positive". Otherwise, the samples were designated as "negative". The evaluation was performed by two senior pathologists. HP was stained as a gray colored S-shaped mass or rod-shaped structure.

*Real-time PCR*

Total RNA was extracted from the cells and tissues using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The isolated RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (50 U; Life Technologies). PCR was performed with 1 μl cDNA by using HDAC6-specific primers, and β-actin was used as the control. The HDAC6-specific primers were 5'-AGTCCATCGCAGATACTGGC-3' (forward) and 5'-TTAGTCTGG CCTGGAGTGGA-3' (reverse). The β-actin-specific primers were 5'-TTAGTT GCGTTACACCCTTTC-3' (forward) and 5'-ACCTTCACCGTT CCAGTTT-3' (reverse). The lengths of HDAC6 and β-actin PCR products were 216 bp and 150 bp, respectively. To differentiate HDAC6 from the other HDAC family members, HDAC1 (class I HDAC), HDAC4 (class IIa HDAC) and SIRT1 (class III HDAC), primers were designed as follows: HDAC1 (231 bp), forward primer (F), 5'-TGCTAAAGTATCACCAGAGGGT-3', reverse primer (R), 5'-TGGCCTCATAGGACTCGTCA-3'; HDAC4 (264 bp), F: 5'-CCCTCTACACATCGCCATCC-3'; R: 5'-CTGTGACGAGGGGTGCTTG-3'; SIRT1 (401 bp), F: 5'-GCGGTTCCTACTGCGCGA-3', R: 5'-TGTGACAGAGAGATGGCTGG A-3'. Gene expression was analyzed with a ABI 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) with SYBR green (TransGen Biotech Co., Ltd., Beijing, China). One microliter of cDNA and 1 μl of primers were mixed to a final volume of 12 μl. Briefly, the final q-PCR conditions were as follows: a pre-denaturation at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and extension at 60°C for 30 s. β-actin was the endogenous control. Each reaction was performed in triplicate.

*Cell lines*

The GC cell lines BGC823, MGC803, SGC7901 and the immortalized normal human gastric epithelial cell line (GES1) were obtained from the Cell Bank of Shanghai (Shanghai, China). The GC cell lines AGS and N87 were purchased from the American Type Culture Collection (Manassas, VA, USA). *H. pylori*-SS1 bacterial strains were obtained from Beijing Cancer Hospital. The SS1 strain of *H. pylori* was a CagA+ and VacA+ strain, which was isolated from a patient with atrophic gastritis. Under the condition of microaerophilic, brain heart infusion was used for bacterial culture in agar plates. Agar plates which was containing 7% horse blood were incubated at 37°C for 72 h. Bacteria was observed by naked eyes in colony morphology, Gram staining and microscopy observation of bacterial morphology. HP strain were finally confirmed by Rapid urease test and scraped bacteria in a PBS buffer. The concentration of bacteria was determined by ultraviolet spectrophotometer (1 $OD_{600} = 3\times10^{8}$ cfu/ml).The concentration of bacterial suspension was $3\times10^{8}$ cfu/ml. Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Life Technologies, Grand Island, NY, USA) was used for cell culture. The GES1 cells were co-cultured with SS1 for 24 h with the addition of 0 μl, 100 μl, 500 μl, 1000 μl of the bacterial culture to 2 ml of the cell culture medium. Furthermore, the GES1 cells were co-cultured with 500 μl of SS1 for 0 h, 6 h, 12 h, 24 h and 48 h. The variation of HDAC6 expression was determined using real-time PCR in triplicate.

*Western blotting*

The protein expression levels were evaluated by Western blotting. Antibodies against HDAC1 (AH379), HDAC4 (AH388) and SIRT1 (AF0282) were purchased from Beyotime Ltd., China, those against HDAC6 was obtained from Thermo Fisher Scientific (PA1-31473). Antibody against β-actin (A2228) was obtained from Sigma. β-actin was used as the internal control. SDS-PAGE gels were used for separating protein samples. Then, the separated proteins were transferred to PVDF membranes. The membranes were washed for 5 min at room temperature in PBS-Tween and blocked with 5% non-fat milk. The membranes were probed with following primary antibodies overnight: rabbit polyclonal antibody against HDAC6 (1:1000 dilution), HDAC1 (1:600 dilution), mouse monoclonal antibody against HDAC4 (1:600 dilution), rabbit polyclonal



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antibody against SIRT1 (1:600 dilution) and antibody against β-actin (1:10000 dilution). After washing 3 times with PBS-Tween, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h, and then the proteins were detected.

*Determination of HDAC6 expression in response to HP in vivo using KM mice*

Male 6-week-old KM mice (n=60) were purchased from Beijing Vital River Laboratory Animal Technology. The mice were housed at 25°C in 50-55% humidity and with a 12 h light/dark cycle. The animals were randomly separated into control groups and HP infection groups. The animals were fasted for 8 h before the intragastric (ig) administration of 500 µl *H. pylori* strain-SS1 every other day for a total of 6 times. Two hours after ig delivery, food and water were given to the mice. The animals were sacrificed 3 weeks later, and the gastric mucosal samples were collected to detect the status of HP infection by the rapid urease test (HPUT-H102, Fjsanqiang. Ltd. Fujian Province) or by using primary antibody for the IHC staining of HP. HDAC1, HDAC4, HDAC6 and SIRT1 expression were detected by real-time PCR and Western blotting.

*Transmission Electron Microscopy (TEM)*

A small piece of the gastric mucosa of no more than 1 $mm^3$ was removed from the KM mice after 3 weeks of HP infection. The specimens were fixed in 2.5% glutaraldehyde and 1% osmic acid for 2 h. Graded alcohols were used for dehydrating the specimens. Epoxy resin was used to retain the cellular structure. Then, the specimens were oven dried at 37°C (12 h), 45°C (12 h) and 60°C (36 h). The specimens were cut into utrathin sections of 50-70 nm (with LKB Ultrotome, Sweden). A transmission electron microscope (HT7700 Hitachi, USA) was used to observe the samples.

*Statistical analyses*

All statistical analyses were performed with IBM SPSS 20.0 software (IBM Inc,, Armonk, USA). The correlation between HDAC6 expression and other clinicopathologic features and between HDAC6 expression and different gastric mucosa lesions were performed with the Chi-square test. A non-parametric test was used to analyze the RT-PCR data from the cell lines and tissues. The $2^{-\Delta\Delta CT}$ method was used for quantifying the Real-time PCR results in the gastric cell lines and tissues. Survival analyses were performed with the Kaplan-Meier method. *p*-values of 0.05 or less were considered significant.

## Results

*The expression of HDAC6 in different types of gastric mucosal lesions*

To investigate the expression of HDAC6 in different gastric mucosal lesions, 364 samples comprising CSG, CAG, IM, DYS, and GC were immunostained with the antibody against HDAC6. Positive staining for HDAC6 was detected in 30.8% of GC samples, compared to 85.7% of the normal tissues. As shown in the Table 1 and Fig. 1, the expression of HDAC6 during progression from precancerous conditions to GC was reduced compared with the normal tissues. The expression of HDAC6 was present in 85.7% (18/21), 62.5% (25/40), 18.9% (20/106), 26.6% (25/94), 31.2% (20/64), 30.8% (12/39) of the normal tissues, CSG, CAG, IM, DYS and GC, respectively.

*Association between HP and HDAC6 expression*

HP-induced gastritis is the single strongest risk factor for gastric cancer. HP infection affects the expression of several gastric housekeeping genes and influences others via epigenetic modification. To investigate the potential correlation between HDAC6 expression and HP infection in different gastric mucosal lesions, HP staining was identified in 338 cases from the cohort, and there was an

**Table 1.** Expression of HDAC6 in different gastric mucosal lesions

| | HDAC6 expression | | |
|---|---|---|---|
| Variables | Negative (%) | Positive (%) | Cases |
| Normal tissue | 3 (14.3) | 18 (85.7) | 21 |
| Superficial gastritis | 15 (37.5) | 25 (62.5) | 40 |
| Atrophic gastritis | 86 (81.1) | 20 (18.9) | 106 |
| Intestinal metaplasia | 69 (73.4) | 25 (26.6) | 94 |
| Dysplasia | 44 (68.8) | 20 (31.2) | 64 |
| Gastric carcinoma | 27 (69.2) | 12 (30.8) | 39 |
| Total | 244 (67.0) | 120 (33.0) | 364 |



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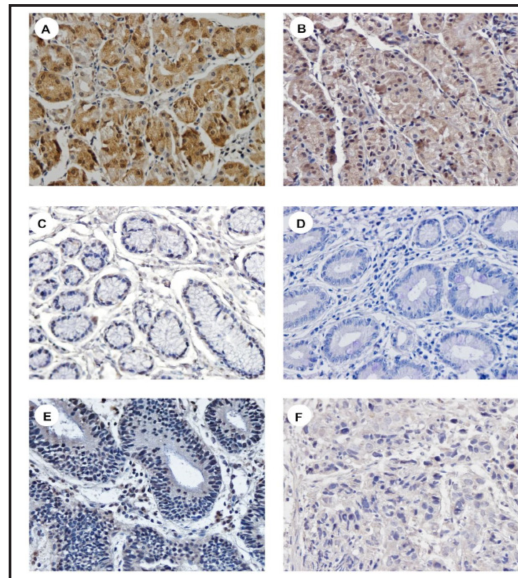


**Fig. 1.** Differential expression of HDAC6 in various gastric mucosal lesions by IHC staining (Original magnification 400×). The level of HDAC6 was reduced during the progression of gastric cancer. (A) HDAC6 expression in normal tissues. Most intracytoplasm brown granulars were observed by immunostaining. (B) HDAC6 was positive in chronic superficial gastritis. Brown granulars of HDAC6 was located in cytoplasm. (C) Negative expression of HDAC6 in chronic gastric atrophy. (D) Negative expression of HDAC6 in intestinal metaplasia. (E) Negative expression of HDAC6 in dysplasia. (F) Negative expression of HDAC6 in gastric cancer.

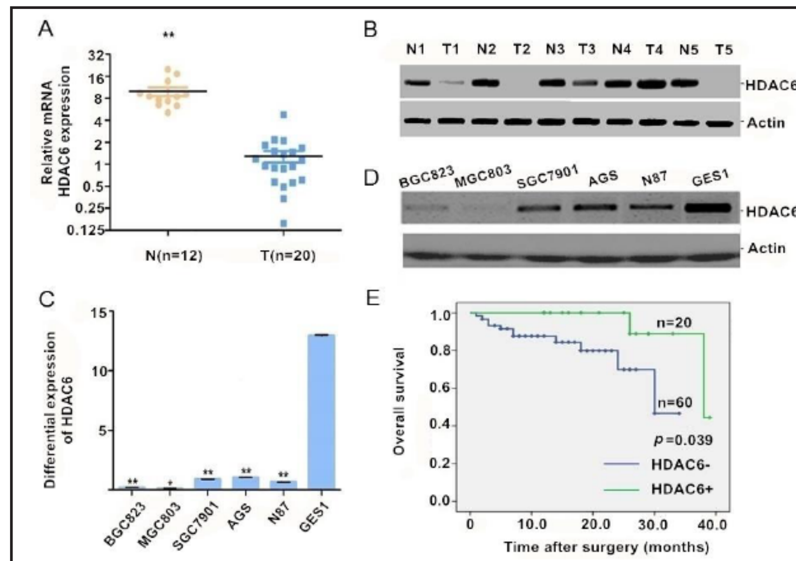


**Fig. 2.** Decreased HDAC6 expression was associated with poor over-all survival in GC patients. (A) Differential expression of HDAC6 mRNA in human GC (C) and normal tissues (N) by real-time PCR. (B) HDAC6 expression in normal human tissues (N) and GC tissues (T) by Western blotting. (C) HDAC6 expression was detected by real-time PCR in cancerous and normal gastric cell lines. (D) HDAC6 expression was detected by Western blotting in cancerous and normal gastric cell lines. (E) GC patients with high levels of HDAC6 (HDAC6+) had better overall survival than those with low levels of HDAC6 (HDAC6-) (*$p$<0.05, **$p$<0.01).

inverse correlation between HDAC6 expression and HP infection (Table 2, $p$<0.01, Fig. 3). The data indicated that HDAC6 was associated with the development of gastric cancer.

*Clinical significance of HDAC6 in the prognosis of GC patients*

An additional 80 GC patients were examined to identify whether HDAC6 could be a candidate prognostic biomarker based on univariate and multivariate regression. Several clinical features were associated with the survival time, such as age, sex, TNM stage, differentiation and HDAC6 expression. The patients with HDAC6-positive expression GC had better prognosis than those with HDAC6-negative GC ($p$ = 0.039, Table 4). In addition, other clinical features, including sex, age and differentiation did not contribute to the outcome of the GC patients ($p$ > 0.05, Table 4). No obvious association was found between HDAC6 expression and other factors including gender, age, differentiation or TNM stage in GC (Table 3). To further assess the clinical value of HDAC6 expression for the prognosis of GC patients, we used multivariate Cox regression to analyze the clinicopathological characteristics that were associated with the survival time in the 80 GC patients. As shown in Table 5, HDAC6



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**Table 2.** The association between HP and HDAC6 expression. * compared with normal tissues. HDAC6-: Negative; HDAC6+: Positive

| Gastric status | | HP+ | | | |
|---|---|---|---|---|---|
| | HP+ (%) | HDAC6- (%) | HDAC6+ (%) | Counts | *p*-value |
| Normal | 21 (100) | 3 (14.3) | 18 (85.7) | 21 | |
| CSG | 29 (72.5) | 12 (41.4) | 17 (58.6) | 40 | 0.039* |
| CAG | 97 (91.5) | 81 (83.5) | 16 (16.5) | 106 | <0.01* |
| IM | 90 (95.7) | 67 (74.4) | 23 (25.6) | 94 | <0.01* |
| DYS | 63 (98.4) | 43 (68.3) | 20 (31.7) | 64 | <0.01* |
| Cancer | 38 (97.4) | 27 (71.1) | 11 (28.9) | 39 | <0.01* |
| Total | 338 (92.9) | 233 (68.9) | 105 (31.1) | 364 | <0.01 |

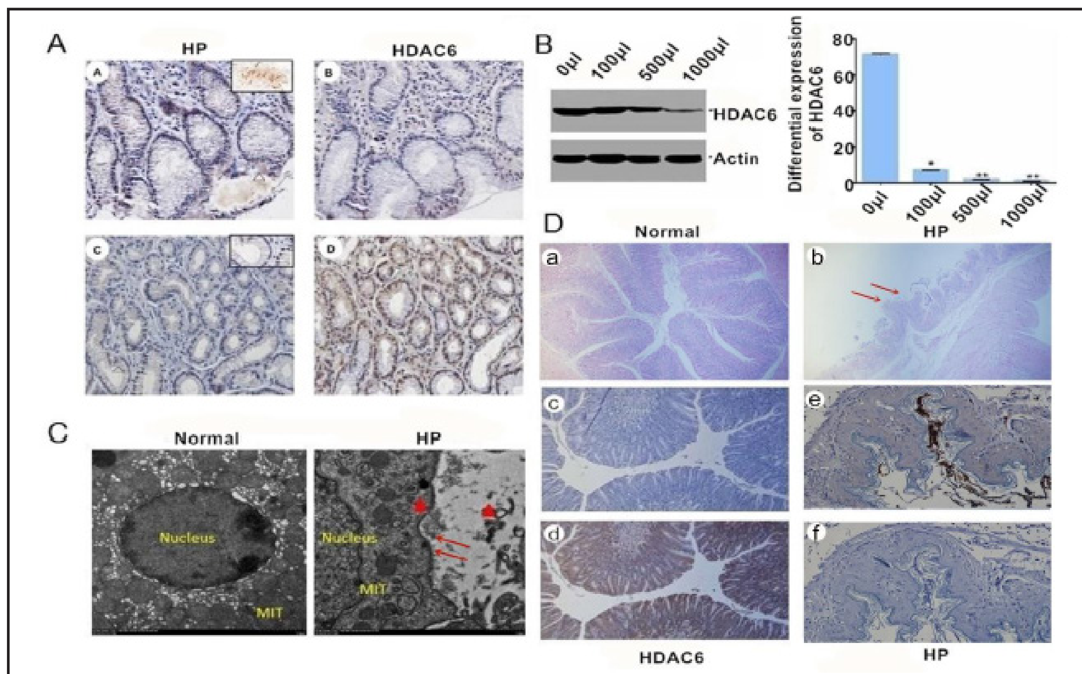


**Fig. 3.** Inverse correlation between HDAC6 and HP infection *in vitro* and *in vivo.* (A) Correlation between HP and HDAC6, as shown by immunostaining (Original magnification 400×) a: HP-positive. b: HDAC6-negative. c: HP-negative. d: HDAC6-positive. (B) HDAC6 protein and mRNA levels were reduced after HP infection in a dose-dependent manner in the GES1 cells co-cultured with 0μl, 100μl, 500μl, 1000μl SS1 for 24 h by Western blot and qRT-PCR, respectively (*$p<0.05$, **$p<0.01$). (C) Electron microscopy of gastric mucosa from the control and HP-infected mice after 3 weeks of infection. ▲Ulcer induced by HP infection. → A single epithelial cell and interface with stomach lumen present with HP infection. (D) Association between HP and HDAC6 was detected in the animals. (a, b) HP infection was measured by H&E straining after 3 weeks of chronic infection. a: HP infection was not detected by H&E straining in the normal tissues of control groups. b: Ulcer was detected by H&E straining in HP infection groups. (c, d, e, f) Inverse association between HP and HDAC6 were observed by immunostaining. c, e : After 3 weeks of chronic infection, HDAC6 negative expression and HP positive expression were detected in HP infection groups. d, f: HDAC6 positive expression and HP negative expression were detected in control groups after 3 weeks of chronic infection.

expression was an independent prognostic biomarker in GC ($p = 0.04$). The patients with higher levels of HDAC6 exhibited better survival according to the Kaplan-Meier analysis ($p = 0.039$, Fig. 2E). Collectively, HDAC6 expression was a protective factor for the gastric mucosa.

*Reduced HDAC6 expression in GC tissues and cells*

The level of HDAC6 mRNA was evaluated by qRT-PCR in 20 GC tissues and 12 normal tissues. As shown in Fig. 2A, a higher level of HDAC6 was found in the normal tissues than in the GC samples ($p < 0.01$). Furthermore, similar results were identified in 5 matched GC



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samples by Western blot (Fig. 2B). Reduced HDAC6 mRNA and protein levels were also observed in the BGC823, MGC803, SGC7901, AGS and N87 cells (Fig. 2C, 2D). Interestingly, a high level of HDAC6 expression was detected in GES1 cells, which is a normal immortalized epithelial gastric mucosal cell line. The other HDAC members, including HDAC1, HDAC4, and SIRT1, had different patterns of expression in the gastric cell lines (Fig. 4A). This implied that HDAC6 was unique, and its level was reduced in several GC cell lines.

**Table 3.** Correlation between HDAC6 expression and clinicopathological features of GC patients

| Variables | | Cases | HDAC6 expression Negative (%) | Positive (%) | *p-value* |
|---|---|---|---|---|---|
| Gender | Female | 20 | 15 (75.0) | 5 (25.0) | >0.999 |
| | Male | 60 | 45 (75.0) | 15 (25.0) | |
| Age (years) | ≥60 | 45 | 33 (73.3) | 12 (26.7) | 0.696 |
| | <60 | 35 | 27 (77.1) | 8 (22.9) | |
| Differentiation | Well/moderately | 19 | 16 (84.2) | 3 (15.8) | 0.373 |
| | Poorly | 61 | 44 (72.1) | 17 (27.9) | |
| TNM stage | I-II | 27 | 18 (66.7) | 9 (33.3) | 0.219 |
| | III-IV | 53 | 42 (79.2) | 11 (20.8) | |

**Table 4.** Univariate analysis of the overall survival of GC patients

| Clinical features | Classification | Cases | Average survival time | *p*-value |
|---|---|---|---|---|
| Sex | Male | 60 | 31.20 ± 3.39 | 0.291 |
| | Female | 20 | 32.61 ± 2.02 | |
| Age | ≥60 | 45 | 29.63 ± 2.30 | 0.096 |
| | <60 | 35 | 32.18 ± 1.25 | |
| TNM | Stage I+II | 27 | 31.85 ± 1.11 | 0.048* |
| | III+IV | 53 | 29.63 ± 2.25 | |
| Differentiation | Poorly | 61 | 33.92 ± 1.67 | 0.364 |
| | Well/moderately | 19 | 28.85 ± 3.24 | |
| HDAC6 expression | Negative | 60 | 27.01 ± 1.78 | 0.039* |
| | Positive | 20 | 37.11 ± 1.35 | |

*Downregulation of HDAC6 mediated by HP infection in GES1 cells*

As shown in Fig. 3B and 4C, real-time PCR results demonstrated a reduction in HDAC6 expression in the GES1 cells co-cultured with SS1 strains (HP) in a time- and dose-dependent manner. Similar data were obtained in our animal model by Western blotting and IHC staining of the stomach (Table 7 and Fig. 4B and 4D). This suggested that HP reduced the expression of HDAC6 at the transcriptional level via genetic or epigenetic regulation. Further studies are needed.

**Table 5.** Multivariate Cox regression analysis of the clinicopathological features of GC patients

| Clinical features | 95.0% CIs | *p*-value |
|---|---|---|
| Sex | 0.100-1.251 | 0.107 |
| Age | 0.944-21.584 | 0.059 |
| Differentiation | 0.347-4.214 | 0.765 |
| TNM stage | 0.753-47.597 | 0.091 |
| HDAC6 | 0.012-0.902 | 0.040* |

*Differential expression of HDAC family members in vitro and in vivo*

Greater expression of HDAC6 was observed in the GES1 cells compared with the gastric cancer cell lines (Fig. 2D). The MGC803 cells had the lowest expression of HDAC6. The expression of HDAC1 was higher in BGC823, SGC7901, and AGS cells compared with N87 and GES1 cells (Fig. 4A). The N87 cells had the lowest levels of HDAC4 and SIRT1. The expression of HDAC4 and SIRT1 was not significantly different between GES1 and the GC cell lines. A reduction in HDAC6 protein by Western blot was observed after HP infection, indicating that the expression of HDAC6 is inhibited HP in a dose-dependent and time-dependent manner (Fig. 3B). The expression of HDAC6 was higher in GES1 than the GC cell lines. There was no consistent pattern of expression in the other members of HDAC family. We confirmed the results in the animal model. The expression of HDAC6 was different from that of the other members of the HDAC family.

*Relationship between HP and HDAC family members in the animal model*

The data from the HP-infected groups that were treated with ig administration of HP and the control groups significantly different in terms of the HP infection rate and HDAC6 expression (Table 6, 7). H. pylori rapid urease testing showed positive results in 90.0%



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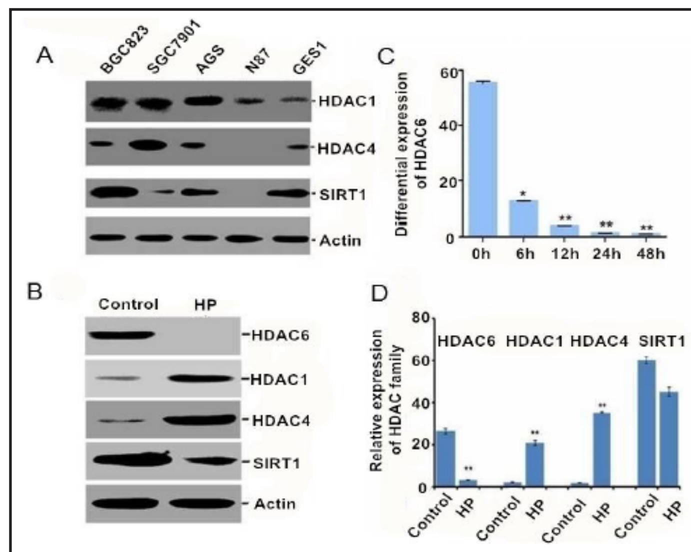


**Fig. 4.** Differential expression of HDAC family members in gastric cell lines and animal model. (A) Expression of HDAC family members in gastric cell lines by Western blotting. (B) Expression of HDAC family members in the animal model by Western blotting. (C) The differential expression of HDAC6 mRNA in the GES1 cells co-cultured with 500 μl SS1 with in a time-dependent manner by qRT-PCR (*$p$<0.05, **$p$<0.01). (D) Differential expression of HDAC family members in the animal model by qRT-PCR (*$p$<0.05, **$p$<0.01).

**Table 6.** Pathological outcomes of KM mice lavaged with vehicle control or HP strains-SS1 after 3 weeks of chronic infection

| Groups | Original average weight (g) | Average weight (after 6 times ig) | Average weight | Rapid urease test | HDAC6 expression |
|---|---|---|---|---|---|
| Control | 45.63 ± 2.68 | 47.68 ± 2.56 | 49.05 ± 2.73 | 0/30 | 30/30 |
| HP | 45.28 ± 2.53 | 44.67 ± 2.47 | 48.25 ± 2.57 | 27/30 | 6/30 |

**Table 7.** Association between HP and HDAC6 expression in KM mice after 3 weeks of chronic infection

| HP infection | HDAC6 expression | | *p*-value |
|---|---|---|---|
| | Negative | Positive | |
| Negative | 0 | 30 | |
| Positive | 24 | 3 | 0.005 |

(27/30) of the HP-infected animals and in 0.0% (0/30) of the control animals. HDAC6 expression was detected in 20.0% (6/30) of the HP-infected animals and in 100.0% (30/30) of the control animals. Interestingly, expression of HDAC1 and HDAC4 at both mRNA and protein levels were detected in the HP-infected group (Fig. 4D). There was no obvious difference in the expression of SIRT1 between the HP-infected group and the negative control group. According to Western blotting results, the most ulcerous lesions in the gastric mucosa occurred in the HP-infected group, and the high infection rate of HP was correlated with the downregulation of HDAC6 expression (Fig. 4B).

## Discussion

At present, effective treatment and diagnosis of advanced GC is still difficult, Meanwhile, the 5-year survival rate after surgery is 90% or more in early GC [17]. This implies that more attention should be given to the early diagnosis and detection of GC. In this study, we discovered that HDAC6 is downregulated in GC compared with precancerous lesions. HDAC6 is thus an independent predictor of prognosis in GC patients. Furthermore, knockdown of HDAC6 expression was shown to decrease migration and invasion of the prostate cancer cell line IA8 *in vitro* [18]. By investigating the mechanism of reduction in HDAC6 expression, we found inverse correlation between HDAC6 and HP infection in clinical samples. Further support for the potential regulation of HDAC6 by HP infection was identified by real-time PCR, immunostaining and Western blot *in vitro* and *in vivo*. Our data revealed that HP inhibited the expression of HDAC6 at both the mRNA and protein levels. Hence, the downregulation of HDAC6 may have been associated with HP infection *via* epigenetic or genetic regulatory mechanisms. In hepatocellular carcinoma (HCC) samples, HDAC6 was found to act as a



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tumor suppressor gene, and reduced HDAC6 expression was associated with poor prognosis and reduced rates of 5-year overall, disease-free and recurrence-free survival in HCC patients [9]. Cell proliferation and metastasis were suppressed after the ectopic expression of HDAC6 in HCC cells by the activation of the JNK/Beclin 1 autophagic cell death pathway. Furthermore, clinical data showed that the level of HDAC6 in HCC was significantly lower than that in hepatitis and liver fibrosis. Opposite results have also been reported in HCC patients [19]. Upregulated HDAC6 were detected in HCC tissues and cell lines at the mRNA and protein levels. The ectopic expression of HDAC6 promoted the proliferation of HCC cells. Knockdown of HDAC6 inhibited cell proliferation. HDAC6 was shown to play an important role in cell proliferation through the NF-κB signaling pathway. Activation of NF-κB leads to the overexpression of HDAC6 at the transcriptional level since the promoter of HDAC6 contains a binding site of NF-κB [19]. Thus, the role of HDAC6 in the development of HCC is controversial, and the association of HDAC6 expression with GC is also unclear. Further studies are needed to define the role of HDAC6 and its involvement in gastric tumorigenesis.

In our study, HDAC6 was expressed both in the cytoplasm and nuclei. Cytoplasmic HDAC6 was mostly observed in normal and CSG samples. Interestingly, higher nuclear HDAC6 staining was observed in the GC samples than in the normal or CSG precancerous lesions. This implied that the cytoplasmic or nuclear localization may play different roles during the malignant progression of gastric cancer. It is possible that nuclear HDAC6 is involved in tumorigenesis. Similar data have been reported in lung cancer [8]. Nuclear HDAC6 was correlated with poor prognosis and distant metastasis. In our study, cytoplasmic HDAC6 was observed in the early gastric lesions. An elevated level of nuclear HDAC6 was observed in GC. The relevant mechanisms should be studied in the future. Studies of how the nuclear localization of HDAC6 can accelerate tumor development and progression provide a novel topic of research. A study by J.A. demonstrated that the sensitivity of androgen receptor could be enhanced by HDAC6 and that the nuclear localization of HDAC6 was regulated by acetylation of HSP90 [20]. Targeting HDAC6 may be a new approach to cure castration-resistant prostate cancer. However, the mechanism regulating the sub-cellular localization of HDAC6 is still unclear.

Other members of the HDAC family have also been studied by many researchers. In our study, we found that HDAC1, HDAC4, HDAC6 and SIRT1 have different expression patterns in gastric cell lines and animal models with HP infection. In particular, reduced HDAC6 levels were detected in several GC cell lines and gastric mucosa infected with HP. However, high levels of HDAC1 and HDAC4 were observed in GC cells and in the animal model. The expression of SIRT1 was not different among the cells or in the animal model. HDAC10, a tumor suppressor gene in GC also belonging to the HDAC IIb family, only localized to the cytoplasm, and its level was noticeably reduced in GC compared with the adjacent normal tissues [5]. Upregulated HDAC4 levels were detected in GC [21]. Overexpression of HDAC4 enhanced proliferation of the SGC7901 cells, whereas the knockdown of HDAC4 expression caused apoptosis. A high level of SIRT1 was correlated with progression, lymphatic invasion and poor prognosis in GC [22]. SIRT1 acts as an oncogene in GC. The study of the expression of different HDACs requires further exploration and may be beneficial for applications in precision medicine.

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### Disclosure Statement

The authors declare that they have no conflicts of interest.



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