ORIGINAL ARTICLE

Basic Study

Helicobacter pylori promotes invasion and metastasis of gastric cancer by enhancing heparanase expression

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Abstract

AIM

To detect the mechanisms of *Helicobacter pylori* (*H. pylori*) infection in the invasion and metastasis of gastric cancer (GC).

METHODS

Specimens from 99 patients with GC were collected. The

correlation among *H. pylori* infection, heparanase (HPA) and mitogen-activated protein kinase (MAPK) expression, which was determined by immunohistochemistry, and the clinical features of GC was analysed using SPSS 22.0. Overall survival (OS) and relapse-free survival (RFS) of GC patients were estimated by the Kaplan-Meier method. Independent and multiple factors of HPA and MAPK with prognosis were determined with COX proportional hazards models. HPA and MAPK expression in MKN-45 cells infected with *H. pylori* was analysed using Western blot.

RESULTS

H. pylori infection was observed in 70 of 99 patients with GC (70.7%), which was significantly higher than that in healthy controls. *H. pylori* infection was related to lymph metastasis and expression of HPA and MAPK ($P < 0.05$); HPA expression was relevant to MAPK expression ($P = 0.024$). HPA and MAPK expression in MKN-45 cells was significantly upregulated following *H. pylori* infection and peaked at 24 h and 60 min, before decreasing ($P < 0.05$). SB203580, an inhibitor of MAPK, significantly decreased HPA expression. HPA was related to lymph metastasis and invasive depth. HPA positive GC cases and *H. pylori* positive GC cases showed poorer prognosis than HPA negative cases ($P < 0.05$). COX models showed that the prognosis of GC was connected with HPA expression, lymph metastasis, tissue differentiation, and invasive depth.

CONCLUSION

H. pylori may promote the invasion and metastasis of GC by increasing HPA expression that may associate with MAPK activation, thus causing a poorer prognosis of GC.

Key words: Gastric cancer; *Helicobacter pylori*; Heparanase; Mitogen-activated protein kinase; Overall survival; Relapse-free survival

Core tip: The mechanism of *Helicobacter pylori* (*H. pylori*) infection in the invasion and metastasis of gastric cancer (GC) is still unknown. This paper studied heparanase (HPA) and mitogen-activated protein kinase (MAPK) expression in GC tissues and GC cells and their relationship with *H. pylori* infection. *H. pylori* infection may promote the invasion and metastasis of GC by increasing the expression of HPA that may be increased by activation of MAPK signal and HPA expression in GC tissue. *H. pylori* positive GC had a poorer prognosis.

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been classified as a class Ⅰ carcinogen by the International Agency for Research on Cancer[1]. *H. pylori* can live in the acidic environment of the stomach for a long time, and its prolonged infection can destroy the gastric mucosa and result in changes in the release of gastric mucosal hormones, thus affecting the physiological state of the stomach. Therefore, *H. pylori* infection represents the most significant risk factor for malignant gastric tumours[2,3]. Approximately 50% of the world's population are infected with *H. pylori*[4,5]. The infection rate in China may be as high as 73.3%[6], especially in the Beijing region, where the infection rate is as high as 83.4%[7]. Although there have been increasing numbers of studies indicating that *H. pylori* infection can result in gastric cancer (GC), the underlying mechanism is still unknown.

Heparanase (HPA) is an endoglycosidase that is capable of degrading heparan sulfate (HS) in the extracellular matrix (ECM) and basement membrane (BM)[8,9], the process of which releases many types of biological mediators, such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF), in response to local or systemic signals[10,11]. Thus, HPA is involved in tissue remodelling and cell migration, which lead to inflammation, angiogenesis, and tumour metastasis[12-15]. The degradation of the ECM is one of the key steps involved in the invasion and metastasis of malignant tumours, and matrix degradation primarily depends on proteolytic enzymes. An increasing number of studies have demonstrated that the invasion and metastasis of tumour cells are closely associated with HPA production[16-18], including those derived from the stomach, pancreas, colon, and bladder. In addition to its enzymatic activity, recent studies have shown that the non-enzyme activity of HPA promotes the aggregation of heparan sulfate proteoglycans (HSPGs), causing a cascade of intracellular signal amplification that results in the activation of protein kinase C (PKC), Src, and Rac. HSPGs act on HPA receptors located on the cell surface, such as 6-phosphate mannose receptor (MPR), cationic non-6-phosphate-dependent mannose receptor (CD222), and low-density lipoprotein receptor-related protein (LRP), to cause signalling cascades. In addition, HPA plays an important role in inflammation and autoimmune diseases (*e.g.*, colitis, arthritis, psoriasis, and sepsis)[19,20].

Some studies have shown that *H. pylori* infection leads the development of gastric adenocarcinoma by activating mitogen-activated protein kinase (MAPK)[21,22]. Once activated, MAPK is translocated to the nucleus and leads to the activation of transcription factors, such as NF-κB[23,24]. A recent study[25] has also shown that the activation of the MAPK pathway is closely related to the expression of HPA. However, it is not clear whether MAPK is involved in the regulation of HPA expression following *H. pylori* infections that lead to GC.

The present research aimed to explore the role of *H.*

pylori infection in GC and analyse the connections among *H. pylori* infection, HPA and MAPK expression, and the pathological state of GC. By detecting the expression of HPA and MAPK and *H. pylori* in GC tissue and the expression of HPA and MAPK in MKN-45 cells infected by *H. pylori*, we analysed their associations with the clinical and pathological features of GC, and the survival and prognosis of *H. pylori* or HPA/MAPK positive GC.

MATERIALS AND METHODS

Cells and H. pylori strain

MKN-45 (human GC cell line) cells were obtained from the Chinese Academy of Sciences (Shanghai, China), preserved in the Key Laboratory of Digestive System Tumours of the Second Clinical Medical School of Lanzhou University, and cultivated in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, United States) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.), 100 U/mL penicillin, and 100 mg/mL streptomycin (North China Pharmaceutical Co., Inc., Shijiazhuang, China) in humidified air containing 5% carbon dioxide at 37 ℃.

H. pylori NCTC11637 was provided by the Key Institute of Digestion and Oncology of Gansu Province and was cultured on Columbia agar plates (Solarbio, Shanghai, China) containing 7% filtered goat blood in an anaerobic tank.

Infection of MKN-45 cells with H. pylori

After digestion, MKN-45 cells were inoculated in three culture dishes with an equal cell volume and cultured under normal growth conditions until they reached the logarithmic growth phase, at which point the old medium was discarded and replaced with culture medium containing serum and antibiotics. *H. pylori* was cultured at 37 ℃ for 72 h under microaerophilic conditions using an anaerobic box. *H. pylori* was then added to the above cell medium at a bacteria:cell ratio of 100:1. The bacteria and cells were co-cultured for 6, 12, 24, and 48 h at 37 ℃ in 5% CO_2 and saturated humidity to detect HPA and MAPK expression.

Western blot assay

Total protein of the treated cells was extracted using RIPA lysis buffer (Beyotime Biotechnology, Haimen, China), phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology), and a protein phosphatase inhibitor (Solarbio Biotechnology Co., Shanghai, China) after being washed with ice-cold phosphate-buffered saline (PBS). The protein concentration was measured by the BCA protein assay (Beyotime Biotechnology) after centrifugation at 14000 rpm for 30 min. The protein was separated on a 10% SDS polyacrylamide gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Solarbio Biotechnology), which was then blocked in the blocking solution for 2 h at a constant temperature. Then, the above membrane was incubated

with the following primary antibodies: anti-heparanase1 (Abcam Biotechnology, Cambridge, United Kingdom), anti-phospho-p38, anti-p38 (Abcam Biotechnology), and anti-β-actin (Zhongshan Golden Bridge Biotech, Beijing, China). After overnight incubation, the membrane was washed three times with Tris Buffered Saline-Tween (TBST) (Solarbio Biotechnology) and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biotech; dilution 1:10000) at room temperature for 1 h. The SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Inc., Rockford, IL, United States) was used to detect signals, which were displayed with a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States). Data were analysed using Bio-Rad Quantity One Software v4.62 (Bio-Rad Laboratories Co., Ltd.). To further illustrate whether the *H. pylori*-induced upregulation of HPA was mediated through the MAPK pathway, 20 μmol/L of the MAPK inhibitor SB203580 (Selleck, United States) was added to MKN-45 cells for 2 h before they were cultured with *H. pylori*.

Transwell assay

A Transwell assay was used to detect the invasion capability of the tumour cells. A 1:8 mixture of Matrigel gel:RPMI-1640 medium (100 μL) was added to the lower chamber, which was placed in 24-well plates at 37 ℃ for 24 h. A serum-free cell suspension (200 μL) at a concentration of 2.5×10^5 cells/mL was added to the upper chamber, while serum-containing medium was added to the lower chamber. Then, the cells were cultured at 37 ℃ in an atmosphere with 5% CO_2 and saturated humidity for 24 h. The remaining cells in the upper chamber were carefully wiped off, and the lower chamber was rinsed twice with PBS and fixed in methanol for 15 min. The cells were stained with crystal violet solution in methanol for 30 min, and then excess crystal violet was washed off. The cells were observed and images were obtained using a microscope. The number of migrated cells was counted in several fields of view.

Scratch test

The scratch test was used to detect the migration ability of tumour cells. Horizontal lines were drawn every 0.5-1 cm on the back of a 6-well plate and 5×10^5 cells were added to each well. The cells were incubated overnight and when cells were 100% confluent, a cut was made across the dish with a 200-mL pipette. The cells were rinsed twice with PBS, serum-free medium was added, and the cells were incubated at 37 ℃ in an atmosphere with 5% CO_2. Following the incubation, images of the cells were obtained and the migration distance was measured.

Patients and clinicopathological characteristics

Ninety-nine cases of pathologically diagnosed and surgically resected primary gastric carcinoma tissues

Table 1 Heparanase and mitogen-activated protein kinase expression in specimens of gastric cancer *n* (%)

	Cases (*n*)	Immunohistochemical result				HR (*P* value)
		-	+	+ +	+ + +	
HPA						
Gastric cancer	99	30 (30.3)	16 (16.2)	25 (25.3)	28 (28.3)	35.547 (0.000)
Para-cancer	99	57 (57.6)	11 (11.1)	17 (17.2)	16 (16.2)[b]	
Normal tissue	25	24 (96.0)	1 (4.0)	0 (0.0)	0 (0.0)[bd]	
MAPK						
Gastric cancer	99	49 (49.5)	12 (12.1)	19 (19.2)	19 (19.2)	33.303 (0.000)
Para-cancer	99	82 (82.8)	5 (5.1)	6 (6.1)	6 (6.1)[b]	
Normal tissue	25	23 (92.0)	2 (8.0)	0 (0.0)	0 (0.0)[b]	

P values < 0.05 were determined using one way analysis of variance (ANOVA): [b]*P*: Comparison with gastric cancer; [d]*P*: Comparison with para-cancer tissue. HPA: Heparanase; MAPK: Mitogen-activated protein kinase; HR: Hazard ratio.

and tumour-adjacent tissues (> 5 cm from the edge of the neoplastic foci and non-tumour tissue confirmed by pathology) without preoperative chemotherapy or radiotherapy from the Department of Gastroenterological and Oncological Surgery of the First Hospital of Lanzhou University were collected between June 2013 and June 2014. Patients with GC were aged between 31 and 77 years, with a mean age of 59.5 ± 9.8 years, and included 61 males and 38 females. Of the 99 cases of GC, there were 48 cases of highly and moderately differentiated carcinoma, 51 cases of poorly differentiated carcinoma, 55 cases of lymph node metastasis, and 44 cases without lymph node metastasis. With respect to the extent of infiltration, there were 42 cases of the T1/2 stage and 57 cases of the T3/4 stage. The clinicopathological characteristics are shown in Table 1. All cases of GC had received D2 radical surgery, and paraffin sections of GC tissue were taken from the Department of Pathology, First Hospital of Lanzhou University. The patient's clinical data, pathological results, and follow-up data were all recorded in detail. Except for stage Ia patients with GC, patients were given postoperative chemotherapy regimens of 6 cycles of XELOX. A total of 25 healthy controls were selected from the Department of Gastroenterology, who required gastroscopy to exclude digestive system tumours and diseases, including 16 males and 9 females, with an average age of 40 years. The follow-up of patients with GC was performed by a specialist. Overall survival time was measured from the date of surgery to the date of death due to any cause. All postoperative cases were followed for 3-60 mo. All research complied with the "Methods for Ethical Review of Biomedical Research Involving Human Beings (Trial)" and the Declaration of Helsinki. Prior written informed consent was obtained from every subject, and the study was approved by the Ethics Review Board of the First Hospital of Lanzhou University.

Immunohistochemistry
Protein expression of HPA and MAPK was detected by immunohistochemical staining (the SP method) in GC and para-carcinoma tissues. Formaldehyde fixed and paraffin embedded sections of samples were dewaxed, rehydrated with different concentrations of alcohol,

and prepared for antigen retrieval with citrate by the high-temperature and high-pressure method. Primary antibodies were added to the sections and incubated at 37 ℃ in the dark for 1 h. Then, secondary antibodies were added for 30 min. Next, DAB chromogenic reagent was added to develop and hematoxylin was added to stain. The working concentrations of the primary antibodies against HPA and MAPK were both 1:200, and the negative control group used PBS instead of the primary antibody. Positive staining for HPA and MAPK was both primarily located in the cytoplasm. The semi-quantitative scoring criteria were as follows: according to the percentage of positive cells and staining intensity of each slice, the positive staining cell ratio was recorded as 0 points, 1 point, 2 points and 3 points for < 5%, 5% to 25%, 26% to 50%, and > 50%, respectively; the staining intensity was defined as 0 points, 1 point, 2 points and 3 points for no staining, pale yellow, brownish yellow and tan, respectively.

H. pylori infection status
To identify the infection status of *H. pylori* in GC and adjacent tissues, immunohistochemistry was used to test for *H. pylori* infection with a rabbit polyclonal anti-*H. pylori* antibody (DAKO). All diagnosed GC patients underwent a C13 breath test before surgery to check for *H. pylori* infection. If the clinical C13 exhalation test and immunohistochemical staining of the surgical pathology section were both confirmed to be positive for *H. pylori*, the patient was defined as *H. pylori* positive.

Statistical analysis
Data were analysed using SPSS 22.0 statistical software (IBM, Amonk, NY, United States). The classified data are described as the number of cases and rate or constituent ratio (%). The Chi-square test was used to compare classified disordered data groups. The Kruskal-Wallis H test was used to compare the classified ordered data. Association analysis between *H. pylori* infection and the expression of HPA and MAPK was analysed by the chi-square test, and the contingency coefficient was calculated. The survival rates of the different groups were analysed by the Kaplan-Meier method and log-rank (mantel-COX) test. Univariate and multivariate COX
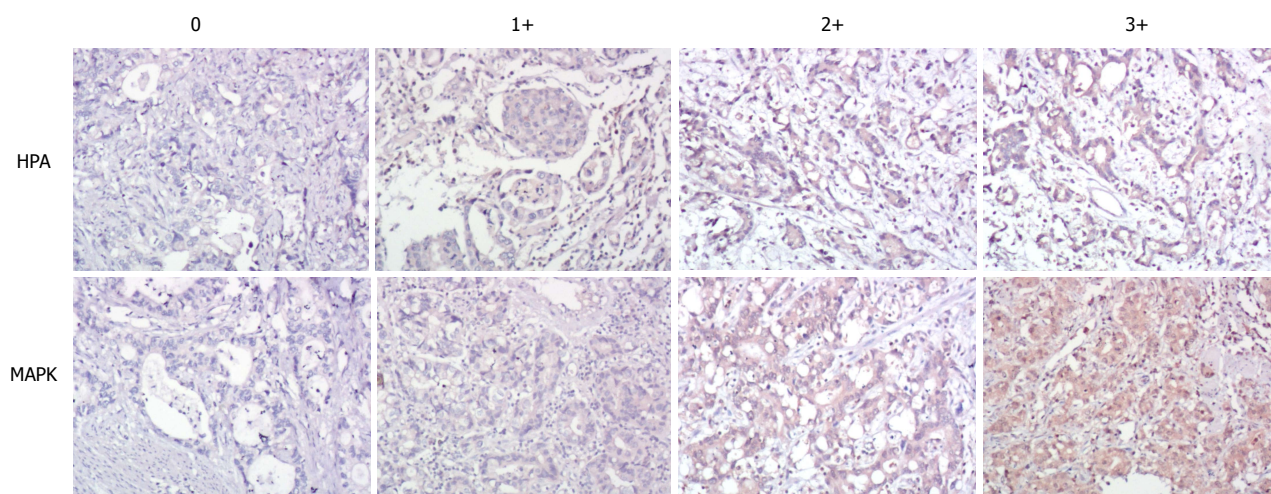
Figure 1 Immunohistochemical analysis of heparanase and mitogen-activated protein kinase protein expression in gastric cancer. Expression of heparanase (HPA) and mitogen-activated protein kinase (MAPK) was detected by immunohistochemical staining in normal gastric tissue. Representative immunohistochemical staining images are shown (magnification, 200 ×). HPA: Heparanase; MAPK: Mitogen-activated protein kinase.

regression analyses were used to explore the influencing factors of the survival time. *P* < 0.05 was considered statistically significant.

RESULTS

HPA and MAPK protein expression in GC tissue is higher than that in para-carcinoma tissue and normal gastric tissue

To detect HPA and MAPK protein expression in GC, immunohistochemical staining was carried out. Representative results from the immunohistochemical staining are shown in Figure 1 and detailed data are shown in Table 1. As shown in Figure 1, HPA and MAPK were not basally expressed in normal gastric tissue and were seldom expressed in para-carcinoma tissue; however, expression of HPA and MAPK was significantly positive in GC tissue. As seen in Table 1, HPA and MAPK were not basally expressed in normal gastric tissue, but expression of HPA and MAPK was positive in GC and para-carcinoma tissues, and expression of HPA and MAPK in GC tissue was significantly higher than that in para-carcinoma and normal tissues.

H. pylori infection in GC is relevant to the expression of HPA and MAPK and leads to higher expression than that in normal gastric tissue

To detect the *H. pylori* infection levels, the clinical C13 exhalation test and immunohistochemical staining of surgical sections were used. There were 70 cases of *H. pylori* infection out of 99 GC cases, with a positive rate of 70.71%, and 10 cases of *H. pylori* infection out of 25 healthy control cases, with a positive rate of 40.0%. There was a significant difference between the levels of *H. pylori* infection in GC and those of normal gastric tissue (*P* < 0.05), as revealed in Table 2. This result suggests that there is a significant correlation between *H. pylori* infection and GC.

Correlation between H. pylori infection, expression of HPA and MAPK proteins, and pathological characteristics of GC

To ascertain the effects of *H. pylori* infection and HPA and MAPK protein expression in GC, the associations among the clinical pathological characteristics of gastric cancer and the above factors were analysed. Positive *H. pylori* infection and positive expression of HPA were both associated with lymph node metastasis (*P* < 0.05), but not with age, gender, diameter of tumour, or differentiation degree (*P* > 0.05). Similarly, positive HPA expression and positive MAPK expression were both associated with the depth of invasion (*P* < 0.05), as shown in Table 3.

There were 54 cases of HPA positive expression in 70 *H. pylori* positive GC cases, with a positive rate of 54.5%, and 15 cases of HPA positive expression in 29 *H. pylori* negative GC cases, with a positive rate of 15.2%. Obviously, significantly higher levels of HPA positive expression in *H. pylori* positive GC cases were observed than those in *H. pylori* negative GC cases (*P* < 0.05), as shown in Table 4. This result suggests that there is significant HPA expression in GC with *H. pylori* infection.

There were 40 cases with MAPK positive expression in 70 *H. pylori* positive GC cases, with a positive rate of 40.4%, and 10 cases of MAPK positive expression in 29 *H. pylori* negative GC cases, with a positive rate of 10.1%. Significantly higher levels of MAPK positive expression in *H. pylori* positive GC cases were observed than those in *H. pylori* negative GC cases (*P* < 0.05), as shown in Table 5.

There were 40 cases of MAPK positive expression in 69 HPA positive GC cases, with a positive rate of 39.4%, and 10 cases of MAPK positive expression in 30 HPA negative GC cases, with a positive rate of 11.1%. Significantly higher MAPK positive expression in HPA positive GC cases were observed than those in HPA negative GC cases (*P* < 0.05), as shown in Table 6. This result suggests that there is a significant correlation between HPA expression and MAPK expression in GC.

Table 2 Correlation between *Helicobacter pylori* infection and gastric cancer *n* (%)

H. pylori infection	Gastric cancer (*n* = 99)	Normal tissue (*n* = 25)	χ^2	*P* value	C
Negative	29 (29.29)	15 (60.00)	8.221	0.004	0.229
Positive	70 (70.71)	10 (40.00)			

H. pylori: Helicobacter pylori.

Table 3 Correlation analyses of *Helicobacter pylori* infection, heparanase and mitogen-activated protein kinase expression, and gastric cancer pathological characteristics *n* (%)

Parameter	Cases (*n*)	*H. pylori* infection		*P* value	HPA expression		*P* value	MAPK expression		*P* value
		Positive	Negative		Positive	Negative		Positive	Negative	
Age (yr)										
< 60	46	32 (32.3)	14 (14.1)	0.816	31 (31.3)	14 (14.1)	0.8731	22 (22.2)	24 (24.2)	0.619
≥ 60	53	38 (38.4)	15 (15.2)		38 (38.4)	16 (16.2)		28 (28.3)	25 (25.3)	
Gender										
Male	61	46 (46.5)	15 (15.2)	0.193	42 (42.4)	18 (18.2)	0.935	27 (27.3)	34 (34.3)	0.115
Female	38	24 (24.2)	14 (14.1)		27 (27.3)	12 (12.1)		23 (23.2)	15 (15.2)	
Lymph node metastasis										
Without	42	24 (24.2)	18 (18.2)	0.011	23 (23.2)	19 (19.2)	0.006	24 (24.2)	18 (18.2)	0.257
With	57	46 (46.5)	11 (11.1)		46 (46.5)	11 (11.1)		26 (26.3)	31 (31.3)	
Tumour diameter										
< 40 mm	57	40 (40.4)	17 (17.2)	0.892	39 (39.4)	17 (17.2)	0.989	25 (25.3)	32 (32.3)	0.123
≥ 40 mm	42	30 (30.3)	12 (12.1)		30 (30.3)	13 (13.1)		25 (25.3)	17 (17.2)	
Differentiation degree										
Poorly	40	28 (28.3)	12 (12.1)	0.899	26 (26.3)	14 (14.1)	0.402	23 (23.2)	17 (17.2)	0.252
Highly and moderately	59	42 (42.4)	17 (17.2)		43 (43.3)	16 (16.2)		27 (27.3)	32 (32.3)	
Invasive depth										
T1/2	39	31 (31.3)	8 (8.1)	0.122	33 (33.3)	6 (6.1)	0.009	24 (24.2)	15 (15.2)	0.077
T3/4	60	39 (39.4)	21 (21.2)		36 (36.4)	24 (24.2)		26 (26.3)	34 (34.3)	

HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

Table 4 Correlation between heparanase expression and *Helicobacter pylori* infection in gastric cancer

H. pylori infection	HPA expression		χ^2	*P* value	C
	Negative	Positive			
Negative	14 (14.1)	15 (15.2)	6.273	0.012	0.244
Positive	16 (16.2)	54 (54.5)			

HPA: Heparanase; *H. pylori*: *Helicobacter pylori*.

H. pylori mediates the increase of HPA expression in MKN-45 cells via the MAPK signalling pathway

To detect the effect of *H. pylori* infection on HPA expression in GC cells, *H. pylori* and MKN-45 were co-cultured at a ratio of 100 bacteria: 1 cell for 0 h, 6 h, 12 h, 24 h, and 48 h. Western blot assay confirmed the enhancement of HPA expression at the protein level, and this level peaked at 24 h in *H. pylori*-infected GC cells (Figure 2A and B). To illustrate whether MAPK signalling is involved in *H. pylori*-induced expression of HPA, the expression of phosphorylated p38 MAPK (p-p38MAPK) was detected by Western blot analysis when *H. pylori* and MKN-45 were co-cultured for 0 min, 30 min, 60 min, 120 min, and 480 min. The expression of p-p38MAPK was significantly higher after 30 min and peaked at 60 min, whereas the total amount of p38MAPK remained unchanged (Figure 2C and D). To further illustrate

whether *H. pylori*-induced upregulation of HPA is mediated through the MAPK pathway, 20 μmol/L of the MAPK inhibitor SB203580 was added to MKN-45 cells for 2 h before they were cultured with *H. pylori*. The expression of HPA protein was significantly higher when *H. pylori* infected MKN-45 cells, but that upregulation was significantly inhibited by SB203580 (Figure 2E and F). The Transwell invasion (Figure 2G and H) and scratch test migration (Figure 2I and J) assays confirmed that the addition of SB203580 to *H. pylori*-infected MKN-45 cells markedly decreased the invasion and migration abilities of MKN-45 cells.

H. pylori infection, HPA, and prognosis

HPA positive expression in GC significantly predicted poor overall survival (*P* = 0.000) and poor relapse-free survival (*P* = 0.006). Especially in *H. pylori*-infected GC, HPA positive expression was a more significant factor for predicting poor prognosis (overall survival, *P* = 0.000; relapse-free survival, *P* = 0.007) (Figure 3).

H. pylori infection, MAPK, and prognosis

MAPK positive expression in GC cannot predict overall survival (*P* = 0.063) or relapse-free survival (*P* = 0.163). However, in *H. pylori*-infected GCs, MAPK positive expression was a relatively significant factor for predicting poor prognosis (overall survival, *P* = 0.007), but did not

Table 5 Correlation between mitogen-activated protein kinase expression and *Helicobacter pylori* infection in gastric cancer

| H. pylori infection | MAPK expression | | χ^2 | P value | C |
	Negative	Positive			
Negative	19 (19.2)	10 (10.1)	4.212	0.04	0.202
Positive	30 (30.3)	40 (40.4)			

MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

Table 6 Correlation between heparanase expression and mitogen-activated protein kinase expression in gastric cancer

| HPA expression | MAPK expression | | χ^2 | P value | C |
	Negative	Positive			
Negative	20 (19.2)	10 (11.1)	5.077	0.024	0.221
Positive	29 (30.3)	40 (39.4)			

HPA: Heparanase; MAPK: Mitogen-activated protein kinase.

predict relapse-free survival (*P* = 0.557) (Figure 4).

Prognostic analysis using the COX regression model
Univariate COX regression analysis: By applying the COX regression model to single factor prognostic analysis, a stepwise regression approach was used. The results showed that there was no correlation between gender, age, and tumour diameter. However, lymph node metastasis in GC patients had a significant influence on prognosis (*P* = 0.003). The degree of histological differentiation had a positive impact on the prognosis of patients with GC (*P* = 0.003); the tumour invasion depth was also significantly associated with the prognosis of GC patients (*P* = 0.015). The level of HPA expression also had a very important role in the prognosis of GC patients (*P* = 0.000), and MAPK expression also had an influence on the prognosis of GC patients (*P* = 0.031). Whether *H. pylori* infection was present in patients with GC had no correlation with prognosis (*P* = 0.849), but *H. pylori* infection may play an important role in the initiation of GC. Therefore, the univariate COX regression analysis showed that lymph node metastasis, tissue differentiation, depth of invasion, and HPA expression in GC tissues were significantly correlated with the prognosis of patients (Table 7).

Multivariate COX regression analysis: The COX regression multivariate model was used for analysis. The results showed that age, sex, tumour diameter, MAPK expression, and *H. pylori* infection were all removed from the model and that lymph node metastasis, tissue differentiation, depth of invasion, and HPA expression were independent prognostic factors that affected the overall survival rate of GC patients. The relative risk of lymph node metastasis in GC tissues [hazard ratio (HR) = 4.443, *P* = 0.000] indicated that there was a higher risk of death (increased by 6.52 times) in GC patients with lymph node metastasis compared with those

without. The lower the tissue differentiation of patients with GC, the higher the risk of death (HR = 3.452, *P* = 0.013). The greater the invasion of GC, the higher the risk of death [risk ratio (RR) = 2.542, *P* = 0.015]. The higher the expression of HPA protein in GC tissues, the shorter the survival time of patients (RR = 2.463, *P* = 0.015) (Table 8)

DISCUSSION

HPA has been documented in many primary human tumours[26-28], including GC[29], and is known to have multiple vital functions in accelerating tumour growth, angiogenesis, and tumour metastasis[30-32]. MAPK, including JNK, ERK, and p38 kinase, plays pivotal roles in proliferation, invasion, and migration of cancer cells[33-36]. There are many studies[37-39] that have shown that *H. pylori* infection leads to increased p38MAPK in GC cells, and it has also been shown[40] that p38MAPK leads to HPA elevation. This study showed that HPA and MAPK expression was significantly higher in patients with GC than that in para-carcinoma and normal gastric tissues. Our research also confirmed that HPA was highly expressed in GC cells, as has been previously reported in the literature[41,42]. To explain the correlation between HPA and MAPK expression and the clinical pathology of GC cases, we first investigated the clinicopathological characteristics. Positive expression of HPA was associated with lymph node metastasis and depth of invasion, but not with age, gender, tumour diameter, or differentiation degree. In addition, positive MAPK expression was only associated with depth of invasion, which illustrates that HPA is involved in the invasion and metastasis of GC and that MAPK is primarily involved in the proliferation of GC cells. This research also suggests that there is a significant correlation between HPA expression and MAPK expression in GC.

H. pylori, as a class I carcinogen, causes invasion, proliferation, and metastasis of GC cells[43,44], but its specific mechanism of action remains unclear. It has been reported[45,46] that infections can cause a HPA elevation. *H. pylori*, as a bacterium, is associated with various human gastric diseases, especially gastritis and GC, but there has been no evaluation of whether *H. pylori* infection causes an increase in HPA in GC, which would contribute to the invasion and metastasis of GC. In the present study, it was revealed that *H. pylori* infection is significantly associated with HPA expression and that a positive *H. pylori* infection is connected to lymph node metastasis. To further elucidate the effect of *H. pylori* infection leading to heparanase elevation in GC, MKN-45 GC cells were infected by *H. pylori*. We showed that HPA expression was the highest at 24 h post *H. pylori* infection in these GC cells and the abilities of invasion and metastasis were increased when GC cells were infected by *H. pylori*.

There are many studies[37-39] showing that *H. pylori* infection leads to increased p38MAPK in GC cells, and it has been shown that[40] p38MAPK leads to elevation of
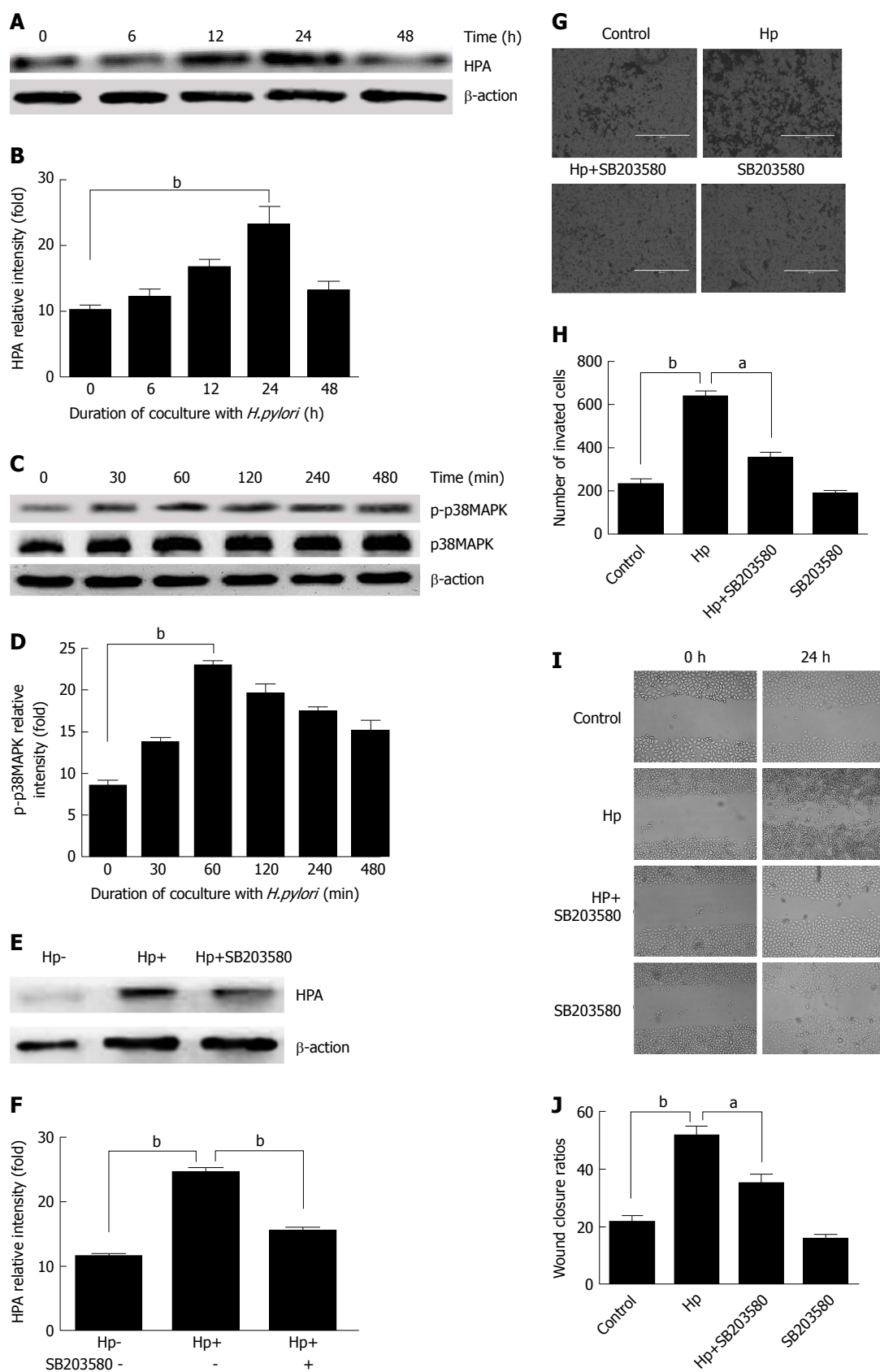
Figure 2 Heparanase protein expression following *Helicobacter pylori* infection in MKN-45 gastric cancer cells *via* the mitogen-activated protein kinase signaling pathway. A: Heparanase (HPA) expression was determined by Western blot at 0, 6, 12, 24, and 48 h after *Helicobacter pylori* (*H. pylori*) infection; B: Quantitative Western blot results of HPA; C: p-p38MAPK expression was determined by Western blot at 0, 30, 60, 120, and 480 min after *H. pylori* infection; D: Quantitative Western blot results of p-p38MAPK; E: HPA expression when the MAPK inhibitor SB203580 was given to MKN-45 cells before *H. pylori* infection; F: Quantitative Western blot results of HPA when the MAPK inhibitor SB203580 was given. [b]$P < 0.01$ compared with the value at 0 h. G, H: Cell invasion rates in the three groups detected using a Transwell invasion assay. I, J: Migration rates in the three groups detected using a scratch migration assay. [a]$P < 0.05$, [b]$P < 0.01$. HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori: Helicobacter pylori*.

Table 7 Univariate COX regression analysis

Variable	Overall survival		
	HR	95%CI	*P* value
Gender (man *vs* women)	0.952	0.498-1.821	0.882
Age (≥ 60 *vs* < 60)	1.113	0.581-2.130	0.747
Tumour diameter (≥ 40 mm *vs* < 40 mm)	1.285	0.684-2.413	0.436
Lymph metastasis (yes *vs* no)	2.667	1.381-5.128	0.003
Tissue differentiation (high and medium *vs* low)	4.156	1.622-10.656	0.003
Invasive depth (T3/4 *vs* T1/2)	2.464	1.189-5.104	0.015
HPA expression (positive *vs* negative)	3.282	1.968-7.475	0.000
MARK expression (positive *vs* negative)	2.083	1.068-4.065	0.031
H. pylori (positive *vs* negative)	1.065	0.556-2.045	0.849

HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*; HR: Hazard ratio; CI: Confidence interval.
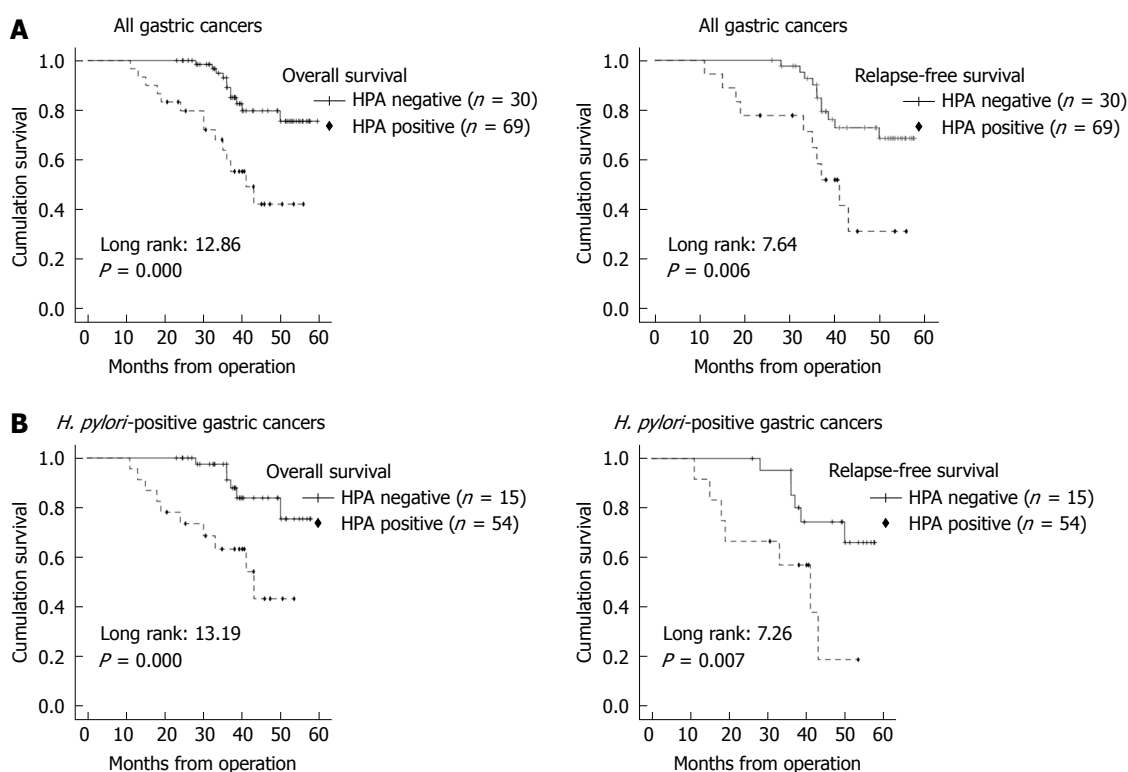
Figure 3 Kaplan-Meier survival plots for overall survival and relapse-free survival according to heparanase expression and *Helicobacter pylori* infection status. A: Heparanase (HPA) expression status (negative or positive) and the prognosis of all gastric cancer cases. HPA positive staining includes all cases of 1+, 2+, and 3+. HPA positive expression detected by immunohistochemical staining significantly predicts poor overall survival and relatively poor relapse-free survival; B: Kaplan-Meier survival according to HPA status in *Helicobacter pylori* positive gastric cancer cases. HPA positive expression significantly predicts poor overall survival as well as relapse-free survival. HPA: Heparanase; *H. pylori*: *Helicobacter pylori*.

HPA. Therefore, we hypothesized that *H. pylori* infection causes an increase in HPA in GC *via* the MAPK pathway. In this study, it was demonstrated that *H. pylori* infection was significantly associated with MAPK expression and that there was a significant correlation between HPA expression and MAPK expression in GC. In MKN-45 GC cells infected by *H. pylori*, *H. pylori* infection significantly enhanced the expression of MAPK. MAPK expression peaked at 60 min post *H. pylori* infection in MKN-45 cells. Inhibition of MAPK by SB203580 significantly decreased the expression of HPA and the invasion and metastasis of MKN-45 cells infected by *H. pylori*. Therefore, we speculate that *H. pylori* infection in GC activates MAPK

signalling, leading to the activation of HPA.

HPA expression is a poor prognostic factor in some cancers[47-49], including GC[50,51]. In the present study, it was revealed that positive expression of HPA was able to predict the malignancy of GC due to its correlation with lymphatic metastasis and invasive depth. Beyond that, positive expression of HPA was a poor prognostic factor for overall survival and relapse-free survival compared with HPA negative cases, which was consistent with previously published reports[52]. Especially in GC patients with an obvious *H. pylori* infection, HPA positive expression indicated a poorer prognosis both in overall survival and in relapse-free survival, which illustrates

Table 8 Multivariate COX regression analysis

Variable	Overall survival		
	HR	95%CI	*P* value
Lymph metastasis (yes *vs* no)	4.443	2.185-9.036	0.000
Tissue differentiation (high and medium *vs* low)	3.452	1.299-9.176	0.013
Invasive depth (T3/4 *vs* T1/2)	2.542	1.198-5.392	0.015
HPA expression (positive *vs* negative)	2.463	1.195-5.076	0.015

HPA: Heparanase; HR: Hazard ratio; CI: Confidence interval.
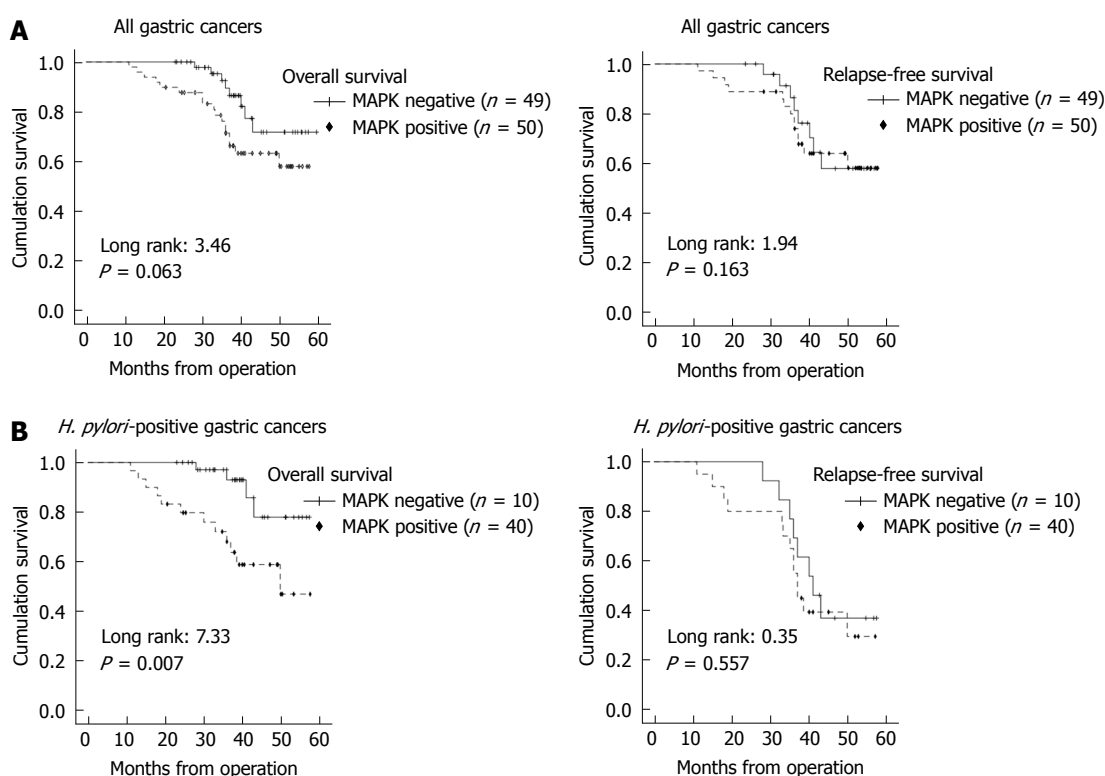
Figure 4 Kaplan-Meier survival plots for overall survival and relapse-free survival according to mitogen-activated protein kinase expression and *Helicobacter pylori* infection status. A: Mitogen-activated protein kinase (MAPK) expression status (negative or positive) and prognosis of all gastric cancer cases. MAPK positive staining includes all cases of 1+, 2+, and 3+. MAPK positive expression detected by immunohistochemical staining cannot predict overall survival or relapse-free survival; B: Kaplan-Meier survival according to MAPK status in *Helicobacter pylori* (*H. pylori*) positive gastric cancer cases. MAPK positive expression significantly predicts poor overall survival in *H. pylori* positive gastric cancer cases, but does not predict relapse free survival. MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

that HPA is an important factor for the prediction of prognosis and relapse of GC and that *H. pylori* infection leads to an increase of HPA expression, which can worsen the prognosis of GC and make recurrence more likely. Compared to HPA, positive MAPK expression only predicted prognosis in overall survival of GC patients, which was consistent with previous reports[53], but MAPK expression could not predict a relapse in *H. pylori*-infected GC. Thus, MAPK expression cannot be used to determine the prognosis of GC patients with *H. pylori* infection, but a poor prognosis of GC patients with positive HPA expression is associated with *H. pylori* positive cases, which suggests that therapy against HPA should be taken into account when GC patients are infected with *H. pylori*.

Univariate COX regression analysis showed that lymph node metastasis, degree of histological differen-

tiation, and invasive depth in GC patients had a significant influence on prognosis, which was identical to the results of previously published reports[54]. Moreover, in the univariate COX regression analysis, the levels of HPA and MAPK expression also had an influence on the prognosis of GC patients. Similarly, multivariate COX regression analysis showed that lymph node metastasis, tissue differentiation, depth of invasion, and HPA protein expression level were independent prognostic factors that affected the overall survival rate of GC patients. In addition to using the clinical characteristics to judge prognosis, such as lymph node metastasis, tissue differentiation, and depth of invasion, HPA is still an important factor as a biomarker to judge the prognosis of GC, which is consistent with the report by Takaoka *et al*[51].

In conclusion, the results of the current study demonstrate that *H. pylori* infection is not only the primary factor

involved in GC but is also involved in the invasion and metastasis of GC by upregulating HPA expression, which is likely mediated *via* activation of the MAPK signalling pathway. HPA is an important factor for predicting the prognosis and relapse of GC, and *H. pylori* infection increases HPA expression, which makes the prognosis of GC more aggressive and recurrence more likely, suggesting that therapy against HPA should be taken into consideration when GC patients are infected with *H. pylori*.

ARTICLE HIGHLIGHTS

Research background

The underlying mechanism that *Helicobacter pylori* (*H. pylori*) infection results in gastric cancer (GC) is still unknown. Heparanase (HPA) leads to the invasion and metastasis of GC. However, it is not clear whether *H. pylori* infection in GC increases HPA expression. Such finding suggests that HPA may become a therapeutic target for GC with *H. pylori* infection.

Research motivation

Although there have been increasing numbers of studies indicating that *H. pylori* infection results in GC, the underlying mechanism is still unknown. HPA is expressed in many tumours and leads to the invasion and metastasis of tumour, especially in GC. *H. pylori* infection can induce the development of GC by activating mitogen-activated protein kinase (MAPK) which is closely related to the expression of HPA. However, it is not clear whether MAPK is involved in the regulation of HPA expression following *H. pylori* infection that leads to GC.

Research objectives

To detect the mechanisms of *H. pylori* infection in the invasion and metastasis of GC.

Research methods

Immunohistochemistry method was used to detect *H. pylori* infection and HPA and MAPK expression in GC tissue, and their association with the clinical features of GC was analysed with SPSS 22.0. Kaplan-Meier method and COX proportional models were used to analyse prognosis. HPA and MAPK expression in MKN-45 cells infected with *H. pylori* was analysed using Western blot.

Research results

This study demonstrates that *H. pylori* infection increases HPA expression in GC, which is likely mediated *via* activation of the MAPK signaling pathway.

Research conclusions

The current study shows that *H. pylori* infection is involved in the invasion and metastasis of GC by upregulating HPA expression, which is likely mediated *via* activation of the MAPK signaling pathway. HPA is an important factor for predicting the prognosis and relapse of GC with *H. pylori* infection.

Research perspectives

HPA may become a therapeutic target for GC with *H. pylori* infection.

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