

Helicobacter pylori upregulates TRPC6 via Wnt/ β -catenin signaling to promote gastric cancer migration and invasion

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background: *Helicobacter pylori* infection is recognized as a major risk factor for gastric cancer (GC) progression; however, the underlying molecular mechanisms have remained to be fully elucidated.

Methods: qPCR and Western blot were used to detect mRNA level and relative protein expression. Wound healing assay and transwell were used to determine migration and invasion of cells. Calcium imaging was used to determine calcium signaling in cells. Luciferase reporter assay and immunohistochemistry were performed.

Results: In the present study, it was demonstrated that *H. pylori* infection in GC is closely associated with the depth of tumor invasion, lymph node metastasis, tumor-nodes-metastasis stage, and distant metastasis. Migration and invasion assays indicated that *H. pylori* infection enhanced the migration and invasion of GC cells in a Ca^{2+} -dependent manner. Calcium imaging was applied to detect intracellular Ca^{2+} and revealed that *H. pylori* induced an increase of intracellular Ca^{2+} in GC cells through release from Ca^{2+} stores and extracellular Ca^{2+} influx. Further study indicated that *H. pylori* infection led to an upregulation of the expression of transient receptor potential cation channel subfamily C member 6 (TRPC6) and induced an increase of Ca^{2+} through the TRPC6 channel. Furthermore, *H. pylori* increased TRPC6 transcription through the Wnt/ β -catenin pathway, and Wnt/ β -catenin/TRPC6 signaling was identified to be at least in part responsible for *H. pylori*-induced GC migration and invasion. Finally, it was observed that TRPC6 expression was significantly associated with the *H. pylori* infection status in GC tissues, and *H. pylori* infection was associated with metastasis and poor prognosis for GC patients.

Conclusion: The present results indicate that *H. pylori* causes an upregulation of TRPC6 expression through the Wnt/ β -catenin pathway to promote GC progression, and this interaction may serve as a promising target for GC therapy.

Keywords: helicobacter pylori, TRPC6, Wnt/ β -catenin, calcium signaling, gastric cancer

Introduction

Helicobacter pylori persistently colonizes in the stomach, and has been classified as a type-I carcinogen that induces the development of gastric cancer (GC) by the World Health Organization and the International Agency for Research of Cancer.^{1,2} The ability of *H. pylori* to colonize in the human stomach and to promote GC development may be attributed to the production of multiple virulence factors,^{3,4} which modulate the pathogen–host interaction and determine the degree of bacterial pathogenicity. *H. pylori* exerts multiple effects on cell proliferation, apoptotic pathways, cytokine secretion, and DNA damage.^{5–8} Numerous studies have

indicated that *H. pylori* infection drives GC progression,^{9–11} while the associated molecular mechanisms implicated in GC migration and invasion remain to be fully elucidated.

For the progression of cancer, it is required that tumor cells acquire migratory and invasive abilities, and Ca^{2+} is a critical regulator of cell migration and invasion.¹² Calcium is responsible for the transmission of signals from the microenvironment into cells, and calcium signaling is an important step in cancer cell migration and invasion.^{13,14} Numerous studies have indicated that Ca^{2+} channels are essential for the progression of various tumor types.^{15–18} Among these, transient receptor potential (TRP) channels are non-selective Ca^{2+} entry channels. Several families of TRP channels exist in humans, among which TRP cation channel subfamily C member 6 (TRPC6) is greatly associated with the progression of GC. TRPC6 is significantly upregulated in human GC, and TRPC6 inhibition was reported to lead to growth suppression of GC cells.¹⁹ However, to date, the functions of TRPC6 in *H. pylori* infection in GC and the underlying and mechanisms have remained largely elusive.

In the present study, *H. pylori* was identified to upregulate TRPC6 expression through the Wnt/ β -catenin pathway, and TRPC6 then mediates the extracellular Ca^{2+} influx in GC cells to finally promote GC cell migration and invasion in a Ca^{2+} -dependent manner. The present study indicates that the Wnt/ β -catenin/TRPC6 signaling cascade is responsible for the mediation of *H. pylori*-induced human GC progression, providing novel insight that may be utilized to develop strategies for preventing GC development.

Materials and methods

Cell culture

The human GC cell line MKN45 was obtained from the Chinese Academy of Sciences, and the AGS cell line was purchased from the American Type Culture Collection (ATCC). MKN45 and AGS cells were cultured in RPMI 1640 medium (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37°C.

Analysis of TRPC6 expression in GC specimens

Tissue samples were collected from GC patients at the Department of General Surgery, Chinese PLA General Hospital. The written informed consent was obtained from all patients. This study was conducted in accordance

with the Declaration of Helsinki. The use of the clinical specimens was approved by the Clinical Research Ethics Committee of Chinese PLA General Hospital. Tissues were obtained from each patient and blocked with 2.5% hydrogen peroxide in methanol. All the GC patients enrolled in our study were tested for *H. pylori* infection using a rapid urease test before the resection. Patients were regarded as positive for *H. pylori* infection if they were positive by the rapid urease test. Then, TRPC6 expression in gastric tissues was detected using the immunohistochemical staining. A polyclonal rabbit anti-TRPC6 antibody (Santa Cruz Biotechnology, CA, USA) was used for the staining. Then, the semi-quantitative analysis was performed. Briefly, images of all tissue samples were acquired with a constant set of imaging parameters on the microscope and imaging software. The images were then subjected to optical density (OD) analysis by Image-Pro Plus software. Adjustments to the background and color intensity range were performed. The intensity range selection was based on histograms, with the intensity (I) and saturation (S) set at maximum hue (H) was set at a range in which most of the brown DAB color was selected. These settings were saved and subsequently applied to all images analyzed. After defining the area of interesting, the mean OD of the selected area [integrated optical density/unit area] was determined by the software and represents the immunoreactivity of the candidate protein within tumor tissue. The acquired score of the OD was standard normalized and subjected to ImagePro Plus (Media Cybernetics, MD, USA) for further analysis. Then, patients were evaluated in terms of sex, age, tumor differentiation, depth of invasion, lymph node metastasis, TNM stage, distant metastasis, and survival.

H. pylori culture and infection in GC cells

The CagA- and VacA- positive *H. pylori* strain NCTC11637 was obtained from ATCC, which was grown on Columbia agar (Oxoid, Basingstoke Hampshire, UK) plates containing 5% sheep blood and incubated at 37°C under microaerophilic conditions for 48–72 hrs. *H. pylori* was collected from the plates with PBS and then resuspended in RPMI 1640 medium without antibiotics. The densities of bacteria were measured by the OD at 660 nm [$1 \text{ OD}_{660} = 1 \times 10^8$ colony-forming units (CFU)/mL]. Cultured AGS and MKN45 cells were seeded on plates and grown to 80% confluency. Then, the cells were infected with the bacteria at a bacteria-to-cell ratio of 100:1 in the culture media.

Real-time quantitative PCR

Total RNA was extracted by TRIZOL Reagent Kit (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed to cDNA using PrimeScript RT-polymerase (Takara, Japan). Quantitative real-time PCR was performed using SYBR Green Mix (Takara, Japan) and primers specific for TRPC6 and GAPDH. GAPDH was used as an internal control. All the experiments were performed in triplicate.

Western blotting

Cells were lysed with a lysis buffer, and protein concentration was measured using a BCA Protein Assay Kit (Beyotime, Beijing, China). Protein expression levels were detected using the Western blotting analysis. Primary antibodies: anti-TRPC6, anti-GAPDH were purchased from Cell Signal Technology (CST, USA). Then, secondary antibodies conjugated with horseradish peroxidase were used. The relative levels were quantified and normalized against GAPDH in the same sample.

Luciferase reporter vectors and Luciferase assays

TRPC6 promoter was amplified from human cDNA, and cloned into the KpnI and BglII sites of a PGL3-basic vector (Promega, USA). Similar plasmids containing mutations in the putative TCF/LEF-binding sites, named Mut, were constructed. AGS and MKN45 cells were co-transfected with the constructed luciferase vectors. Then, *H. pylori* was added to the cells. After 24 hrs, the cells were harvested in 1×Reporter Lysis Buffer (Catalog no.: E1960, Promega), and luciferase activities were measured using the Dual-Luciferase Reporter Assay System Kit (Promega, WI, USA). The luciferase activity for each lysate was normalized to the renilla luciferase activity.

Scratch wounding assay

GC cells were seeded into 24-well plates as a monolayer at 24 hrs. Cells were scraped with a 10 μ L pipette tip. After scratching, the cells were washed to remove the detached cells. *H. pylori* was added to the cells. The extent of cell migration was observed, and images were captured at 0 and 24 hrs under a microscope. In each group, at least three parallel wells were utilized for testing.

Cell invasion assay

The invasive potential of GC cells treated by *H. pylori* was assessed using a transwell invasion assay. Matrigel Basement Membrane Matrix (BD Biosciences, San Diego, CA, USA)

was added to each well and incubated at 37°C for 4 hrs to assess tumor cell invasion. Cells (approximately 1×10^4), in 200 μ L of serum-free RPMI-1640 medium, were added into the upper compartment of the chamber. A total of 500 μ L of RPMI-1640 medium with 10% fetal bovine serum were placed in the bottom compartment of the chamber. Then, *H. pylori* and the inhibitors were added to the upper chamber. After 24 hrs of incubation at 37°C with 5% CO₂, the medium was removed from the upper chamber, and invading cells were fixed and stained with crystal violet. Then, the number of invasive cells were analyzed using a microscope (Olympus, Japan). Triplicate experiments were performed.

Measurement of $[Ca^{2+}]_{cyt}$ by calcium imaging

GC cells were cultured on coverslips and loaded with Fura-2AM (Invitrogen, NY, USA) at 37°C for 45 mins and then washed with PBS for 30 mins. After loading, cells were transferred to a standard perfusion chamber on the stage of a TMD inverted microscope (Nikon, Japan). $[Ca^{2+}]_i$ measurements were made at 340/380 nm excitation and 510 nm emission wavelengths from an SLM-Aminco spectrophotometer (Rochester, NY). F340/380 ratios were used to represent $[Ca^{2+}]_{cyt}$. For the Ca²⁺-free solution, the CaCl₂ was replaced with NaCl. *H. pylori* and the correspond inhibitors or treatments were applied as described.

TOP/FOP flash reporter assay

A TOP/FOP flash assay was used for Wnt signaling pathway analysis. Briefly, the TCF/LEF-responsive luciferase construct was made under the control of minimal TK promoter and tandem repeats of the TCF/LEF transcriptional response element. GC cells were seeded in a 24-well plate and co-transfected with the pRL-TK plasmid (10 ng/well) and either TOP flash plasmid or FOP flash plasmid (200 ng/well), and then treated by *H. pylori* and inhibitors as indicated. At 48 hrs later, the cells were analyzed using the Dual-Luciferase Reporter Assay System (Promega, E1910). The ratio of TOP Flash activity and FOP Flash activity represented the results of the activity.

Statistical analysis

Data are expressed as means \pm standard error (SE). A two-tailed Student's *t*-test or Mann–Whitney test was used to test the difference. The Kaplan–Meier curves and log-rank test were applied to assess survival. The associations between the protein expression and clinicopathological parameters were analyzed with Pearson's χ^2 test. SPSS Statistics software (version 19.0, SPSS Inc., Chicago, IL, USA) was used

for statistical analysis, and the graphs were generated using GraphPad Prism 6.0 (Graphpad Software Inc, CA, USA).

Results

H. pylori infection is correlated with the progression of GC. To investigate the correlation between *H. pylori* infection and the clinicopathological features of GC patients, *H. pylori* colonization was examined in 360 GC tissues. As presented in Table 1, *H. pylori* colonization was present in 44.2% (159/360) of GC tissues. Analysis of the potential correlation between *H. pylori* infection and the clinicopathological features indicated that *H. pylori* infection in GC was not associated with sex, age, or degree of differentiation, while it was associated to the depth of invasion, TNM stage and lymph node or distant metastasis (Table 1). These results suggest that *H. pylori* infection has an important role in GC metastasis and is correlated with an advanced stage of GC.

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

Clinicopathological factors	n	<i>H. pylori</i> positive (n=159)	<i>H. pylori</i> negative (n=201)	P
Sex				
Female	168	78	90	0.4-19
Male	192	81	111	
Age (years)				
≥65	146	65	81	0.9-11
<65	214	94	120	
Differentiation				
Differentiated	212	86	126	0.1-00
Undifferentiated	148	73	75	
Depth of invasion				
T1-T2	175	54	121	<0.001
T3-T4	185	105	80	
Lymph metastasis				
Negative	256	92	164	<0.001
Positive	104	67	37	
TNM stage				
I - II	224	86	138	0.0-05
III - IV	136	73	63	
Distant metastasis				
Negative	276	102	174	<0.001
Positive	84	57	27	

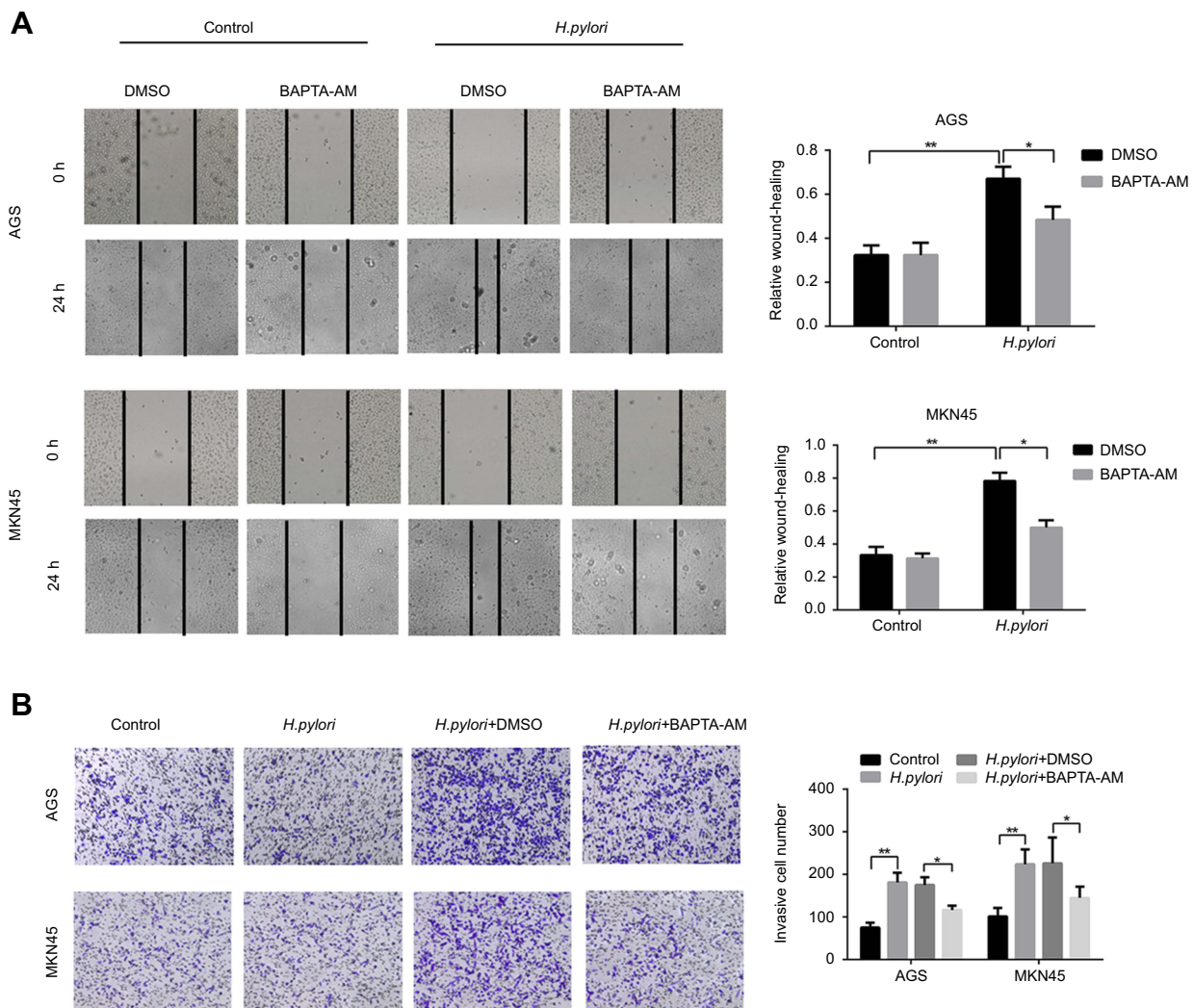
H. pylori enhances GC cell migration and invasion in a Ca^{2+} -dependent manner. To assess the effect of *H. pylori* infection on the migratory and invasive capacities of GC cells, AGS and MKN45 cells were infected with *H. pylori* NCTC 11637. The results indicated that *H. pylori* infection increased the migration and invasion of GC cells (Figure 1A and B). Since Ca^{2+} signaling is important in tumor cell migration, it was further explored whether Ca^{2+} was involved in *H. pylori*-induced GC cell migration and invasion. The results suggested that pre-treatment of GC cells with the Ca^{2+} chelator BAPTA-AM significantly attenuated *H. pylori*-induced GC cell migration and invasion (Figure 1A and B). Taken together, it was demonstrated that *H. pylori* infection promotes the migration and invasion of GC cells in a Ca^{2+} -dependent manner.

H. pylori induces intracellular Ca^{2+} signaling through intracellular Ca^{2+} release and Ca^{2+} influx. The present study further assessed whether *H. pylori* infection increases the intracellular Ca^{2+} concentration of GC cells. Various amounts of *H. pylori* were added to the cell culture and the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was measured. As presented in Figure 2A, *H. pylori* induced a dose-dependent increase in $[Ca^{2+}]_i$ in GC cells, comprising a biphasic $[Ca^{2+}]_i$ increase ("peak" phase), followed by a descent to a $[Ca^{2+}]_i$ baseline ("plateau" phase).

The Ca^{2+} source of the *H. pylori*-evoked increase in $[Ca^{2+}]_i$ in GC cells was then determined. One of the major intracellular Ca^{2+} sources is the sarcoendoplasmic reticular Ca^{2+} store (SERCA). GC cells were pretreated with thapsigargin, a Ca^{2+} -ATPase inhibitor in the SERCA, followed by addition of *H. pylori* and measurement of $[Ca^{2+}]_i$. The result indicated that thapsigargin significantly reduced the $[Ca^{2+}]_i$ peak levels, while it had no effect on the $[Ca^{2+}]_i$ plateau change (Figure 2B).

The role of calcium channels in the cell membrane in the changes in $[Ca^{2+}]_i$ was then investigated. When *H. pylori* was first applied in Ca^{2+} -free solutions and with pre-treatment using thapsigargin, a marked increase in $[Ca^{2+}]_i$ was observed in GC cells, and re-addition of extracellular Ca^{2+} further induced an increase in $[Ca^{2+}]_i$ (Figure 2C). Furthermore, the *H. pylori*-induced rise in $[Ca^{2+}]_i$ was inhibited by 2-aminoethoxydiphenyl borate (2-APB, 100 μ M; Figure 2D), a non-specific inhibitor of TRP channels. Taken together, these results suggest that the *H. pylori*-induced peak $[Ca^{2+}]_i$ is caused by Ca^{2+} release from intracellular Ca^{2+} stores, while the *H. pylori*-induced plateau $[Ca^{2+}]_i$ change is caused by Ca^{2+} influx.

H. pylori upregulates the TRPC6 channel and induces Ca^{2+} influx through TRPC6. To further identify the



potential calcium channels involved in *H. pylori*-induced Ca^{2+} influx, various membrane Ca^{2+} -permeable channels reported in GC cells were screened. It was indicated that SN-6, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1 inhibitor, and RN1734 (10 μM), a selective TRPV4 inhibitor, did not alter the *H. pylori*-induced increase in $[\text{Ca}^{2+}]_i$ (Figure 3A and B). However, SKF96365 (10 μM), a blocker for TRPC channels, attenuated the *H. pylori*-induced rise in $[\text{Ca}^{2+}]_i$ in GC cells (Figure 3C). Overall, these results suggest that TRPC channels may be the target calcium channels mediating *H. pylori*-induced Ca^{2+} entry in GC cells. Since several TRPC channel subtypes are present in the cell membrane, it was attempted to identify the subtype of TRPC channel involved in *H. pylori*-induced Ca^{2+} influx in GC cells. GC

cells were infected with the *H. pylori* strain, and the expression of different subtypes of TRPC channel in response to *H. pylori* treatment was examined. TRPC6 expression was significantly increased in *H. pylori*-infected GC cells (Figure 3D and E). Therefore, it was indicated that TRPC6 is involved in the *H. pylori*-induced Ca^{2+} influx. Furthermore, knockdown of TRPC6 significantly decreased the *H. pylori*-induced increase in $[\text{Ca}^{2+}]_i$ in GC cells (Figure 3F). These results strongly indicate that *H. pylori* increases TRPC6 channel expression to induce Ca^{2+} entry in GC cells via this channel.

H. pylori upregulates TRPC6 expression via Wnt/ β -catenin signaling. Next, it was investigated how *H. pylori* treatment causes an upregulation of TRPC6 expression in GC cells. It is

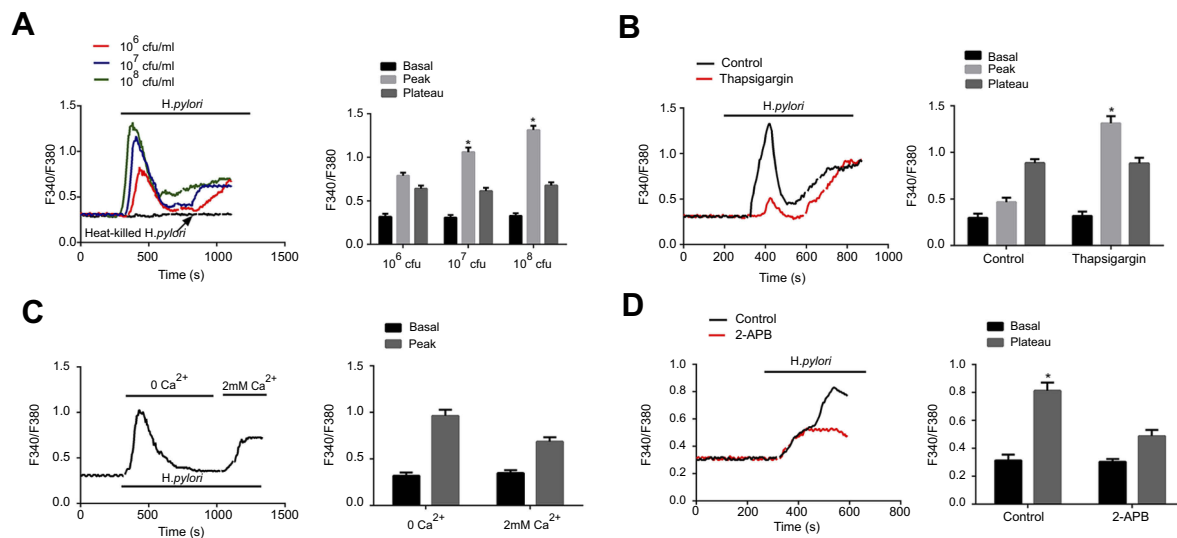


Figure 2 *H. pylori* induces intracellular Ca^{2+} increase through intracellular Ca^{2+} release and extracellular Ca^{2+} influx. **(A)** Representative tracings of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes with varying concentrations [1×10^6 – 1×10^8 colony-forming units (CFU)/mL] of *H. pylori* on Fura 2-loaded gastric cancer cells. The addition of *H. pylori* produced a concentration-dependent biphasic change in $[Ca^{2+}]_i$, which was followed by a return to baseline $[Ca^{2+}]_i$ levels. Histogram at the right shows the summary data for peak and plateau changes of Fura-2 fluorescence ratio. Heat killed *H. pylori* was used as a negative control. * $P < 0.05$. **(B)** Effects of intracellular Ca^{2+} depletion on *H. pylori*-induced $[Ca^{2+}]_i$ changes in MKN45 cells. Fura 2-loaded human gastric cancer cells were pretreated for 30 mins with 500 nM thapsigargin to release intracellular Ca^{2+} . The *H. pylori* strains (1×10^8 CFU/mL) were then added, the peak and plateau changes were recorded. Histogram at the right shows the summary data for peak and plateau changes of Fura-2 fluorescence ratio. * $P < 0.05$. **(C)** Effects of extracellular Ca^{2+} -free Ringer on *H. pylori*-induced $[Ca^{2+}]_i$ changes in gastric cancer cells. Fura 2-loaded cells were first exposed to extracellular Ca^{2+} -free Ringer for 15 mins, *H. pylori* strains (1×10^8 CFU/mL) were added and $[Ca^{2+}]_i$ was recorded. Then, 2 mM Ca^{2+} was readded to the solution and $[Ca^{2+}]_i$ was recorded again. Right panel: Summary data for peak changes of Fura-2 fluorescence ratio. **(D)** Effects of 2-APB on *H. pylori*-induced $[Ca^{2+}]_i$ changes gastric cancer cells. Fura 2-loaded human gastric cancer cells were pretreated for 30 mins with 500 nM thapsigargin for depletion of intracellular Ca^{2+} . Then, time course of $[Ca^{2+}]_i$ changes induced by *H. pylori* in the absence and presence of 100 nM 2-APB was recorded. Histogram at the right shows the summary data for basal and plateau changes of Fura-2 fluorescence ratio. * $P < 0.05$. For all tracings, n=10 independent experiments.

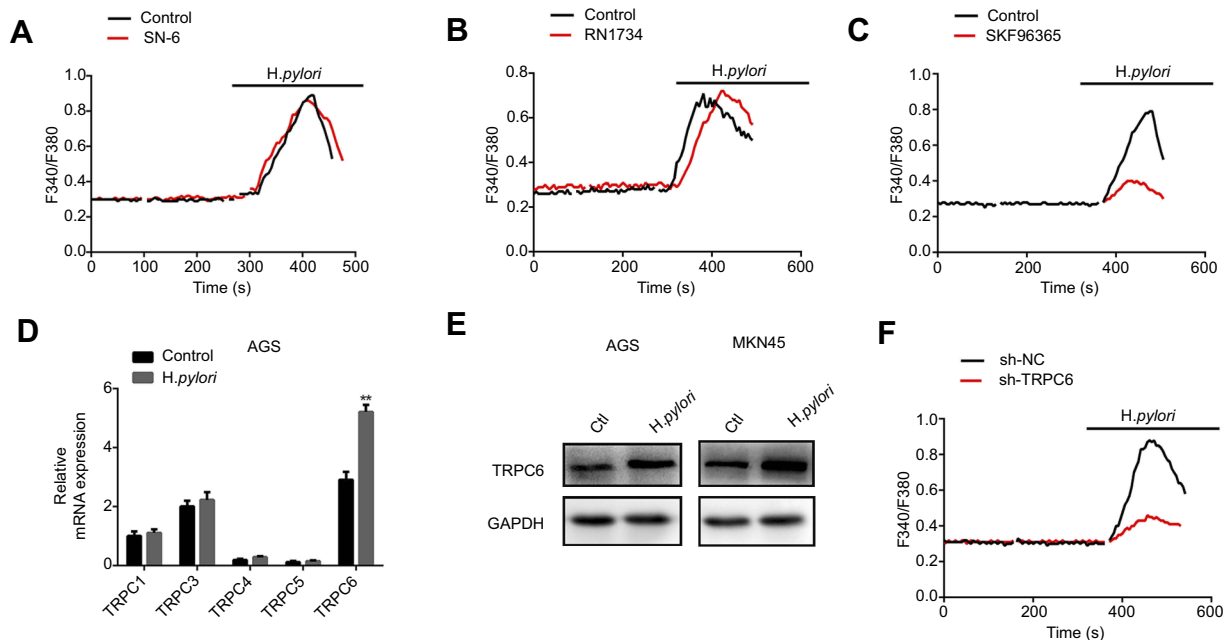


Figure 3 *H. pylori* increases TRPC6 expression and induces extracellular Ca^{2+} influx through TRPC6 channel. **(A–C)** Cells were pretreated with 500 nM thapsigargin for 30 mins for the depletion of intracellular Ca^{2+} . Then, SN-6 (10 μ M) **(A)**, RN1734 (10 μ M) **(B)**, and SKF96365 (10 μ M) **(C)** were added to detect *H. pylori*-induced $[Ca^{2+}]_i$ changes in MKN45 cells. **(D)** The relative mRNA expression of subtypes of TRPC channels induced by *H. pylori* in AGS cells was measured by qPCR. **(E)** *H. pylori*-induced TRPC6 protein expression was evaluated by Western blot analysis. **(F)** Effect of TRPC6 knockdown on *H. pylori*-induced $[Ca^{2+}]_i$ changes in MKN45 cells. For all tracings, n=10 independent experiments.

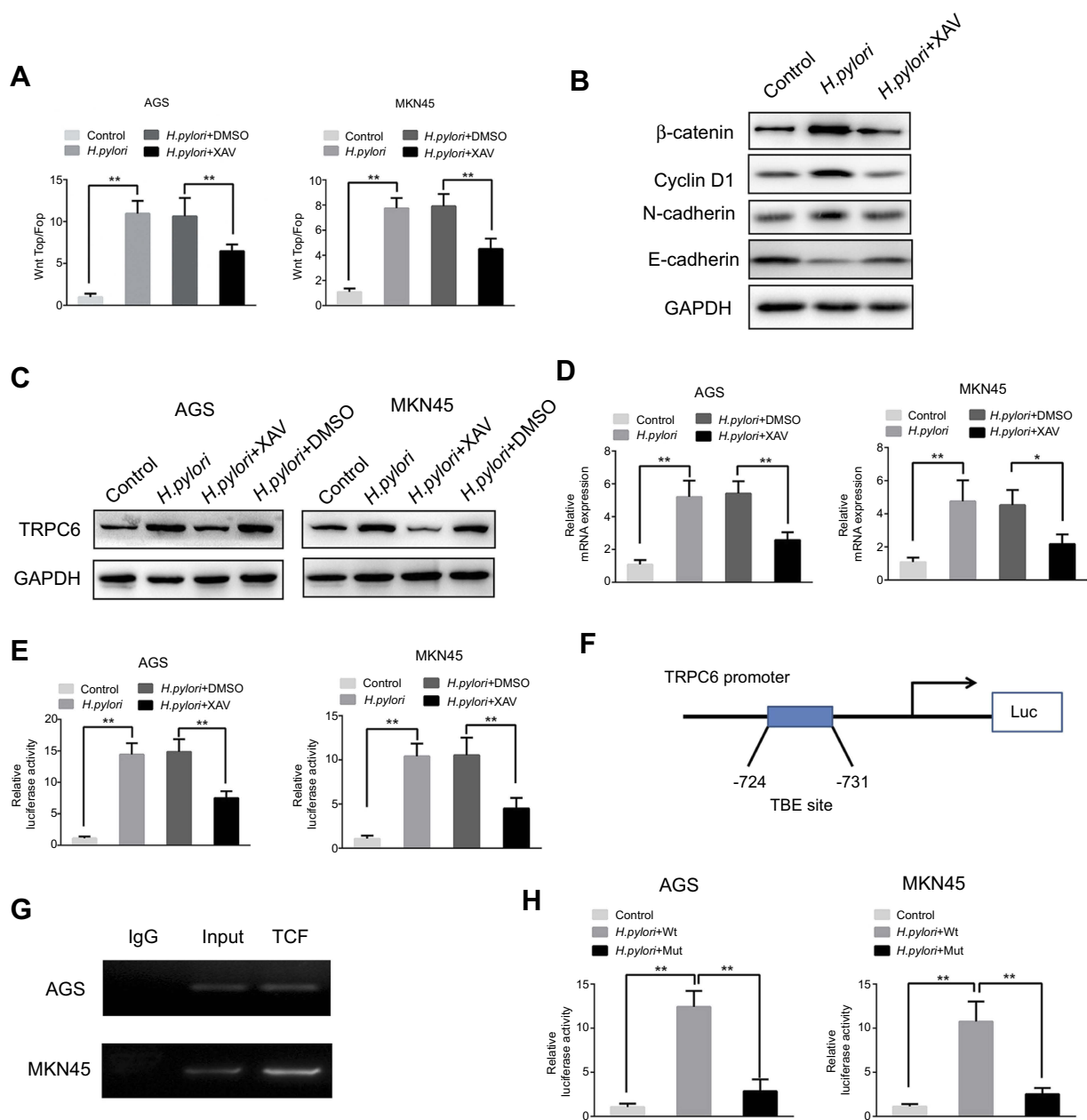


Figure 4 Wnt/ β -catenin signaling is involved in *H. pylori*-induced transcriptional expression of TRPC6 in gastric cancer cells. **(A)** AGS and MKN45 cells were transfected with luciferase reporter constructs containing TCF/LEF-binding motifs (Top-Flash) or mutated TCF/LEF sites (Fop-Flash). Then, *H. pylori* was added to cells pretreated with XAV (10 μ M) or DMSO for 2 hrs, luciferase activity was measured to assess β -catenin transcriptional activity. ** $P < 0.01$. **(B)** Various markers of Wnt signaling were detected by Western blot in *H. pylori* treated cells with or without XAV. **(C)** AGS and MKN45 cells were pretreated with XAV or DMSO, and *H. pylori* was incubated with cells for 24 hrs. The TRPC6 protein expression was examined by Western blot analysis. **(D)** AGS and MKN45 cells were pretreated with XAV or DMSO, then *H. pylori* was incubated with cells for 24 hrs. The relative mRNA expression of TRPC6 was detected by qPCR analysis. ** $P < 0.01$. **(E)** AGS and MKN45 cells were pre-transfected with luciferase reporter constructs containing the TRPC6 promoter for 4 hrs. Luciferase activity was measured to assess promoter activity in response to *H. pylori* infection in the absence or presence of XAV. ** $P < 0.01$. **(F)** The potential TCF binding element (TBE) was identified within the TRPC6 promoter. **(G)** Chromatin immunoprecipitation (ChIP) assays were conducted in AGS and MKN45 cells using an antibody against TCF or a control IgG antibody. **(H)** The TBE binding site was mutated in the TRPC6 promoter, the mutated and wild type of TRPC6 promoter activity induced by *H. pylori* infection was assessed. ** $P < 0.01$.

known that *H. pylori* infection activates a series of signaling pathways, among which the Wnt/ β -catenin pathway is involved in *H. pylori*-mediated tumor progression.^{20–22}

Therefore, the present study aimed to investigate whether *H. pylori* promotes TRPC6 expression through the Wnt/ β -

catenin pathway. The results of the present study also suggested that CagA positive *H. pylori* enhanced the TOP/FOP activity in GC cells (Figure 4A and B), while CagA negative *H. pylori* could not enhance TOP/FOP activity (Figure S1A). GC cells were then pretreated with XAV, an inhibitor of Wnt/ β -

catenin signaling. The results indicated that XAV significantly attenuated CagA positive *H. pylori*-induced expression of TRPC6 in GC cells (Figure 4C and D).

Next, it was examined whether Wnt/ β -catenin controls the expression of TRPC6 transcription. A luciferase reporter assay revealed that CagA positive *H. pylori* enhanced the promoter activity of TRPC6, whereas XAV attenuated the *H. pylori*-induced promoter activity (Figure 4E). Nevertheless, CagA negative *H. pylori* could not enhance TRPC6 promoter activity and expression (Figure S1B and C). Furthermore, the gene encoding TRPC6 was analyzed using the University of California Santa Cruz (UCSC) genome browser (<https://genome.ucsc.edu>). One putative transcription factor (TCF) binding site was identified within the TRPC6 promoter (Figure 4F). The chromatin immunoprecipitation assay showed that TCF could directly

bind to the promoter of TRPC6 (Figure 4G). Luciferase reporter vectors including the wild type or mutant binding site were then constructed and it was demonstrated that after infection with *H. pylori*, the luciferase activity of the vector with the mutant binding site was decreased compared with that of the vector with the wild-type promoter (Figure 4H). These results clearly suggest that *H. pylori* increased the transcription of TRPC6 in GC cells through the Wnt/ β -catenin pathway.

Wnt/ β -catenin/TRPC6 signaling is responsible for H. pylori-induced GC progression. The role of TRPC6 in *H. pylori*-induced GC migration and invasion was further examined by performing TRPC6 knockdown, leading to a significant reduction in *H. pylori*-induced migration and invasion (Figure 5A and B). Taken together, the present results indicate that Wnt/ β -catenin/TRPC6 signaling is involved in *H. pylori*-induced GC progression.

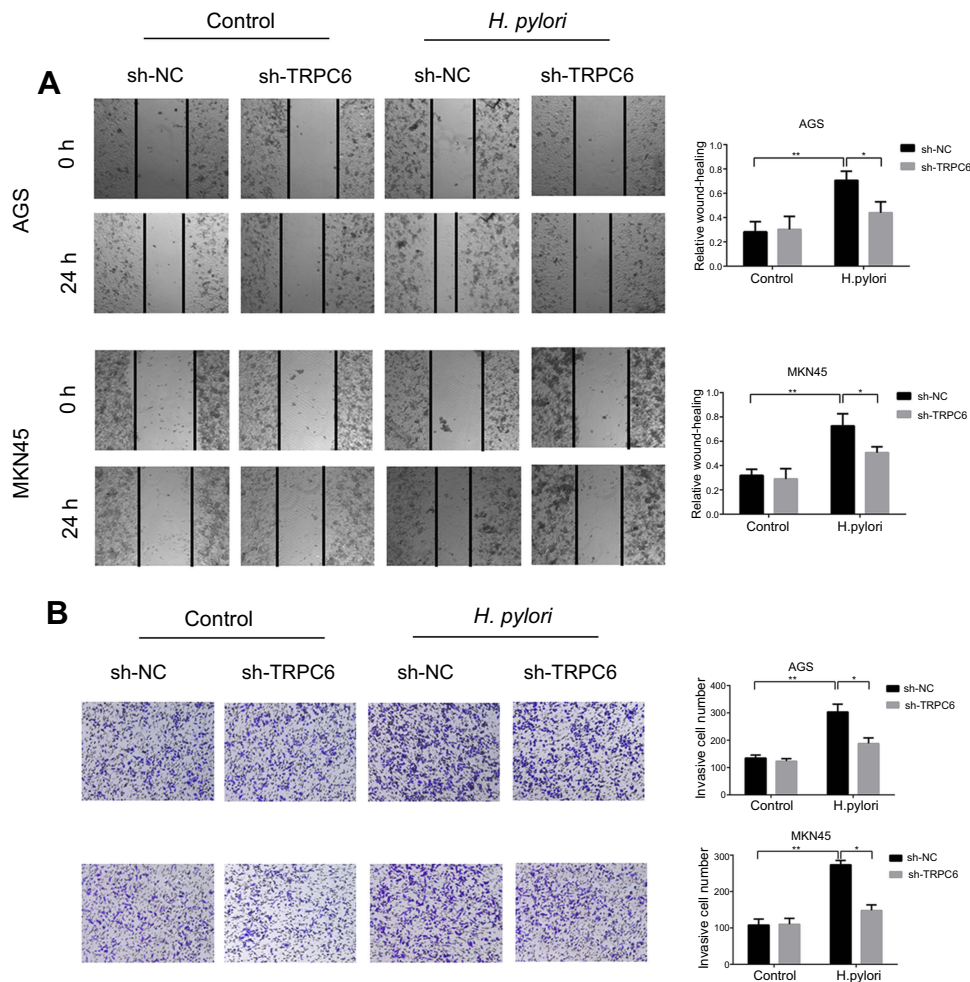


Figure 5 Wnt/ β -catenin/TRPC6 signaling is involved in *H. pylori*-induced gastric cancer migration and invasion. (A and B) AGS and MKN45 cells were pre-transfected with sh-NC or sh-TRPC6, and then infected with *H. pylori* for 24 hrs. Scratch wounding assay (A) and transwell invasion assay (B) were performed to evaluate the migration and invasion of GC cells. * $P < 0.05$; ** $P < 0.01$.

H. pylori infection increases TRPC6 expression in GC tissues and is associated with poor prognosis of GC patients. Since *H. pylori* infection caused an increase in TRPC6 expression in GC cells in vitro, TRPC6 expression was further examined in *H. pylori*-infected GC tissues. qPCR analysis of freshly resected GC tissues indicated that TRPC6 expression in *H. pylori*-positive GC was markedly higher than that in *H. pylori*-negative GC (Figure 6A). The expression of TRPC6 was then assessed in 360 GC tissues by using immunohistochemistry. The results suggested an obvious upregulation of TRPC6 expression in *H. pylori*-positive GC tissues compared with that in *H. pylori*-negative tissues (Figure 6B, Table S1). Furthermore, survival analysis demonstrated that *H. pylori* infection was highly associated with poor survival (Figure 6C). Taken together, the present results indicated that *H. pylori* infection was associated with increased TRPC6 expression and poor prognosis of GC patients.

Discussion

H. pylori infection-associated chronic inflammation is an important factor for the development of GC.^{23,24} Several previous studies have also suggested that *H. pylori* infection

is closely associated with GC progression.^{23–27} The present study indicated that *H. pylori* infection is associated with invasion and lymph node metastasis of GC in vivo, and it was demonstrated that *H. pylori* promoted GC cell migration and invasion in vitro. However, the molecular mechanisms of how *H. pylori* causes GC progression remain largely elusive. The present study revealed a novel mechanism, namely that *H. pylori* activates Wnt/ β -catenin to transcriptionally upregulate TRPC6 expression and eventually induce Ca^{2+} -dependent GC cell migration and invasion.

Calcium signaling is an important regulator for cell migration and invasion.¹³ In the present study, calcium signaling was demonstrated to be involved in *H. pylori*-induced GC cell migration and invasion. A previous study revealed that *H. pylori* promoted a dose-dependent $[\text{Ca}^{2+}]_i$ increase in human gastric mucous epithelial cells.²⁸ However, the mechanisms and exact dynamics of how *H. pylori* infection induces calcium mobilization in GC cells remain elusive. The present study indicated that *H. pylori* induced a biphasic $[\text{Ca}^{2+}]_i$ increase (transient “peak”) and a $[\text{Ca}^{2+}]_i$ baseline value (“plateau” phase). The SERCA Ca^{2+} store is an important intracellular Ca^{2+} store.^{29,30} The present study indicated that pre-treatment

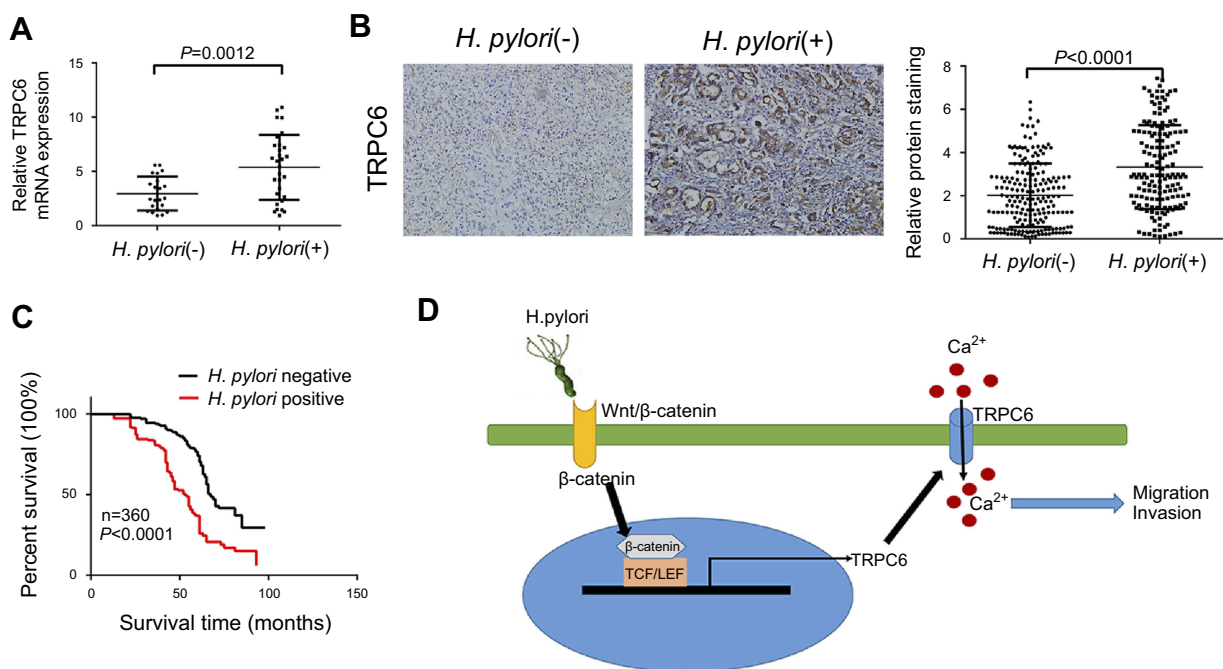


Figure 6 *H. pylori* infection was associated with increased expression of TRPC6 in GC tissues and poor prognosis of GC patients. (A) The relative mRNA expression of TRPC6 in gastric cancer tissues from *H. pylori*-positive (n=30) and *H. pylori*-negative (n=22) patients. (B) Representative immunohistochemical staining for TRPC6 in gastric cancer tissues from *H. pylori*-positive (n=159) and *H. pylori*-negative (n=201) patients. Right panel: TRPC6 expression levels were scored with semi-quantitative immunohistochemical analysis. (C) Kaplan–Meier curves comparing overall survival in GC patients with positive and negative infection of *H. pylori* (n=360; $P<0.0001$). (D) Schematic model of *H. pylori*-induced TRPC6/ Ca^{2+} signaling through activation of the Wnt/ β -catenin signaling pathway, which promotes migration and invasion of human gastric cancer cells.

with the SERCA inhibitor thapsigargin did not affect the $[Ca^{2+}]_i$ plateau change. Furthermore, in a Ca^{2+} -free solution, *H. pylori* induced a $[Ca^{2+}]_i$ peak change, while re-addition of extracellular Ca^{2+} produced a plateau change, suggesting that the *H. pylori*-induced $[Ca^{2+}]_i$ peak change is based on intracellular Ca^{2+} release, whereas the plateau $[Ca^{2+}]_i$ change is dependent on Ca^{2+} influx.

There are several major classes of plasma membrane Ca^{2+} -permeable channels, which mediate Ca^{2+} influx in response to various activating stimuli.⁸ In the present study, a panel of membrane Ca^{2+} -permeable channels was screened, which have been previously reported in GC cells, and it was revealed that only TRPC channel blocker SKF96365 attenuated the *H. pylori*-induced increase in $[Ca^{2+}]_i$ in GC cells. Since the family of TRPC channels has seven members,^{31,32} the expression of each subtype in response to *H. pylori* infection was further evaluated by qPCR. The results suggested that *H. pylori* infection induced TRPC6 expression and that *H. pylori* may induce the increase in $[Ca^{2+}]_i$ by activating the expression of TRPC6. The present study then attempted to investigate the possible mechanisms of how *H. pylori* induces TRPC6 expression.

Previous studies have indicated that *H. pylori* activates various intracellular pathways.^{33–36} The Wnt/ β -catenin pathway is critical to tumorigenesis and cancer development,^{37,38} and is also responsible for *H. pylori*-induced gastric carcinogenesis.^{11,39,40} In the present study, it was demonstrated that *H. pylori* activated Wnt/ β -catenin signaling and induced the transcription of TRPC6 in GC cells. A search with the UCSC genome browser indicated that the TRPC6 promoter had a TCF binding site, and mutation of this binding site significantly attenuated *H. pylori*-induced TRPC6 promoter activity. Furthermore, inhibition of Wnt/ β -catenin or TRPC6 markedly inhibited *H. pylori*-induced migration and invasion of GC cells. In addition, the clinical analysis revealed that TRPC6 levels in GC samples from *H. pylori*-positive patients were higher compared with those from *H. pylori*-negative patients.

In conclusion, the present study indicated that *H. pylori* promotes GC migration and invasion through the Wnt/ β -catenin/TRPC6/ Ca^{2+} signaling pathway (Figure 6D). It provides a novel molecular target for developing effective strategies to prevent GC progression.

Acknowledgment

This work was supported by Hainan Provincial Natural Science Foundation of China (grant number 20158275).

Disclosure

The authors report no conflicts of interest in this work.

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