

# World Journal of *Gastrointestinal Oncology*

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WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Basic Study

## Effects of *Helicobacter pylori* and Moluodan on the Wnt/ $\beta$ -catenin signaling pathway in mice with precancerous gastric cancer lesions

Yi-Mei Wang, Zheng-Wei Luo, Yu-Lin Shu, Xiu Zhou, Lin-Qing Wang, Chun-Hong Liang, Chao-Qun Wu, Chang-Ping Li

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### Abstract

#### BACKGROUND

*Helicobacter pylori* (*H. pylori*) is the primary risk factor for gastric cancer (GC), the Wnt/ $\beta$ -Catenin signaling pathway is closely linked to tumorigenesis. GC has a high mortality rate and treatment cost, and there are no drugs to prevent the progression of gastric precancerous lesions to GC. Therefore, it is necessary to find a novel drug that is inexpensive and preventive to against GC.

#### AIM

To explore the effects of *H. pylori* and Moluodan on the Wnt/ $\beta$ -Catenin signaling pathway and precancerous lesions of GC (PLGC).

#### METHODS

Mice were divided into the control, N-methyl-N-nitrosourea (MNU), *H. pylori* + MNU, and Moluodan groups. We first created an *H. pylori* infection model in the *H. pylori* + MNU and Moluodan groups. A PLGC model was created in the remaining three groups except for the control group. Moluodan was fed to mice in the Moluodan group ad libitum. The general condition of mice were observed during the whole experiment period. Gastric tissues of mice were grossly and microscopically examined. Through quantitative real-time PCR (qRT-PCR) and Western blotting analysis, the expression of relevant genes were detected.

#### RESULTS

Mice in the *H. pylori* + MNU group showed the worst performance in general condition, gastric tissue visual and microscopic observation, followed by the MNU group, Moluodan group and the control group. QRT-PCR and Western blotting analysis were used to detect the expression of relevant genes, the results



showed that the *H. pylori* + MNU group had the highest expression, followed by the MNU group, Moluodan group and the control group.

## CONCLUSION

*H. pylori* can activate the Wnt/ $\beta$ -catenin signaling pathway, thereby facilitating the development and progression of PLGC. Moluodan suppressed the activation of the Wnt/ $\beta$ -catenin signaling pathway, thereby decreasing the progression of PLGC.

**Key Words:** *Helicobacter pylori*; Gastric cancer; Wnt/ $\beta$ -catenin signaling pathway; Moluodan

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**Core Tip:** *Helicobacter pylori* (*H. pylori*) is a pathological bacteria. We explored the effects of *H. pylori* and the traditional Chinese medicine Moluodan on the Wnt/ $\beta$ -Catenin signaling pathway and precancerous lesions of GC (PLGC). Our experiments successfully established a mouse model of *H. pylori* infection and PLGC, which serves as a reference for others. Through the gene expression assay, we concluded that *H. pylori* accelerates the progression of PLGC by promoting the expression of Wnt/ $\beta$ -Catenin signaling pathway, epidermal growth factor (EGF) and c-Myc. Meanwhile, Moluodan can repair the gastric mucosa and delay the progression of PLGC by inhibiting the Wnt/ $\beta$ -Catenin signaling pathway and the expression of EGF and c-Myc.

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## INTRODUCTION

Gastric cancer (GC) is a significant health issue in China that is characterized by its complex etiology, high prevalence and mortality rates, and challenges in treatment[1]. GC ranks among the top three cancers in terms of mortality worldwide [2]. Unfortunately, early symptoms of GC are often inconspicuous, leading to delayed detection and missed opportunities for timely treatment. Notably, the development of GC involves stages such as chronic atrophic gastritis, heterogeneous hyperplasia, and intestinal epithelial hyperplasia, which are all considered as precancerous lesions[3,4]. Thus, implementing proactive interventions during the early stages of the disease can effectively impede the progression of precancerous lesions of GC (PLGC), thereby reducing the incidence of GC.

*Helicobacter pylori* (*H. pylori*) is a type of bacteria that thrives in the stomach and damages the gastric mucosa primarily through the production of urease and induction of immune responses[5]. Numerous reports, including the Kyoto Global Consensus, have identified *H. pylori* as a leading risk factor for GC[6]. Consequently, the timely eradication of *H. pylori* has become crucial in delaying the progression to GC. The Wnt/ $\beta$ -catenin signaling pathway is a ubiquitous intracellular signaling pathway that plays a role in early embryonic development by promoting cell proliferation and epithelial mesenchymal transition (EMT); this pathway has also been implicated in the development of various tumors[7,8]. Notably, the Wnt/ $\beta$ -catenin signaling pathway has a strong association with GC. Studies have demonstrated that this pathway can facilitate the development of GC through mechanisms involving microRNA, ligands, receptors, and other factors[9-11]. The relationship between *H. pylori* and signaling pathways has garnered increasing attention, which this study aimed to investigate. Traditional Chinese medicines have gained popularity among medical practitioners and have been extensively used in clinical practice, particularly in infection, tumors, and digestive, nervous, and cardiovascular systems[12,13]. One such medicine is Moluodan, which is commonly used in the treatment of digestive system disorders such as chronic gastritis and indigestion[14]. However, its role in PLGC and GC prevention as well as its relationship with the Wnt/ $\beta$ -catenin signaling pathway have not been explored. Therefore, the second objective of this study was to examine the association between Moluodan and the Wnt/ $\beta$ -catenin signaling pathway and its potential preventive effects against the development and progression of PLGC. This research aimed to provide a novel approach for the prevention and treatment of GC.

## MATERIALS AND METHODS

### *H. pylori* recovery and succession

*H. pylori* Sydney strain SS1 (donated by the Third Military Medical University) was retrieved from a  $-80^{\circ}\text{C}$  ultra-low temperature refrigerator (Thermo). The strain was then resuscitated at room temperature and mixed using a pipette gun.



Subsequently, 100  $\mu\text{L}$  of the bacterial solution was inoculated onto a solid culture medium. The bacterial solution was allowed to dry on the surface of the medium before being inverted and placed in a  $\text{CO}_2$  incubator for incubation at  $37^\circ\text{C}$  with 5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , and 85%  $\text{N}_2$ , creating a microaerobic environment. After 48–72 h, pinpoint-sized transparent colonies were observed. The presence of *H. pylori* was confirmed through HE staining, rapid urease testing, and catalase testing. A sterile inoculation loop was used to scrape a small quantity of cultured *H. pylori* colonies, which were evenly mixed with a PBS buffer. Subsequently, 100  $\mu\text{L}$  of the mixture was aspirated and inoculated onto the next solid medium; the growth of colonies was observed within 48–72 h. Prior to administration in mice *via* gavage, *H. pylori* colonies were selected and dissolved in a PBS buffer. The concentration of the resulting liquid was controlled using turbidimetric methods to achieve a density of  $1 \times 10^9$  colony forming units per milliliter of *H. pylori* mixture.

### Configuration of N-methyl-N-nitrosourea solution

The powdered form of N-methyl-N-nitrosourea (MNU) was stored in a refrigerator at  $4^\circ\text{C}$ , ensuring protection from light. For the experiments, a solution with a concentration of 240 ppm (0.024%) was prepared by diluting the MNU in distilled water. The pH of the solution was maintained at 4.5 using a citric acid configuration solution. Fresh solution was prepared twice a week, and the bottle containing the solution was stored away from light to ensure the well-being of the free-range mice.

### Experimental animals

In this study, 14 SPF-grade Balb/c male mice, aged 6–8 wk, were obtained from Changzhou Cavins Experiment Co. They were allocated into four groups: A control group consisting of 3 mice, the MNU group consisting of 4 mice, the *H. pylori* + MNU group consisting of 4 mice, and the Moluodan group consisting of 3 mice. The mice were housed in a sterile laboratory with laminar air flow for 1 wk. The mice were subjected to a 12-h light–dark cycle, and the room temperature was maintained at  $22\text{--}24^\circ\text{C}$  with a humidity of 40%–50%. The mice were provided with standard mouse maintenance chow and had unrestricted access to water. Then the subsequent treatments were administered as follows: (1) Control group: On the second week, mice were fasted for 24 h, gavaged with 0.5 mL of saline, and resumed their normal diet after 4 h. The procedure was repeated every 2 d for five consecutive times. On the third week, the mice were fed with normal maintenance chow and sterile distilled water; (2) MNU group: The first 2 wk were similar to that of the control group. From the third week, the mice were given 240 ppm of MNU solution to drink freely. They were also protected from light and fed with 0.03% ranitidine (Beijing Jingming Biotechnology Co.) with their diet for 2 d followed by fasting for 1 d. On the fasting day, 10% of NaCl solution was heated to  $56^\circ\text{C}$ , and mice were gavaged at a dose of 10 mL/kg. On week 23, one mouse was randomly sacrificed, and stomach tissues were taken, stained with HE, and microscopically observed to confirm the development of PLGC. The rest of the mice were continuously fed with normal chow and sterile distilled water until week 29; (3) *H. pylori* + MNU group: Mice were fasted for 12 h at the beginning of the second week. Each mouse received 0.2 mL of 2%  $\text{H}_2\text{NCO}_3$  solution followed by 0.4 mL of *H. pylori* solution for 1 h; normal diet was resumed after 4 h. This procedure was repeated once every 2 d for five consecutive times. From the third week until the end of the 29<sup>th</sup> week, the feeding regimen of the MNU group was adopted. On week 10, one mouse was sacrificed; stomach tissues were taken, stained with HE and Giemsa, and tested with the rapid urease assay to confirm *H. pylori* infection; and (4) Moluodan group: Mice in this group were fed the same way as those in the *H. pylori* group for the first 23 wk. After successful PLGC model creation, distilled water was substituted for a solution of 10 g/L Moluodan small honey pills (Handan Pharmaceutical Co.) in combination with normal chow until the end of the 29<sup>th</sup> week.

### Sample collection

At the end of the 29<sup>th</sup> week, mice were fasted for 24 h. The mice were then intraperitoneally injected with 10% trichloroacetaldehyde hydrate at a dosage of 3 mL/kg. Gastric tissues were then exposed, isolated, and incised along the greater curvature of the stomach and thoroughly rinsed with 0.9% NaCl solution. Subsequently, tissues were grossly observed for histopathological and morphological changes. To confirm *H. pylori* infection, gastric sinus tissues from both the *H. pylori* + MNU and Moluodan groups underwent HE and Giemsa staining as well as rapid urease assay. Additionally, gastric tissues in all mice underwent HE staining after fixation with 10% formaldehyde, allowing for the microscopic observation of pathological changes. A portion of the samples were immediately frozen in liquid nitrogen and stored in a refrigerator at  $-80^\circ\text{C}$  for subsequent molecular testing.

### *H. pylori* detection

**Rapid urease method:** The procedure was performed according to the manufacturer's instructions (Guangzhou Shunaimi Biotechnology Co.). The transparent film covering the urease test paper was carefully removed up to the dotted line. Fresh gastric sinus tissue was obtained, cut into small pieces, and placed in the center of the rapid urease test paper. Subsequently, the film was closed to seal the test paper. A color change from yellow to red in the central area of the paper within 3 min indicated a positive result for *H. pylori*.

**HE stain:** Fresh gastric sinus tissues were processed for histological analysis. Tissues were embedded in paraffin, sectioned, and subsequently deparaffinized. Hematoxylin staining was performed to visualize the nuclei, followed by ammonia rebluing and eosin re-staining. Dehydration and transparency of the tissues were achieved by immersing them in anhydrous ethanol and xylene. Tissue sections were then sealed with neutral tree glue. The presence of short bluish-purple rod-shaped or curved bacteria indicated the presence of *H. pylori*.

**Giemsa stain:** Fresh gastric sinus tissues were fixed using a 10% paraformaldehyde solution and embedded in paraffin. The paraffin-embedded tissues were then sectioned into 4- $\mu\text{m}$  thick slices. To enhance hydrophilicity, sections were

sequentially dewaxed using xylene and alcohol. Giemsa stain was applied dropwise onto the sections and allowed to stand for 30 min at 37°C. Following staining, the slides were washed with distilled water and soaked in xylene for 5 min until they became clear. Finally, the slides were sealed using neutral tree glue.

### **HE-stained light microscopic observation of pathological histological changes in gastric tissues**

Following sacrifice, a segment of stomach tissues of mice was preserved in a 10% formaldehyde solution. Subsequently, the tissues were embedded in paraffin and cut into 4-μm thick sections. After removing the paraffin, the sections were stained in a sequential manner using HE. Following dehydration, the slices were sealed with neutral gum. The gastric mucosa of the mice was then examined under a light microscope to observe any pathological and histological alterations.

### **Relative m-RNA expression of *Wnt1*, *β-catenin*, *cyclinD1*, *epidermal growth factor* and *c-Myc* in the gastric tissues of mice as detected by quantitative real-time PCR**

Mouse stomach tissue specimens were removed from the -80°C ultra-low temperature refrigerator and thawed at room temperature. They were then thoroughly ground in TRIpure Total RNA Extraction Reagent (EP013 ELK Biotechnology) and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was collected and centrifuged again for 10 min with isopropanol, and the supernatant was discarded. The RNA precipitate was washed with 75% ethanol, and 100 μL of RNase-Free Water was added to completely solubilize the RNA. cDNA was synthesized by reverse transcription using the EntiLink™ 1st Strand cDNA Synthesis Super Mix kit (EQ031 ELK Biotechnology) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using the EnTurbo™ SYBR Green PCR Supermix Kit (EQ001 ELK Biotechnology) according to the manufacturer's instructions. Briefly, pre-denaturation was performed at 95°C for 30 s followed by denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The total volume was 10 μL, and 40 cycles were performed. Finally, the relative amount of mRNA for each gene was determined using the  $2^{-\Delta\Delta C_t}$  method. The reverse transcription primers employed are listed in Table 1.

### **Relative expression of *Wnt1*, *β-catenin*, *cyclinD1*, *epidermal growth factor* and *c-Myc* proteins as detected by Western blot analysis**

Gastric tissues were washed several times with PBS buffer, cut into pieces, and added to a tissue protein extraction reagent. The supernatant was collected after lysis in an ice bath. The BCA Protein Concentration Assay Kit (AS1086 ASPEN) was used to determine the protein concentration of the sample. After sample processing, SDS-PAGE electrophoresis and membrane transfer were conducted. The transferred membranes were incubated with the sealing solution for 1 h at room temperature, and the primary antibody (AS1061 ASPEN) was added and incubated overnight at 4°C. The membranes were again incubated for 1 h at room temperature with the sealing solution. After buffer rinsing, the secondary antibody (AS1058 ASPEN) was added, and the solution was re-incubated. Finally, the membranes were exposed, developed, and fixed, and the optical density values of the target bands were analyzed using AlphaEaseFC software.

### **Statistical methods**

Statistical analyses were performed using SPSS 27.0 software, while image depiction was performed using GraphPad Prism 9.0 software. Experimental data are presented as mean ± SD. One-way analysis of variance was conducted for between-group comparisons.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### **Changes in the behavior, physical appearance, and gastrointestinal function of mice**

The control group exhibited an optimal mental condition as characterized by high activity levels, responsive reflexes, normal food consumption, glossy and thick fur, and regular and well-formed stools. Meanwhile, mice in the MNU group displayed inferior conditions than that of the control group. The *H. pylori* + MNU group exhibited the most severe deterioration, including a significantly reduced mental status, dull eyes, decreased activity levels, slow reflexes, decreased food intake, thinning and lackluster fur, and loose and irregular stools. Initially, mice in the Moluodan group showed a similar condition to those in the *H. pylori* + MNU group. However, upon the addition of Moluodan to the animals' diet, the mental state, activity levels, feeding behavior, fur quality, and overall condition of mice improved significantly, and their stools became regular and well-formed.

### **Gross observation of histopathological phantomization of stomach tissues**

Following rinsing of gastric tissues with physiological saline, the gastric mucosa appeared smooth, gastric tissues were elastic, and no abnormalities were detected along the gastric wall. In the MNU group, there was a decrease in mucus secretion in the stomach along with a slightly rough and swollen gastric mucosa, scattered hemorrhages, and thinning and reduced elasticity of the gastric wall. Gastric tissues in the *H. pylori* + MNU group exhibited only a small amount of mucus adhering to the stomach along with a more swollen and rough stomach wall, increased inflammatory manifestations, and decreased elasticity. In comparison, gastric tissues in the Moluodan group showed an improvement in the gastric mucosal condition compared to that of the *H. pylori* + MNU and MNU groups, with increased mucus secretion, mild swelling and scattered inflammatory changes and moderate elasticity of the gastric wall.

Table 1 Reverse transcription primers of each gene

Primer name	Primer information	Base sequences (5'-3')		Tm value	Product length
M-GAPDH	NM_008084.3	Sense	TGAAGGGTGGAGCCAAAAG	58.3	227
		Anti-sense	AGTCTTCTGGGTGGCAGTGAT	58.4	
M-wnt1	NM_021279	Sense	GCAGCCTCTTCTCACTGCAG	61.0	148
		Anti-sense	CCCAGGCTGGCTCTAATAAGT	59.2	
M-β-catenin	NM_007614	Sense	TCCGAGGACTCAATACCATTCC	60.6	250
		Anti-sense	CGCTTCTTGTAATCCTGTGGC	60.0	
M-cyclinD1	NM_007631.2	Sense	GGATGAGAACAAGCAGACCATC	58.6	186
		Anti-sense	AGAAAGTGCCTGTGTGCGGTA	59.2	
M-EGF	NM_010113	Sense	TTATGACCCTGTGGAAAGCAAG	58.6	119
		Anti-sense	CAAGCGTATCTACTCTTCTGTGAT	60.2	
M-c-myc	NM_001177352.1	Sense	GGACTGTATGTGGAGCGGTTT	59.1	208
		Anti-sense	GTTGAGCGGGTAGGGAAGA	59.3	

### Detection of *H. pylori* infection

The findings indicated a positive urease test. HE staining revealed the presence of blue-purple rod-shaped *H. pylori* bacteria (Figure 1A). Additionally, Giemsa staining revealed purple short clostridial *H. pylori* bacteria (Figure 1B). Notably, all mice in both groups exhibited a 100% *H. pylori* infection rate.

### HE staining to observe the pathological changes in the gastric mucosa

Under light microscopy, gastric tissues of mice in the control group exhibited intact gastric mucosal glandular architecture without any infiltration of inflammatory cells, abnormal cell proliferation, or pathological nuclear division (Figure 2A). In contrast, those of mice in the MNU group displayed a small number of heterogeneous cells with enlarged and deeply stained nuclei, moderate infiltration of inflammatory cells, and a few cells with pathological nuclear division (Figure 2B). The gastric glands of mice in the *H. pylori* + MNU group exhibited significant disorganization, evident cellular heterogeneity, an increased nucleoplasmic ratio, pronounced staining of the nucleus, fusion of some cells, extensive inflammatory cell infiltration, and an increase in pathological divisions (Figure 2C). Comparatively, the gastric mucosal condition of mice in the Moluodan group showed improvement compared to that of the *H. pylori* + MNU and MNU groups as characterized by reduced disorganization of the glandular structure and inflammatory cell infiltration as well as the absence of significant cellular anisotropy or pathological nuclear division (Figure 2D).

### M-RNA expression of Wnt1, β-catenin, cyclinD1, epidermal growth factor, and c-Myc in gastric tissues by qRT-PCR

There was significantly increased Wnt1, β-catenin, cyclinD1, epidermal growth factor (EGF), and c-Myc m-RNA expression in the MNU group compared to the Moluodan and control groups ( $P < 0.05$ ; Table 2). Wnt1, β-catenin, cyclinD1, EGF, and c-Myc m-RNA expression in the *H. pylori* + MNU group was significantly greater than that in the MNU, Moluodan, and control groups and was the highest among the four groups ( $P < 0.05$ ). Meanwhile, the Moluodan group showed higher expression levels of Wnt1, β-catenin, cyclinD1, EGF, and c-Myc compared to the control group, but lower levels compared to the MNU and *H. pylori* + MNU groups; all differences were significant ( $P < 0.05$ ; Figure 3).

### Western blotting to detect the expression of Wnt1, β-catenin, cyclinD1, EGF, and c-Myc

There was a significant increase in the expression of Wnt1, β-catenin, cyclinD1, EGF, and c-Myc in the MNU group compared to the Moluodan and control groups ( $P < 0.05$ ; Table 3). Furthermore, Wnt1, β-catenin, cyclinD1, EGF, and c-Myc expression in the *H. pylori* + MNU group was significantly greater than that in the MNU, Moluodan, and control groups and was the highest among all groups ( $P < 0.05$ ). In the Moluodan group, expression levels of Wnt1, β-catenin, cyclinD1, EGF, and c-Myc were higher than those in the control group but lower than those in the MNU and *H. pylori* + MNU groups; all differences were significant ( $P < 0.05$ ; Figure 4).

## DISCUSSION

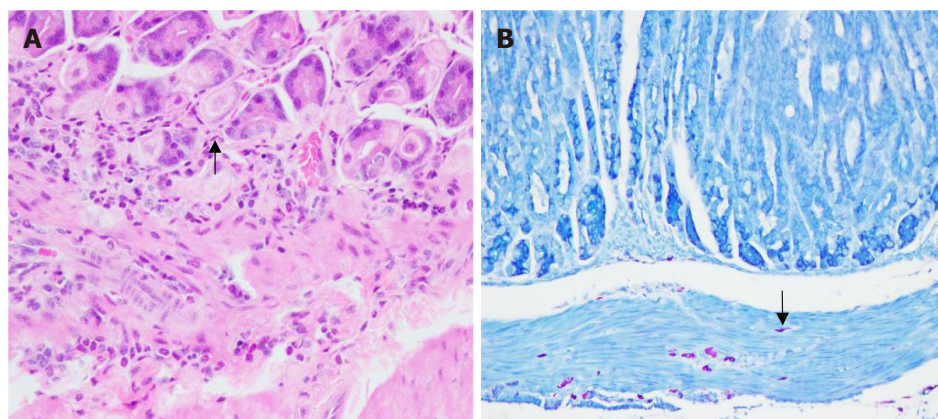
*H. pylori* is highly prevalent and is distributed worldwide[15]. Over 4 billion individuals worldwide are infected with *H. pylori*, with a particularly high prevalence in Asian countries[16,17]. *H. pylori* infection can lead to various diseases, such as indigestion, gastrointestinal ulcers, MALT lymphoma, and even GC. Failure to promptly eradicate *H. pylori* infection can result in significant detrimental effects on human health[18]. Factors, such as the irrational selection of antibiotics, non-standard treatment, and the development of drug resistance, have contributed to an increasing number of cases

**Table 2 Comparison of relative expression of Wnt1,  $\beta$ -catenin, cyclinD1, epidermal growth factor, and c-Myc m-RNA in each group**

Norm	Control group	MNU group	<i>H. pylori</i> + MNU group	Moluodan group	F value	P value
Wnt1	1.10 $\pm$ 0.12	3.69 $\pm$ 0.16 <sup>a</sup>	4.72 $\pm$ 0.06 <sup>a,b</sup>	2.99 $\pm$ 0.19 <sup>a,b,c</sup>	347.51	< 0.001
$\beta$ -catenin	1.03 $\pm$ 0.03	2.74 $\pm$ 0.11 <sup>a</sup>	3.73 $\pm$ 0.12 <sup>a,b</sup>	1.70 $\pm$ 0.06 <sup>a,b,c</sup>	523.67	< 0.001
CyclinD1	1.06 $\pm$ 0.17	3.22 $\pm$ 0.07 <sup>a</sup>	3.84 $\pm$ 0.16 <sup>a,b</sup>	2.17 $\pm$ 0.11 <sup>a,b,c</sup>	248.70	< 0.001
EGF	1.08 $\pm$ 0.07	2.96 $\pm$ 0.14 <sup>a</sup>	4.32 $\pm$ 0.24 <sup>a,b</sup>	2.17 $\pm$ 0.11 <sup>a,b,c</sup>	230.00	< 0.001
c-Myc	1.10 $\pm$ 0.14	2.80 $\pm$ 0.14 <sup>a</sup>	4.40 $\pm$ 0.08 <sup>a,b</sup>	1.97 $\pm$ 0.11 <sup>a,b,c</sup>	411.91	< 0.001

<sup>a</sup>*P* < 0.05 *vs* control group.<sup>b</sup>*P* < 0.05 *vs* N-methyl-N-nitrosourea group.<sup>c</sup>*P* < 0.05 *vs* *Helicobacter pylori* + N-methyl-N-nitrosourea group.EGF: Epidermal growth factor; MNU: N-methyl-N-nitrosourea; *H. pylori*: *Helicobacter pylori*.**Table 3 Comparison of Wnt1,  $\beta$ -catenin, cyclinD1, epidermal growth factor, and c-Myc protein expression in each group**

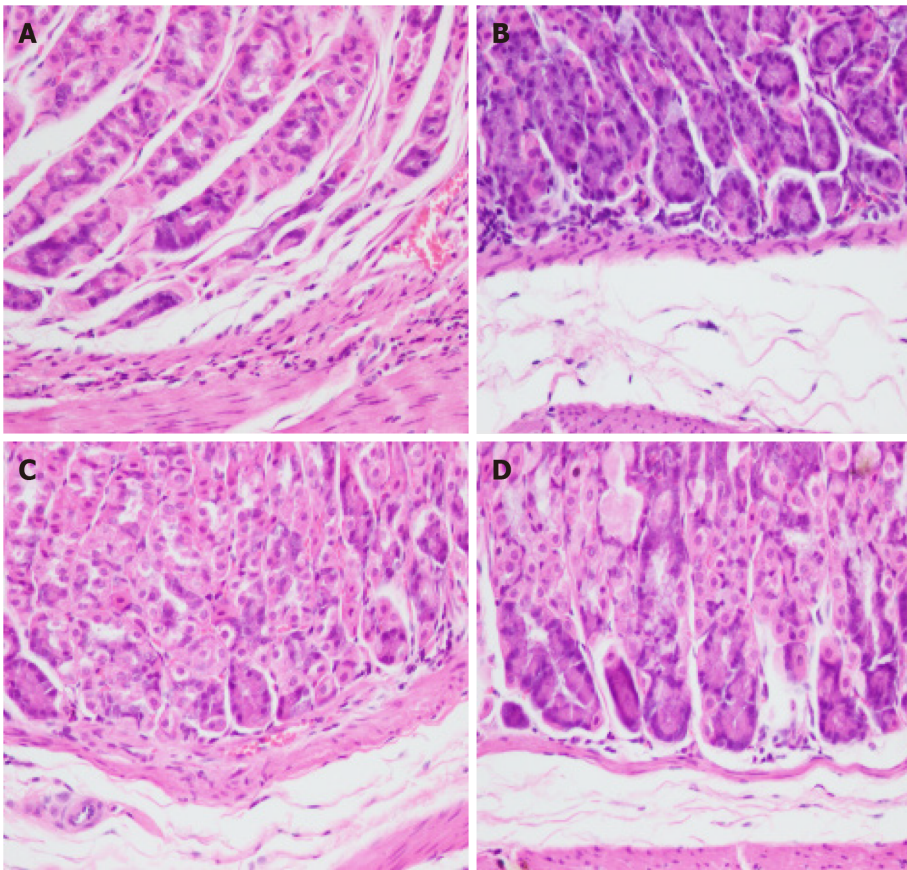
Norm	Control group	MNU group	<i>H. pylori</i> + MNU group	Moluodan group	F value	P value
Wnt1	0.05 $\pm$ 0.01	0.29 $\pm$ 0.02 <sup>a</sup>	0.58 $\pm$ 0.02 <sup>a,b</sup>	0.12 $\pm$ 0.02 <sup>a,b,c</sup>	479.39	< 0.001
$\beta$ -catenin	0.06 $\pm$ 0.04	0.26 $\pm$ 0.08 <sup>a</sup>	0.43 $\pm$ 0.06 <sup>a,b</sup>	0.16 $\pm$ 0.06 <sup>a,b,c</sup>	18.80	< 0.001
cyclinD1	0.09 $\pm$ 0.04	0.31 $\pm$ 0.12 <sup>a</sup>	0.60 $\pm$ 0.10 <sup>a,b</sup>	0.15 $\pm$ 0.07 <sup>a,b,c</sup>	11.59	0.003
EGF	0.06 $\pm$ 0.03	0.17 $\pm$ 0.02 <sup>a</sup>	0.39 $\pm$ 0.08 <sup>a,b</sup>	0.09 $\pm$ 0.02 <sup>a,b,c</sup>	35.26	< 0.001
c-Myc	0.05 $\pm$ 0.01	0.23 $\pm$ 0.06 <sup>a</sup>	0.54 $\pm$ 0.06 <sup>a,b</sup>	0.13 $\pm$ 0.02 <sup>a,b,c</sup>	64.19	< 0.001

<sup>a</sup>*P* < 0.05 *vs* control group.<sup>b</sup>*P* < 0.05 *vs* N-methyl-N-nitrosourea group.<sup>c</sup>*P* < 0.05 *vs* *Helicobacter pylori* + N-methyl-N-nitrosourea group.EGF: Epidermal growth factor; MNU: N-methyl-N-nitrosourea; *H. pylori*: *Helicobacter pylori*.**Figure 1 Staining results of mouse gastric sinus tissue.** A: HE staining of mouse gastric antrum tissue (400  $\times$ ); B: Gimesa-stained image of mouse gastric sinus tissue (200  $\times$ ). The arrows indicate the location of the *Helicobacter pylori*.

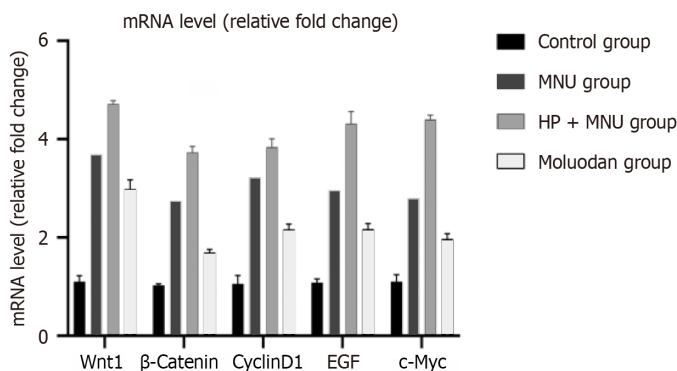
wherein *H. pylori* is refractory to treatment. A study involving 180000 individuals revealed that gender, irregular medication use, smoking, alcohol consumption, previous stomach diseases, and obesity are risk factors for the failure of *H. pylori* eradication, with a higher failure rate observed in men compared to women[19]. Therefore, it is crucial to further investigate the mechanisms of interaction between *H. pylori* and the human body as well as to explore new methods for eradicating *H. pylori* and treating stomach diseases caused by *H. pylori*. In this study, a mouse model of *H. pylori* infection was established. Ultimately, both the *H. pylori* and Moluodan groups were successfully infected with *H. pylori*. The infection mode in mice was similar to that in humans, and we confirmed that after 8 wk of gavage, *H. pylori* could stably colonize the surface of the gastric mucosa and cause damage.

MNU, which is a chemical preparation commonly utilized in conjunction with *H. pylori*, is frequently employed to establish animal models of PLGC and GC. MNU has also been employed as a tumor inducing agent for modeling





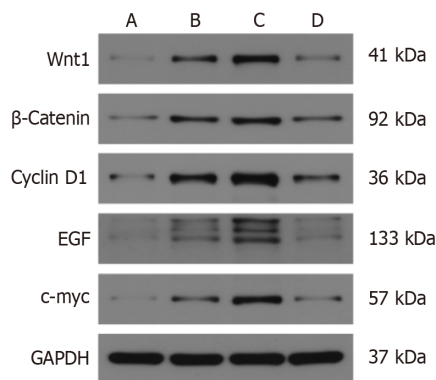
**Figure 2** Pathological observation of HE stain of mouse stomach tissue (400 ×). A: Control group; B: N-methyl-N-nitrosourea (MNU) group; C: *Helicobacter pylori* + MNU group; D: Moluodan group.



**Figure 3** Relative expression of Wnt1, β-catenin, cyclinD1, epidermal growth factor, c-Myc m-RNA in each group. EGF: Epidermal growth factor; MNU: N-methyl-N-nitrosourea; *H. pylori*: *Helicobacter pylori*.

colorectal and prostate cancers, demonstrating its efficacy in this regard[20-22]. Throughout our experiment, when only *H. pylori* infection was induced, mice in the *H. pylori* + MNU and Moluodan groups had poorer diets than those in the other groups accompanied by reduced activity, poor mental status, and irregular and unformed stools. After introducing MNU into the PLGC model, a noticeable decline in the mental state of the mice was observed in all groups except for those in the control group. This decline was characterized by significant weight loss, reduced appetite, hair loss, and decreased activity; these symptoms closely resemble those seen during the chronic progression of human tumors. Additionally, the administration of Moluodan, which is a therapeutic agent that alleviates gastrointestinal bloating and belching, improved symptoms. Specifically, mice in the Moluodan group exhibited increased consumption of the agent when Moluodan was provided in their drinking water, leading to improvements in mental state, bowel movements, and activity levels. These findings further support the notion that Moluodan possesses stomach-protective and gastrointestinal symptom-improving properties.

After the administration of *H. pylori* and MNU to PLGC mice, gastric tissues were obtained and examined, showing that gastric tissues of mice in the *H. pylori* + MNU group exhibited the most pronounced tissue inflammation. Additionally, the gastric wall of was noticeably swollen with red and white spots indicating granular bleeding, consistent



**Figure 4** Western blot of Wnt1,  $\beta$ -catenin, cyclinD1, epidermal growth factor, and c-Myc protein expression in each group. A: Control group; B: *Helicobacter pylori* +N-methyl-N-nitrosourea (MNU) group; C: MNU group; D: Moluodan group.

with chronic atrophic gastritis. Comparatively, gastric tissues in mice in the MNU group showed slightly less severe inflammation and atrophic gastritis. However, upon the addition of Moluodan, there was a significant improvement in the condition of the gastric mucosa accompanied by a reduction in symptoms. Furthermore, the eating and bowel movements of mice also showed significant improvement. These findings provide evidence that *H. pylori* infection can exacerbate damage to the gastric mucosa and accelerate the development and progression of malignant PLGC such as atrophic gastritis. Moluodan may have potential benefits in improving the inflammatory condition of the gastric mucosa by treating gastric mucosal lesions caused by *H. pylori* and potentially preventing or reversing the progression of precancerous lesions. Lee *et al*[23] reported the effects of *H. pylori* and MNU alone and in combination on the induction rate of GC in mice. Their results revealed that the group treated with both *H. pylori* and MNU had the highest induction rate of GC, reaching 37.5%; this was significantly higher compared to that in the other groups. It was suggested that this combination may decrease levels of the Rev-Erb protein and increase levels of IL-1 $\beta$ , thereby promoting the development of GC[23,24].

From a microscopic perspective, the glandular structure of gastric tissues in mice in the *H. pylori* + MNU group exhibited significant disorder as characterized by pronounced nucleolar staining and enlargement. Inflammatory cells were more prevalent with mitotic figures being the most common, and signs of precancerous lesions were highly evident. Conversely, in the MNU group, changes in the microscopic glandular structure, inflammatory infiltration, and pathological mitotic images were less frequent. However, following the administration of Moluodan, inflammatory cell infiltration in the Moluodan group significantly decreased, and the glandular structure reverted to its normal state. Pathological mitotic figures became imperceptible, and nuclei reverted to their normal size. These findings suggest that Moluodan exerts a robust tissue recovering capacity that is capable of visually and microscopically repairing gastric tissue. This mechanism enables the reversal of PLGC, thereby preventing the progression and development of GC. Amieva and Peek[25] conducted a simulation study on the effects of *H. pylori* infection on the gastric mucosal epithelium and discovered that external factors, such as diet, micronutrients, and gastrointestinal microbiota, contribute to changes in the epithelium. Additionally, virulence factors of *H. pylori*, including CagA, are significant pathogenic factors[26]. These findings provide valuable insights for future research and exploration in this field.

The Wnt/ $\beta$ -Catenin signaling pathway plays a crucial role in human growth and development, cell homeostasis, and tissue signal transmission[27,28]. It is also considered the most fundamental signaling pathway in the Wnt family signaling cascade and is involved in various biological processes such as cell proliferation, tissue self-renewal, and EMT, particularly during embryonic development[29,30]. Recently, research on the association between this pathway and the occurrence and progression of tumors, including gastric, endometrial, liver, and adrenal cortical, cancer have gained attention[31-34]. The Wnt/ $\beta$ -Catenin signaling pathway is comprised of the Wnt ligand, Wnt receptor (Frizzled and LRP5/6), intermediate  $\beta$ -Catenin protein, and downstream signaling molecules such as c-Myc and cyclinD1[7,35,36]. Activation of the pathway occurs when Wnt1 or other ligands stimulate the receptor, preventing the phosphorylation degradation of  $\beta$ -Catenin[37,38]. Subsequently,  $\beta$ -Catenin translocates to the nucleus and binds with cytokines TCF/LEF, promoting the transcription and expression of downstream genes[39]. Deletion or inactivation of the adenomatous polyposis coli gene can activate this pathway and is believed to be an initiating factor in the development of colorectal cancer[40]. Wu *et al*[10] confirmed that the long chain non-coding RNA SNHG11 facilitates the activation of the Wnt/ $\beta$ -Catenin signaling pathway by inducing the ubiquitination of the intermediate signal GSK-3 $\beta$ , thereby promoting the progression of gastric tumor cells. In our study, we employed qRT-PCR to detect the expression of mRNA for Wnt1,  $\beta$ -Catenin, and downstream Cyclin D1 signaling molecules on the Wnt/ $\beta$ -Catenin pathway. Additionally, we utilized Western blotting to assess the protein expression of these aforementioned genes. Our results revealed that the transcription of m-RNA and protein expression of these genes were highest in the *H. pylori* + MNU group followed by the MNU group and the Moluodan group, demonstrating that *H. pylori* can enhance the expression of the Wnt/ $\beta$ -Catenin signaling pathway, which decreased after Moluodan administration. However, the specific mechanisms or smaller signaling molecules through which they exert these effects require further investigation.

EGF is a cytokine that plays a crucial role in cellular growth, development, and tumorigenesis. The receptor for EGF, known as EGF receptor (EGFR), has been implicated in the progression and treatment of various types of tumors[41,42]. Sheng *et al*[43] demonstrated that calreticulin facilitates EGF-induced changes in the transformation of pancreatic cancer

cells through the integrin/EGFR-ERK/MAPK signaling pathway. In our study, we assessed the transcription of m-RNA and protein expression levels in relation to EGF expression. Our findings revealed that the *H. pylori* + MNU group exhibited the highest expression of both m-RNA and corresponding proteins followed by the MNU group, while the Moluodan group displayed the lowest expression. This indicates that *H. pylori* enhances the expression of the tumor-associated factor EGF, whereas Moluodan reduces its expression. Additionally, C-Myc, which is an important proto-oncogene, can contribute to tumorigenesis when mutated or overexpressed. c-MYC plays a significant role in various cancer-related processes, such as cell reprogramming, immune evasion, and resistance to chemotherapy, primarily through epigenetic modifications[44,45]. In this study, m-RNA transcription and protein expression levels of c-MYC were assessed as part of the molecular assay. Results showed that the *H. pylori* + MNU group exhibited the highest levels of c-MYC expression followed by the MNU and Moluodan groups. Furthermore, *H. pylori* promoted the expression of c-MYC, while Moluodan inhibited its expression. However, the specific mechanisms or molecules through which these effects occur require further investigation. Moluodan, which is a commonly used Chinese proprietary medicine, is currently being explored for its potential therapeutic applications. Traditional Chinese medicine is gaining recognition on the international stage.

## CONCLUSION

In conclusions, *H. pylori* infection promotes the activation of the Wnt/ $\beta$ -catenin signaling pathway and accelerates the progression of PLGC in mice. Furthermore, Moluodan inhibits the activation of the Wnt/ $\beta$ -catenin signaling pathway, protects the gastric mucosa, treats *H. pylori* -infected gastric mucosal lesions, and halts and reverses the development and progression of PLGC.

## ARTICLE HIGHLIGHTS

### Research background

The early diagnosis of gastric cancer (GC) is difficult. It has the characteristics of high incidence rate and high mortality. Precancerous lesion is an important stage in the development of GC. *Helicobacter pylori* (*H. pylori*) is the primary risk factor of GC, Wnt/ $\beta$ -catenin signaling pathway is closely related to tumor development. So their relationships with precancerous lesion should be further explored in order to explore the specific mechanisms of GC occurrence and find new drugs to prevent the development of GC.

### Research motivation

Constructing a dual model of *H. pylori* infection and precancerous lesions of GC (PLGC) in mice is rare, and we are inspired by some recent researches. Moluodan is a traditional Chinese patent medicine and commonly used to treat digestive tract diseases. It has not yet been used for the prevention and treatment of GC. We can explore its role in the prevention of GC so that to increase its broader clinical pharmacological effects.

### Research objectives

Our aim is to establish a double mouse model of *H. pylori* infection and PLGC, and find a new traditional Chinese patent medicine that can prevent the development of GC.

### Research methods

We established a dual model of *H. pylori* infection and PLGC in mice. After successful modeling, the mice were freely fed with Moluodan aqueous solution to achieve the goal of drug treatment. We observed the general condition of mice throughout the entire experimental period. Subsequently, the mice were killed to detect the infection rate of *H. pylori*, and the pathological changes of the gastric tissue were detected by gross observation and light microscopy. The expression of Wnt/ $\beta$ -Catenin signaling pathway, EGF and c-Myc was detected by quantitative real-time PCR (qRT-PCR) and Western blot analyses.

### Research results

Mice in the *H. pylori* + N-methyl-N-nitrosourea (MNU) group showed the worst performance in general condition, gastric tissue visual and microscopic observation, followed by the MNU group, Moluodan group and the control group. qRT-PCR and Western blotting analysis used to detect the expression of Wnt/ $\beta$ -Catenin signaling pathway, EGF and c-Myc showed that the *H. pylori* + MNU group had the highest expression, followed by the MNU group, Moluodan group and the control group.

### Research conclusions

*H. pylori* can promote the expression of Wnt/ $\beta$ -Catenin signaling pathway, EGF and c-Myc and accelerate the malignant progression of gastric tissues; Moluodan can inhibit the expression of Wnt/ $\beta$ -Catenin signaling pathway, EGF and c-Myc, protect the gastric mucosa, treat *H. pylori* -infected gastric mucous lesions, and prevent the malignant development of gastric tissues.



## Research perspectives

Moluodan has the effect of preventing the progression of PLGC, further in-depth researches can be conducted in the future to explore its deeper mechanisms of action.

## FOOTNOTES

**Author contributions:** Wang YM was responsible for purchasing materials, conducting relevant experiments, and writing articles; Luo ZW was responsible for designing the experimental plan and assisting in completing the experiment and the article; Shu YL, Zhou X, and Wang LQ were responsible for assisting in completing experiments; Liang CH and Wu CQ was responsible for helping to collect data; Li CP was responsible for guiding experiments and methods and editing the article; all authors approved the final version of the article.

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