Molecular Therapy
Oncolytics
Original Article

Helicobacter pylori promotes gastric cancer progression by upregulating semaphorin 5A expression via ERK/MMP9 signaling

Guoqing Pan,[1,4] Xianwen Wang,[2,4] Yingxia Wang,[1,4] Rui Li,[1] Geng Li,[1] Ying He,[1] Shiyue Liu,[1] Yonghui Luo,[1] Liqiong Wang,[3] and Zi Lei[1]

[1]Department of Pathology, The First Affiliated Hospital of Kunming Medical University, No. 295 Xichang Road, Kunming, Yunnan Province 650100, P.R. China; [2]Department of Pathology, The Maternity and Child Care Hospital, Qujing, Yunnan, P.R. China; [3]Department of Pathology, Yan An Hospital, Kunming, Yunnan, P.R. China

***Helicobacter pylori* (*H. pylori*) infection is the strongest risk factor for the occurrence and development of gastric carcinoma. However, the molecular mechanism underlying *H. pylori*-induced pathogenesis has not yet been fully characterized. Here, we explored whether *H. pylori* upregulates semaphorin 5A to promote gastric cancer progression via the extracellular regulated protein kinases/matrix metalloproteinase (ERK/MMP9) signaling pathway. In this study, *H. pylori* upregulated semaphorin 5A expression *in vitro* and *in vivo*. Using the human gastric carcinoma cell lines SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A, our studies showed that *H. pylori* increased the proliferation, growth, migration, and invasiveness of gastric cancer cells via its effects on semaphorin 5A and that *H. pylori* increased the expression of MMP9 in gastric cancer cells via the semaphorin 5A-mediated ERK signaling pathway. Further analysis revealed that the ERK inhibitor PD98059 and MMP9 antibody (Ab) attenuated *H. pylori*-induced gastric cancer cell invasion and metastasis *in vitro* through a semaphorin 5A-dependent mechanism. In conclusion, *H. pylori* could promote gastric cancer progression in a semaphorin 5A-dependent manner via the ERK/MMP9 signaling pathway. Semaphorin 5A and its related signaling molecules potentially represent latent targets for *H. pylori*-related gastric cancer therapy.**

INTRODUCTION

Gastric cancer is one of the most common malignant tumors and seriously threatens human health. Based on global cancer statistics in 2018, an estimated 1,030,000 new stomach cancer cases were diagnosed, and 782,685 deaths occurred.[1] Although great achievements have been made in gastric cancer research and clinical treatment in recent years, patients with advanced gastric cancer have a poor prognosis, with a 5-year survival rate lower than 20%.[2,3] Therefore, the identification of risk factors is of great clinical value to understand their pathogenic effects.

Helicobacter pylori (*H. pylori*) is an important pathogen contributing to the occurrence and progression of gastric cancer. Chronic infection with *H. pylori* leads to atrophic gastritis, along with the development of intestinal metaplasia, dysplasia, and gastric cancer. Therefore, *H. pylori* has been defined as a class I carcinogenic factor in the human stomach by the World Health Organization (WHO) since 1994.[4–6] However, the mechanism underlying the *H. pylori*-induced pathogenesis of gastric cancer remains to be fully elucidated.

Semaphorin 5A, a class 5 semaphorin, is an integral membrane protein with seven characteristic thrombospondin-specific repeats (TSP-1). Initially, the gene was identified to play a crucial role in the guidance of growing axons to their targets during the development of the central nervous system.[7–11] As shown in our previous studies, semaphorin 5A is overexpressed in gastric cancer tissue, which may be closely related to the tumorigenesis and metastasis of gastric cancer.[12–14] In our recent study, semaphorin 5A expression was identified as a potentially important contributor to the occurrence and development of *H. pylori*-related gastric precancerous lesions.[15] However, researchers have not yet determined whether semaphorin 5A is associated with *H. pylori*-related gastric carcinoma. In the present study, we investigated the role of semaphorin 5A in *H. pylori*-induced gastric carcinogenesis and identified a novel intracellular signaling pathway involving semaphorin 5A in *H. pylori*-mediated pathogenesis.

RESULTS

H. pylori Infection is related to the clinicopathological features of gastric cancer

We first observed *H. pylori* colonization in 200 gastric cancer samples to explore the relationship between *H. pylori* infection and the

Received 14 December 2020; accepted 1 June 2021;
https://doi.org/10.1016/j.omto.2021.06.002.

[4]These authors contributed equally

Correspondence: Guoqing Pan, Department of Pathology, The First Affiliated Hospital of Kunming Medical University, No. 295 Xichang Road, Kunming, Yunnan Province 650100, P.R. China.
E-mail: guoqing_pan@163.com
Correspondence: Liqiong Wang, Department of Pathology, Yan An Hospital, Kunming, Yunnan, P.R. China.
E-mail: 1434660283@qq.com
Correspondence: Zi Lei, Department of Pathology, The First Affiliated Hospital of Kunming Medical University, No. 295 Xichang Road, Kunming, Yunnan Province 650100, P.R. China.
E-mail: 11648602@qq.com

Table 1. Correlations of *H. pylori* infection and clinicopathological features of gastric cancer patients

Clinicopathological factors	n	*H. pylori* positive (n = 101)	*H. pylori* negative (n = 99)	p
Sex				
Female	98	53	45	0.197
Male	102	48	54	
Age (years)				
≥65	110	54	56	0.383
<65	90	47	43	
Lauren classification				
Intestinal	100	63	37	<0.001
Diffuse	100	38	62	
Depth of invasion				
T1–T2	103	72	31	<0.001
T3–T4	97	29	68	
Lymph metastasis				
Negative	131	54	77	<0.001
Positive	69	47	22	
TNM stage				
I–II	124	46	78	<0.001
III–IV	76	55	21	

clinicopathological features of patients with gastric cancer. As shown in Table 1, H. *pylori* colonization was present in 50.5% (101/200) of gastric carcinoma specimens. An analysis of the interrelation between *H. pylori* infection and the clinicopathological features showed that *H. pylori* infection in patients with gastric cancer was not related to sex or age, while it was associated with the Lauren classification, depth of invasion, TNM stage, and lymph node metastasis (Table 1). These results imply that *H. pylori* infection is correlated with an advanced stage of gastric cancer.

Semaphorin 5A expression in gastric cancer tissues correlates with the *H. pylori* infection status

We first investigated semaphorin 5A expression in *H. pylori*-infected gastric cancer tissues to explore the mechanism underlying *H. pylori*-induced gastric carcinogenesis. The expression of the semaphorin 5A protein was evaluated in 200 human gastric cancer specimens using immunohistochemistry (IHC). IHC staining of gastric carcinoma specimens showed that the mean staining intensity of semaphorin 5A was higher in *H. pylori*-positive gastric cancer than in *H. pylori*-negative tissues (Figures 1A and 1B; Table 2). Our results suggest that *H. pylori* infection upregulates semaphorin 5A expression, which might play a crucial role in *H. pylori*-induced gastric cancer progression.

Effect of *H. pylori* on semaphorin 5A mRNA and protein levels in SGC7901 and MKN28 cells

We investigated the effect of *H. pylori* infection on semaphorin 5A expression in gastric cancer cells to further confirm semaphorin 5A upregulation in *H. pylori*-infected gastric cancer. After 0, 12, and 24 h of incubation with *H. pylori*, differential upregulation of the semaphorin 5A mRNA was observed in SGC7901 and MKN28 cells (Figure 2A). To evaluate whether *H. pylori*-mediated upregulation of semaphorin 5A also occurred at the protein level, we measured semaphorin 5A expression at 12 and 24 h in *H. pylori*-stimulated or untreated cells using western blotting (WB) (Figure 2B). *H. pylori* significantly increased levels of the semaphorin 5A protein in both gastric cancer cell lines. These data further show that *H. pylori* upregulates semaphorin 5A expression.

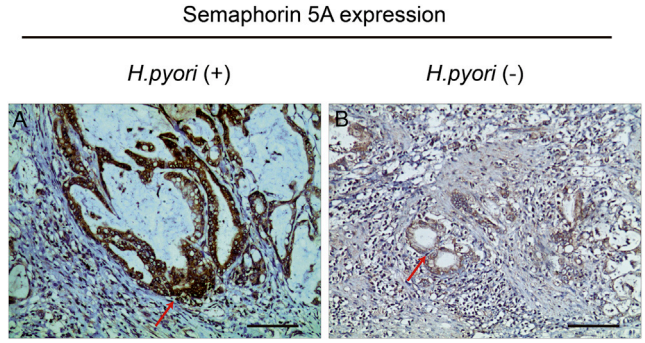

Figure 1. Immunohistochemical (IHC) staining for semaphorin 5A in gastric cancer tissues.

(A) Semaphorin 5A expression in *H. pylori*-positive gastric cancer tissues. (B) Semaphorin 5A expression in *H. pylori*-negative gastric cancer tissues. The red arrow indicates positive staining. Scale bars,100 μm. Note: Samples were obtained from 200 patients who underwent surgical resection for gastric cancer with no preoperative chemotherapy or radiotherapy between March 2019 and July 2020 at the Department of Surgery in the First Affiliated Hospital of Kunming Medical University (P.R. China). By observing the status of *H. pylori* infection, the 200 cases were divided into an infection-positive group and an infection-negative group. All samples underwent Sema5A IHC staining and semiquantitative scoring. Semaphorin 5A expression was associated with *H. pylori* infection ($p < 0.001$) (Table 2).

H. pylori infection upregulates semaphorin 5A expression in an experimental rat gastric *H. pylori* infection model

A Sprague-Dawley rat model colonized with *H. pylori* was established to further assess the relationship between semaphorin 5A expression and *H. pylori* infection. The rapid urease test results confirmed that rats in the infection group were infected with *H. pylori* (Figure S1). The gastric mucosa of the rats in the infection group showed chronic inflammatory cell infiltration (Figure 3B), but marked histological changes were not observed in the control group (Figure 3A). Modified Giemsa and Warthin-Starry staining revealed a number of bacteria that colonized the

Table 2. Semaphorin 5A expression is associated with *H. pylori* infection

	Semaphorin 5A expression						
Variable	0	+	++	+++	Total	χ^2	p
H. pylori-positive group	6	18	34	43	101	71.51	<0.001
H. pylori-negative group	17	63	16	3	99		

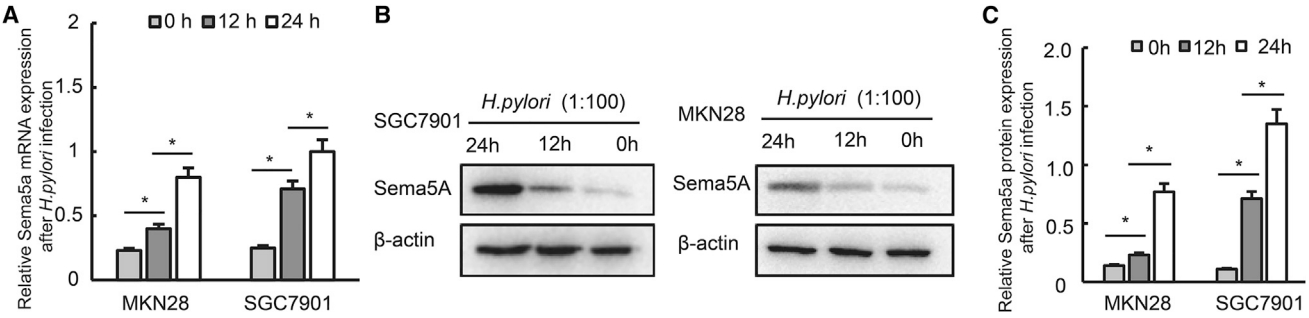

Figure 2. *H. pylori* infection upregulates semaphorin 5A expression levels in SGC7901 and MKN28 cells *in vitro*.
SGC7901 and MKN28 cells were cocultured with *H. pylori* at a ratio of 1:100 for 0, 12, and 24 h. (A) The expression of the semaphorin 5A mRNA was detected using qRT-PCR. (B) The semaphorin 5A protein expression level was detected using western blotting, and β-actin was used as an internal reference. (C) The data shown in the histogram are a summary of three independent experiments. *$p < 0.05$.

lumen of the gastric glands in the infection groups (Figures 3C and 3D), and no bacteria were detected in the control groups (Figure 3B). These phenomena indicated the successful establishment of the experimental animal model of gastric *H. pylori* infection. More importantly, semaphorin 5A was expressed at higher levels in the gastric mucosa of the infection group than in the control group, indicating that semaphorin 5A is involved in *H. pylori* infection (Figures 3E–5G).

H. pylori infection enhances the biofunctions of gastric cancer cells via its effects on semaphorin 5A *in vitro*

As shown above, semaphorin 5A is overexpressed in gastric cancer tissue and gastric cancer cells. However, we have not clearly determined whether semaphorin 5A is involved in *H. pylori*-mediated gastric cancer progression. As shown in Figure 4A, semaphorin 5A knockdown inhibited the *H. pylori*-induced increase in gastric cancer cell proliferation. Similarly, the colony-formation assay indicated that semaphorin 5A knockdown led to a decreased number of colonies after 14 days of coincubation with *H. pylori* (Figure 4B). Next, scratch and Transwell assays were performed *in vitro* to evaluate the effect of *H. pylori* on the migration and invasion of SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells. As illustrated in Figures 4C and 4D, semaphorin 5A depletion resulted in decreased cell migration and invasion induced by *H. pylori*. Together, these data indicate that *H. pylori* enhances the biological function of gastric cancer cells via its effects on semaphorin 5A *in vitro*.

The ERK signaling pathway is responsible for *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A

As reported previously, semaphorin 5A promotes the invasion and migration of gastric cancer cells via activation of the extracellular regulated protein kinase (ERK) signaling pathway. Here, we further investigated whether the ERK signaling pathway was involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A. First, we measured the effect of *H. pylori* on the levels of phosphorylated ERK1/2 in SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells after coinfection with *H. pylori* for 24 h. As shown in Figure 5A, the level of phosphorylated ERK1/2 (pERK) in *H. pylori*-stimulated SGC7901- and SGC7901-siScrambled cells was higher than that in SGC7901-siSema 5A cells, whereas the levels of the total ERK1/2 (tERK) proteins were not altered. Next, we used a Matrigel invasion chamber to determine whether an ERK inhibitor attenuates gastric cancer cell invasion induced by *H. pylori*. SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells were incubated with *H. pylori* in the presence or absence of the ERK inhibitor PD98059. As illustrated in Figure 5B, the ERK inhibitor PD98059 significantly reduced the invasion of *H. pylori*-stimulated cells. The inhibition rates of PD98059 on the invasive abilities of SGC7901- and SGC7901-siScrambled cells were higher than those of SGC7901-siSema 5A cells. Taken together, the ERK signaling pathway may be responsible for *H. pylori*-induced gastric cancer progression through a semaphorin 5A-dependent mechanism.

MMP9 was involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A

Elevated levels of matrix metalloproteinase 9 (MMP9) in tumors are generally associated with cancer cell invasiveness and metastasis.[16] Here, we explored whether MMP9 was involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A. First, the effects of *H. pylori* on the expression level of MMP9 were examined in SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells after a coincubation with *H. pylori* for 24 h. As shown in Figure 6A, the induction of MMP9 expression in SGC7901 and SGC7901-siScrambled cells was more noticeable than that in SGC7901-siSema 5A cells. Next, we performed an invasion assay to examine the effect of an MMP9 antibody (Ab) on the invasion of SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells. As illustrated in Figure 6B, treatment with the MMP9 Ab inhibited the *H. pylori*-induced invasive activities of these cells. The inhibition rates caused by the MMP9 Ab in SGC7901 and SGC7901-siScrambled cells were higher than those in SGC7901-siSema 5A cells. Taken together, our findings suggest that MMP9 is involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A.

H. pylori infection induces MMP9 expression via the semaphorin 5A-mediated activation of the ERK signaling pathway

According to our previous study, semaphorin 5A upregulates MMP9 expression in gastric cancer cells by activating the ERK signaling

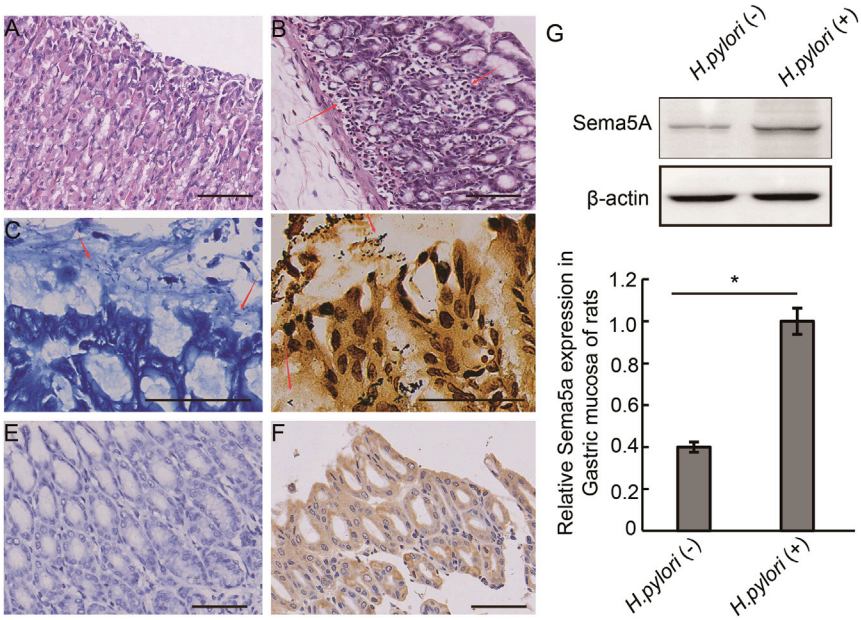

Figure 3. *H. pylori* infection upregulates semaphorin 5A expression in an experimental rat gastric *H. pylori* infection model.

(A and B) Gastric tissue sections from rats stained with hematoxylin and eosin (H&E). The gastric mucosa (B) of rats with *H. pylori* infection showed chronic inflammatory cell infiltration (red arrows). No marked histological changes were observed in the control group (A). Modified Giemsa staining (C) and Warthin-Starry staining (D) were performed to confirm *H. pylori* infection. *H. pylori* (red arrow) colonization was observed in the lumen of the gastric glands. Positive semaphorin 5A immunoreactivity was identified in the gastric mucosa of rats with *H. pylori* infection (F). Semaphorin 5A immunoreactivity was not observed in the gastric mucosa of the control group (E), which was proven by western blotting (G). The data shown in the histogram are a summary of three independent experiments. Scale bars, 100 μm. *p < 0.05.

pathway. To investigate whether activation of MMP9 by *H. pylori* infection was dependent on semaphorin 5A-mediated ERK signaling, SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells were cocultured with *H. pylori* in the presence or absence of the ERK inhibitor PD98059 to investigate whether the induction of MMP9 expression by *H. pylori* infection depended on semaphorin 5A-mediated activation of ERK signaling. As shown in Figure 7, PD98059 reduced levels of both the MMP9 protein and transcript in gastric cancer cells infected with *H. pylori*. These alterations were more significant in SGC7901 and SGC7901-siScrambled cells than in SGC7901-siSema 5A cells. Based on these data, *H. pylori* increased the expression of MMP9 by modulating semaphorin 5A expression and the subsequent activation of the ERK signaling pathway.

MMP9 and semaphorin 5A expression in gastric cancer tissues with *H. pylori* infection

We randomly collected 30 gastric cancer specimens and examined the expression of MMP9 and semaphorin 5A protein using IHC to investigate whether these phenomena occurred *in vivo*; 15 samples were *H. pylori* positive and the other 15 were *H. pylori* negative. As shown in Figures 8A–8E, the intensity of semaphorin 5A and MMP9 immunostaining was stronger in *H. pylori*-positive samples than in *H. pylori*-negative tissue specimens.

DISCUSSION

H. pylori is one of the main factors contributing to the occurrence and development of gastric carcinoma. In our study, *H. pylori* infection was associated with the progression of gastric cancer *in vivo* and promoted the migration and invasion of gastric cancer cells *in vitro*. Although a number of studies have been performed to investigate *H. pylori* pathogenesis and carcinogenesis, the detailed molecular mechanism remains largely elusive. In the present study, we provide the first evidence that *H. pylori* promotes the invasion and metastasis of gastric cancer by upregulating semaphorin 5A expression via the ERK/MMP9 signaling pathway.

Semaphorin 5A, a membrane-bound protein with seven characteristic thrombospondin-specific repeats (TSP-1), contributes to the development of the central nervous system.[9] In our previous studies, we found that semaphorin 5A plays a crucial role in the development and progression of gastric carcinoma, and that *H. pylori* infection upregulates semaphorin 5A expression in gastric precancerous lesions and normal gastric GES-1 cells.[12–15] However, we had not yet completely characterized whether semaphorin 5A is involved in *H. pylori*-related gastric cancer.

In this study, we examined the effect of *H. pylori* infection on semaphorin 5A expression in gastric cancer tissue and gastric cancer cells. Significantly higher semaphorin 5A expression was detected in *H. pylori*-positive gastric cancer tissues than in *H. pylori*-negative gastric cancer tissues, and *H. pylori* infection increased the levels of the semaphorin 5A mRNA and protein in gastric cancer cells in a time-dependent manner. In addition, we established a Sprague-Dawley rat model colonized with *H. pylori* and observed higher semaphorin 5A expression in the gastric mucosa of the *H. pylori* infection group than in the control group, indicating that semaphorin 5A expression is involved in *H. pylori* infection. Together, these observations show that semaphorin 5A activation may be involved in *H. pylori* pathogenesis. However, the mechanism underlying the biological function of semaphorin 5A in *H. pylori*-mediated gastric cancer progression remains to be determined.

In the present study, we performed *in vitro* experiments using gastric cancer cells cocultured with *H. pylori*, which may contribute to the

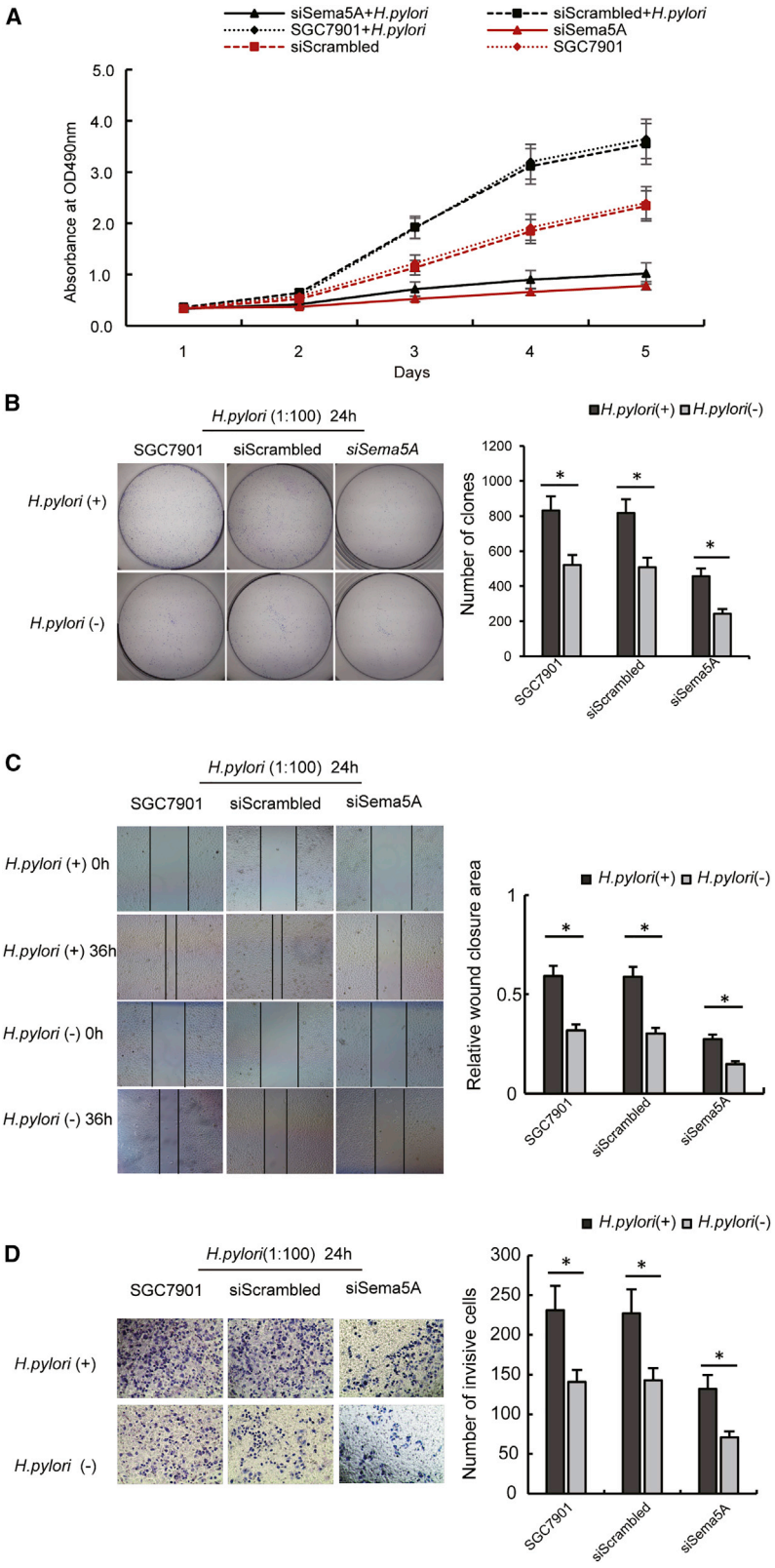

Figure 4. *H. pylori* infection increases the proliferation, growth, migration, and invasion of gastric cancer cells via semaphorin 5A.

SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells were treated with *H. pylori* at a ratio of 1:100 for 24 h. (A) Cell proliferation was detected using MTT assays and is shown in a line chart. (B) Cells were incubated for 14 days in colony-formation assays before colonies were fixed and stained with 0.1% crystal violet. (C) Wound-healing assays were conducted to evaluate the migration of cells after *H. pylori* infection *in vitro*. Photos were captured at 0 and 36 h at 100× magnification. (D) The invasion of gastric cancer cells was evaluated by performing a Matrigel invasion assay. Penetrating cells were stained with hematoxylin, and pictures were captured at 200× magnification. The data shown in the histogram are a summary of three independent experiments. *$p < 0.05$.

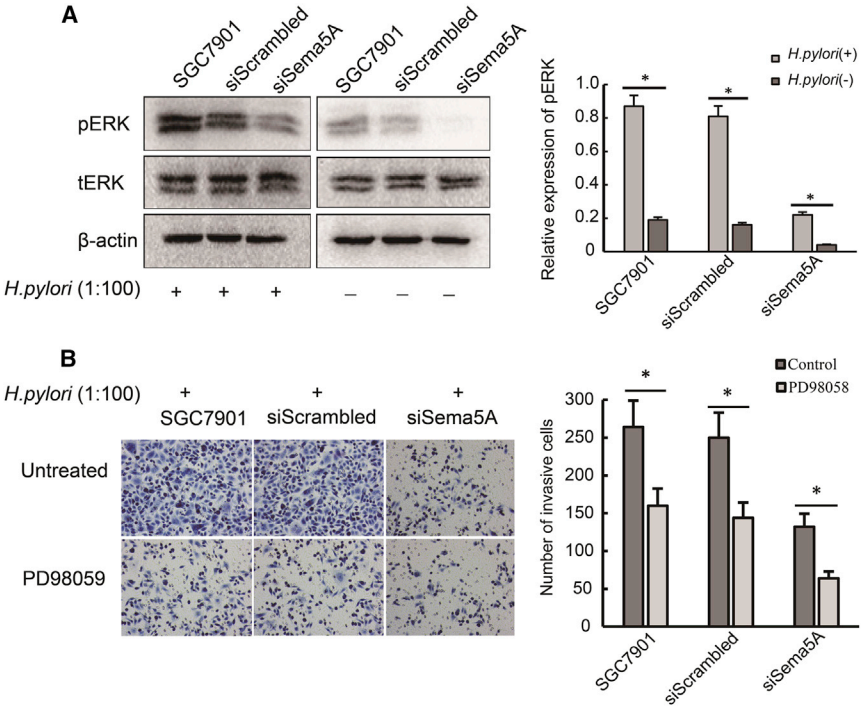

Figure 5. The ERK signaling pathway is responsible for *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A.

SGC7901, SGC7901-siScrambled, and SGC7901-siSema5A cells were cocultured with *H. pylori* at a ratio of 1:100 for 24 h. (A) The effect of *H. pylori* infection on the levels of pERK and tERK was detected using western blotting, and β-actin was used as an internal reference. (B) The ERK inhibitor PD98059 inhibited the invasion of *H. pylori*-infected SGC7901, SGC7901-siScrambled, and SGC7901-siSema5A cells in a semaphorin 5A-dependent manner. The data shown in the histogram are a summary of three independent experiments. *$p < 0.05$.

carcinogenic process. Knockdown of semaphorin 5A attenuated the *H. pylori*-induced proliferation, growth, invasion, and migration of gastric cancer cells. These results suggest that semaphorin 5A may play an important role in the development and progression of *H. pylori*-related gastric cancer. However, the exact mechanism(s) remain(s) unclear.

Matrix degradation by MMPs is critical for tumor cell invasion and metastasis. In this study, *H. pylori* induced MMP9 expression in SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells. In addition, the *in vitro* assay showed that the blockade of MMP9 with an antibody partially reversed the effect of *H. pylori* on increasing the invasion of SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells. Therefore, MMP9 was involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A.

The ERK signaling pathway has been shown to be an important mediator of tumor cell invasion and migration.[17,18] Therefore, we wondered whether the ERK signaling pathway was responsible for *H. pylori*-mediated gastric cancer progression. Using the human gastric cancer cell lines SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A, we found that *H. pylori* increased ERK1/2 phosphorylation, but the addition of PD98059 reduced the ability of *H. pylori* to induce MMP9 expression, migration, and invasion. Consistent with the results obtained from gastric cancer cells, MMP9 expression positively correlated with semaphorin 5A expression in gastric cancer tissues.

Taken together, *H. pylori* promotes the migration and invasion of gastric cancer by upregulating semaphorin 5A expression via the ERK/MMP9 signaling pathway.

To the best of our knowledge, this study is the first to document the functional roles of semaphorin 5A and its molecular mechanism in *H. pylori*-mediated gastric cancer progression, which not only reveals a novel function of semaphorin 5A outside the nervous system but also improves our knowledge of semaphorin 5A. Moreover, *H. pylori* is deemed to be an important contributing factor to the development of gastric cancer. Therefore, this study may provide useful information for the prevention and effective treatment strategies for gastric cancer.

MATERIALS AND METHODS

Human gastric cancer tissue specimens

Tissue specimens were collected from patients with gastric cancer between March 2019 and May 2020 at the Department of Pathology, the First Affiliated Hospital, Kunming Medical University, Kunming, China. The patients were diagnosed by a pathological examination and had not received chemotherapy and radiation therapy before tumor resection. They all provided consent for the use of their tissue specimens for clinical research, which was approved by our University Ethics Committee. The pathological diagnosis was classified based on the diagnostic criteria formulated by the WHO. Of the patients, 101 were confirmed to be *H. pylori*-positive by hematoxylin-eosin staining and IHC.

Cell culture

The human gastric cancer cell lines MKN28, SGC7901, and SGC7901-siScrambled, which was transfected with the scrambled plasmid stably expressing semaphorin 5A, and SGC7901-siSema 5A, which was transfected with siRNA plasmid stably expressing semaphorin 5A siRNA,[12] were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

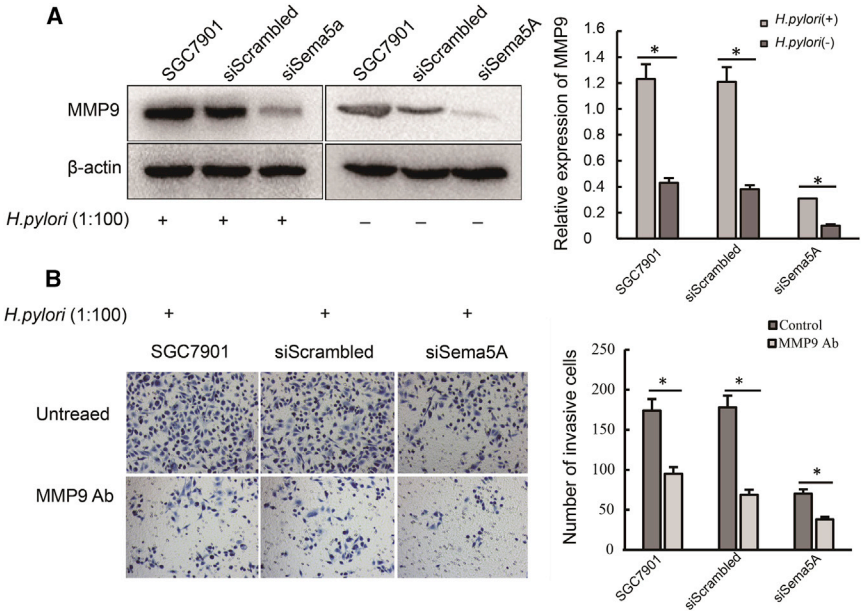

Figure 6. MMP9 is involved in *H. pylori*-induced gastric cancer progression mediated by semaphorin 5A.

SGC7901, SGC7901-siScrambled, and SGC7901-siSema5A cells were cocultured with *H. pylori* at a ratio of 1:100 for 24 h. (A) *H. pylori* upregulated the expression of the MMP9 protein level in gastric cancer cells. (B) An invasion assay using MMP9 Ab-treated SGC7901, SGC7901-siScrambled, and SGC7901-siSema5A cells was performed to observe the effect of the MMP9 Ab on the invasion of those cells. Penetrating cells were fixed, stained with hematoxylin, and imaged at 200× magnification. The data shown in the histogram are a summary of three independent experiments. *$p < 0.05$.

H. pylori culture and infection of gastric cancer cells

H. pylori bacteria were grown on Columbia agar plates (Oxoid, Basingstoke Hampshire, UK) containing 5% sheep blood and incubated at 37°C under microaerophilic conditions. After 72 h, *H. pylori* were harvested and suspended in phosphate-buffered saline (PBS). The *H. pylori* densities were adjusted by measuring the optical density (OD) at 660 nm, and $OD_{660} = 1 \times 10^8$ colony-forming units (CFUs)/mL. *H. pylori* was then coincubated with MKN28, SGC7901, SGC7901-siScrambled, and SGC7901-siSema 5A cells at a cell-to-bacterium ratio of 1:100 for up to 12 or 24 h in the medium.

IHC analysis

Paraffin-embedded gastric tissue samples were cut into 4 μm sections, which were deparaffinized and subjected to antigen retrieval. Sections were incubated with a primary antibody against semaphorin 5A (GeneTex, GTX117160, Irvine, CA, USA), followed by a secondary antibody (GK500705, DAKO A/S, China). Semaphorin 5A expression was determined by scoring the percentage of positive tumor cells (scale, 0%–100%) with a staining intensity from 0–3+. The percentage of cells with positive expression was determined by counting the number of immunopositive cells in three areas randomly chosen in the tissue with a total of 300 cells (0 = 0%, 1 = 1%–25%, 2 = 26%–35%, 3 = 36%–45%, 4 = 46%–65%, and 5 = 66%–100%). The staining intensity was scored (0 = no detectable cell membrane and cytoplasmic staining, 1 = low cell membrane and cytoplasmic staining, 2 = moderate cell membrane and cytoplasmic staining, and 3 = substantial cell membrane and cytoplasmic staining). The two scores were then summed to obtain a final score ranging from 0–8 points. Semaphorin 5A expression levels were divided into 4 groups: 0 (total score = 0), + (total score = 2–3), ++ (total score = 4–5), and +++ (total score = 6–8).

Establishment of a Sprague-Dawley rat model colonized with *H. pylori*

Sprague-Dawley rats (Vitalriver, Nanjing, China; weight, 180 ± 20 g) were used in this study. We randomly divided 30 rats into two groups: the infection group and the control group. The infection group received 5×10^8 CFU/mL *H. pylori* twice per week for 4 consecutive weeks. The control group received 1 mL of PBS each time on the same schedule. After 3 months, rats were sacrificed, and gastric mucosa tissues in the antrum were harvested for semaphorin 5A detection. A rapid urease test, modified Giemsa staining, and Warthin-Starry staining were performed to confirm *H. pylori* infection in the rat model. The animal experiment was approved by our University Ethics Committee.

RT-PCR and qRT-PCR analysis

Total RNA was extracted from samples using TRIzol reagent (Invitrogen). After quantification, RNA was reverse transcribed into cDNAs according to the manufacturer's guidelines. The following primers were used for RT-PCR: semaphorin 5A-f, 5′-AAGATCCAGTAGCG TAGAAGAG-3′, and semaphorin 5A-r, 5′-TGTTGATGTGGTT GGTTATGC-3′; MMP9-f, 5′-CACTGTCCACCCTCAGAGC-3′, and MMP9-r, 5′-GCCACTTGTCGCGATAAGG-3′; and β-actin-f, 5′-CACGCACGATTCCCGCTCGG-3′, and β-actin-r, 5′-CAGGC TGTGCT ATCCTGTAC-3′. PCR was performed at 94°C for 5 min followed by 32 cycles of 94°C for 60 s, 55°C for 30 s, and 72°C for 30 s with a PCR Thermal Cycler Dice (Takara, Otsu, Japan). β-actin served as a loading control. The following primers were used for qRT-PCR: semaphorin 5A-f, 5′-ACCAGTCTTGAACACCAAC-3′, semaphorin 5A-r, 5′-TCGCTAGAACAGTACCGCAT-3′; and GAPDH-f, 5′-GAAAGCCTGCCGGTGACTAA-3′, and GAPDH-r, 5′-GCCC AATACGACCAAATCAGAGA-3′. The expression of the semaphorin5A mRNA was normalized to the expression of the GAPDH mRNA.

Western blot analysis

The cells were homogenized in lysis buffer. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes, which

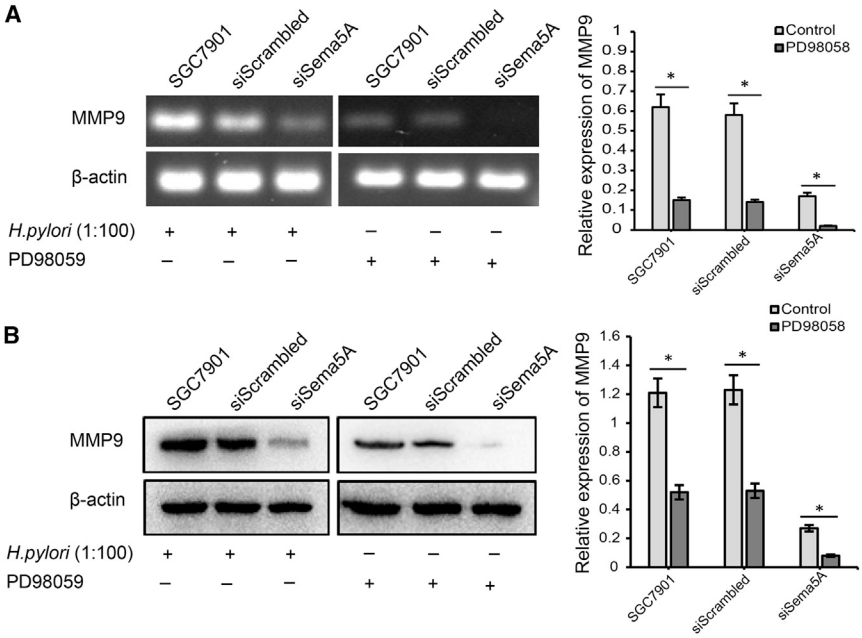

Figure 7. *H. pylori* infection induces MMP9 expression via the ERK signaling pathway in a semaphorin 5A-dependent manner.
SGC7901, SGC7901-siScrambled, and SGC7901-si-Sema 5A cells were cocultured with *H. pylori* in the presence or absence of the ERK inhibitor PD98059. MMP9 expression at the mRNA and protein levels was detected using RT-PCR (A) and western blot (B), respectively. Data shown in the histogram are a summary of three independent experiments. *p < 0.05.

were blocked with 5% non-fat milk and then incubated with primary antibodies against semaphorin 5A, MMP9, pERK1/2, tERK1/2, and β-actin at 4°C overnight. After three washes for 15 min with Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST), the membranes were incubated with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody, followed by enhanced chemiluminescence detection (KPL, Gaithersburg, MD, USA). β-actin served as an internal control.

Cell proliferation and colony-formation assays

Five thousand cells were seeded in each well of 96-well plates and incubated for 0, 24, 48, 72, 96, and 120 h. Then, the MTT (Sigma) stock solution was added to the medium and incubated for an additional 4 h at 37°C in a cell incubator; subsequently, 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken on a rotary platform for 8 min, and then values were measured using a microplate reader at a wavelength of 490 nm to evaluate cell growth. For the colony-formation assay, 2,000 cells from each group were plated in 60 mm dishes. After 14 days of coculture with *H. pylori*, cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet. Colony numbers were measured under a microscope, and photos were acquired.

Wound-healing assay

A wound-healing assay was performed to evaluate gastric cancer cell mobility. A total of 50,000 of each cell line were plated on a six-well plate coated with fibronectin. When cells reached more than 90% confluence after culture, scratch wounds were carefully generated using a sterile pipette tip, and any cellular debris was removed by washing the wells with PBS. Pictures were captured at 0 and 36 h after wounding.

Invasion assay

The examined cells were seeded in Transwell chambers coated with Matrigel to evaluate invasion. The medium in the upper compartment lacked serum, while the medium in the lower compartment contained 10% bovine serum. In some inhibitor experiments, inhibitors and antibodies were also added to the upper chambers. After culture for 24 h at 37°C in a humidified 5% CO_2 incubator, the cells that migrated through the Matrigel were fixed with methanol and stained with hematoxylin. Invasion was

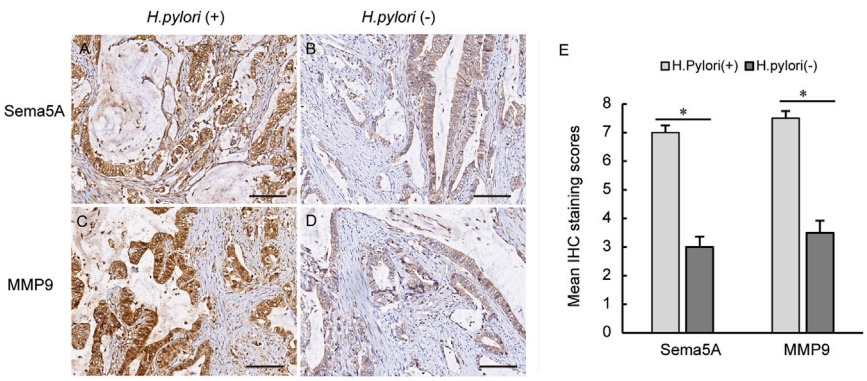

Figure 8. IHC staining for MMP9 and semaphorin 5A in gastric cancer tissues.
The intensity of semaphorin 5A and MMP9 expression was increased in *H. pylori*-positive tissue (A and C) compared with *H. pylori*-negative tissues (B and D). Scale bars,100 μm. Data shown in the histogram are a summary of three independent experiments (E). *p < 0.05.

determined by counting the cells that penetrated the Matrigel matrix under a microscope.

Statistical analysis

The results are presented as the means ± SDs from three independent experiments. The data were analyzed using ANOVA. The statistical analysis was performed using SPSS 13.0 software (SPSS), and $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.06.002.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant no. 81660413), High Level Talent Training Support Plan of Yunnan (project no. RLMY20200016), and Priority Union Foundation of Yunnan Provincial Science and Technology Department and Kunming Medical University (project no.2019FE001(-211)).

AUTHOR CONTRIBUTIONS

G.P., X.W., and Y.W. conceived the study, and S.L., Y.L., R.L., G.L., and Y.H. participated in its design and coordination. L.W. and L.Z. helped to draft the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. *68*, 394–424.
2. Lazăr, D.C., Avram, M.F., Romoşan, I., Cornianu, M., Tăban, S., and Goldiş, A. (2018). Prognostic significance of tumor immune microenvironment and immunotherapy: Novel insights and future perspectives in gastric cancer. World J. Gastroenterol. *24*, 3583–3616.
3. Yaprak, G., Tataroglu, D., Dogan, B., and Pekyurek, M. (2019). Prognostic factors for survival in patients with gastric cancer: Single-centre experience. North. Clin. Istanb. *7*, 146–152.
4. Han, F., Ren, J., Zhang, J., Sun, Y., Ma, F., Liu, Z., Yu, H., Jia, J., and Li, W. (2016). JMJD2B is required for Helicobacter pylori-induced gastric carcinogenesis via regulating COX-2 expression. Oncotarget *7*, 38626–38637.
5. McClain, M.S., Beckett, A.C., and Cover, T.L. (2017). Helicobacter pylori Vacuolating Toxin and Gastric Cancer. Toxins (Basel) *9*, E316.
6. Li, G., Yu, S., Xu, J., Zhang, X., Ye, J., Wang, Z., and He, Y. (2019). The prognostic role of Helicobacter pylori in gastric cancer patients: A meta-analysis. Clin. Res. Hepatol. Gastroenterol. *43*, 216–224.
7. Lordick, F., Shitara, K., and Janjigian, Y.Y. (2017). New agents on the horizon in gastric cancer. Ann. Oncol. *28*, 1767–1775.
8. Nienhüser, H., and Schmidt, T. (2017). Angiogenesis and Anti-Angiogenic Therapy in Gastric Cancer. Int. J. Mol. Sci. *19*, E43.
9. Vrána, D., Matzenauer, M., Neoral, Č., Aujeský, R., Vrba, R., Melichar, B., Rušarová, N., Bartoušková, M., and Jankowski, J. (2018). From Tumor Immunology to Immunotherapy in Gastric and Esophageal Cancer. Int. J. Mol. Sci. *20*, E13.
10. Alto, L.T., and Terman, J.R. (2017). Semaphorins and their Signaling Mechanisms. Methods Mol. Biol. *1493*, 1–25.
11. Koropouli, E., and Kolodkin, A.L. (2014). Semaphorins and the dynamic regulation of synapse assembly, refinement, and function. Curr. Opin. Neurobiol. *27*, 1–7.
12. Pan, G., Lv, H., Ren, H., Wang, Y., Liu, Y., Jiang, H., and Wen, J. (2010). Elevated expression of semaphorin 5A in human gastric cancer and its implication in carcinogenesis. Life Sci. *86*, 139–144.
13. Pan, G., Zhu, Z., Huang, J., Yang, C., Yang, Y., Wang, Y., Tuo, X., Su, G., Zhang, X., Yang, Z., and Liu, T. (2013). Semaphorin 5A promotes gastric cancer invasion/metastasis via urokinase-type plasminogen activator/phosphoinositide 3-kinase/protein kinase B. Dig. Dis. Sci. *58*, 2197–2204.
14. Pan, G., Zhang, X., Ren, J., Lu, J., Li, W., Fu, H., Zhang, S., and Li, J. (2013). Semaphorin 5A, an axon guidance molecule, enhances the invasion and metastasis of human gastric cancer through activation of MMP9. Pathol. Oncol. Res. *19*, 11–18.
15. Pan, G., Su, G., Wang, Y., Xue, F., Yang, Y., Yang, Z., Zhang, Q., Liu, T., Hong, M., and Zheng, J. (2017). Effect of Helicobacter pylori on the expression of semaphorin 5A in patients with gastric precancerous lesions and its clinical significance. Int. J. Clin. Exp. Pathol. *10*, 1403–1409.
16. Farina, A.R., and Mackay, A.R. (2014). Gelatinase B/MMP-9 in Tumour Pathogenesis and Progression. Cancers (Basel) *6*, 240–296.
17. Salaroglio, I.C., Mungo, E., Gazzano, E., Kopecka, J., and Riganti, C. (2019). ERK is a Pivotal Player of Chemo-Immune-Resistance in Cancer. Int. J. Mol. Sci. *20*, E2505.
18. Wee, P., and Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers (Basel) *9*, E52.