

## Connexin 32 and 43 promoter methylation in *Helicobacter pylori*-associated gastric tumorigenesis

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gastric cancer (GC;  $n = 30$ ), as well as specimens of normal gastric mucosa without *H. pylori* infection (NGM;  $n = 25$ ), were confirmed by endoscopy and pathological examination. *Cx32* and *Cx43* mRNA expression was detected by real-time polymerase chain reaction (PCR). *Cx32* and *Cx43* promoter CpG island methylation status was determined by methylation-specific PCR (MSP), bisulfite PCR sequencing (BSP) and MassArray methods.

**RESULTS:** The relative mRNA expression levels in the gastric mucosa of patients with NGM, NAG, CAG, IM, DYS and GC were  $0.146 \pm 0.011$ ,  $0.133 \pm 0.026$ ,  $0.107 \pm 0.035$ ,  $0.039 \pm 0.032$ ,  $0.037 \pm 0.01$  and  $0.03 \pm 0.011$  for *Cx32*; and  $0.667 \pm 0.057$ ,  $0.644 \pm 0.051$ ,  $0.624 \pm 0.049$ ,  $0.555 \pm 0.067$ ,  $0.536 \pm 0.058$  and  $0.245 \pm 0.121$  for *Cx43*, respectively, which were gradually decreasing and significantly different (GC vs NGM:  $P < 0.001$  for *Cx32*,  $P < 0.001$  for *Cx43*). The promoter methylation levels in the gastric mucosa from NGM to GC stages by MSP were  $38.8\% \pm 9.0\%$ ,  $43.1\% \pm 9.4\%$ ,  $56.5\% \pm 3.1\%$ ,  $64.4\% \pm 9.7\%$ ,  $72.5\% \pm 4.2\%$  and  $79.6\% \pm 6.8\%$  for *Cx32*; and  $49.0\% \pm 3.9\%$ ,  $58.1\% \pm 5.0\%$ ,  $66.5\% \pm 7.9\%$ ,  $74.0\% \pm 8.8\%$ ,  $78.3\% \pm 3.6\%$  and  $88.7\% \pm 6.2\%$  for *Cx43*, respectively, which were gradually increasing and significantly different ( $P = 0.039$ ,  $P = 0.019$ ). The promoter methylation levels by BSP and MassArray exhibited similar trends. *Cx32* and *Cx43* mRNA expression was negatively correlated with promoter methylation status and gastric carcinogenesis stages ( $P < 0.001$ ,  $P = 0.016$ ).

**CONCLUSION:** *Cx32* and *Cx43* mRNA expression decreased gradually during *H. pylori* infection-associated gastric carcinogenesis, and it is associated with hypermethylation of these genes' promoter.

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**Key words:** Gastric cancer; *Helicobacter pylori*; *Cx32*; *Cx43*; DNA methylation

**Core tip:** The relationship between *Connexin (Cx) 32*

### Abstract

**AIM:** To explore the mechanism of abnormal *Connexin (Cx) 32* and *Cx43* expression in the gastric mucosa after *Helicobacter pylori (H. pylori)* infection.

**METHODS:** Biopsy specimens of gastric mucosa in different gastric carcinogenesis stages with *H. pylori* infection, that is, non-atrophic gastritis (NAG;  $n = 24$ ), chronic atrophic gastritis (CAG;  $n = 25$ ), intestinal metaplasia (IM;  $n = 28$ ), dysplasia (DYS;  $n = 24$ ), and

and *Cx43* mRNA expression and gene promoter methylation at different gastric carcinogenesis stages with *H. pylori* infection, that is, non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and gastric cancer, is not clear. Here, gastric mucosa biopsy specimens from these carcinogenic stages, as well as normal gastric mucosa without *H. pylori* infection, were examined for *Cx32* and *Cx43* mRNA expression and promoter methylation by real-time polymerase chain reaction and methylation detection. *Cx32* and *Cx43* mRNA expression decreased gradually during gastric carcinogenesis, and it is associated with hypermethylation of these genes' promoter.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infection is an important risk factor for gastric cancer (GC)<sup>[1]</sup>, with its carcinogenic mechanisms not yet fully understood<sup>[2,3]</sup>. *Connexin* (*Cx*) 32 and *Cx43* are key members of gap junctions between gastric epithelial cells, showing a gradual down-regulation trend from normal mucosa to precancerous lesions and GC<sup>[4]</sup>. We have found that the decrease in *Cx32* and *Cx43* expression in precancerous lesions and GC is associated with *H. pylori* infection<sup>[5]</sup>, coculture of gastric epithelial cells with *H. pylori* reduces expression of *Cx43*<sup>[6]</sup>, and eradication of *H. pylori* upregulates *Cx32* and *Cx43* expression in precancerous lesions<sup>[7]</sup>. However, the mechanisms by which *H. pylori* infection decreases *Cx32* and *Cx43* expression are unclear. Inactivation of gastric tumor suppressor genes, such as *CDX2*, *RASSF1A* and *P16(INK4A)*, is induced by promoter hypermethylation<sup>[8-10]</sup>. In this study, we observed *Cx32* and *Cx43* mRNA expression and its relationship with the promoter methylation status in different stages of GC, and from the *Cx32* and *Cx43* gene methylation perspective, to explore the mechanism of abnormal *Cx32* and *Cx43* expression after *H. pylori* infection and its role in the occurrence and development of GC.

## MATERIALS AND METHODS

### Patients and tissues

A total of 1550 patients underwent endoscopic and pathological examinations because of upper gastrointestinal symptoms between September 2011 and April 2012 in the Third Xiangya Hospital, Central South University, Changsha, China. Fifty cases at each stage of gastric carcinogenesis with *H. pylori* infection, *i.e.* non-atrophic gastritis (NAG), chronic atrophic gastritis (CAG), intesti-

nal metaplasia (IM), atypical hyperplasia (dysplasia, DYS) and GC, were screened; and 50 cases of normal gastric mucosa from age-matched subjects without *H. pylori* infection (NGM) in the same period were chosen as controls. Patients with gastric surgery or those taking antibiotics, nonsteroidal anti-inflammatory drugs, proton-pump inhibitors (PPIs), or histamine receptor (H<sub>2</sub>) antagonists within 1 mo before endoscopy were excluded. Signed informed consent was obtained from all patients and controls, and the study was approved by the hospital medical ethics committee. Endoscopic and pathological diagnosis was made according to the 8<sup>th</sup> edition of the Cecil Essentials of Medicine<sup>[11]</sup>, Chinese consensus on chronic gastritis<sup>[12]</sup> and Chinese guidelines for diagnosis and treatment of gastric cancer (2011 edition)<sup>[13]</sup>. Generally, the endoscopic findings of NAG included mucosal congestion and edema, accompanied with little hemorrhage and erosion, and those of CAG included a thinning mucous layer, shallowing or disappearing folds, visibility of the submucosal vascularity, and fine granules on the surface. The pathological findings of NAG included necrosis of the superficial mucosal epithelium and infiltration of lymphocytes and plasma cells in the lamina propria, and those of CAG included shrinking gastric glands with a reduced number and shallowing of gastric pits. IM was identified by replacement of gastric epithelium by intestinal epithelium, accompanied with goblet cells secreting acidic mucus, absorptive epithelial cells with striated edge, and Paneth cells. DYS was identified by proliferation of atypical cells, but it was not sufficient to be diagnosed as cancer. GC was identified as cancerous tissues infiltrating the mucosal, submucosal or entire layers, taking on polypoid, ulcerous, and diffuse infiltrative types.

Any two positives of rapid urease test, <sup>14</sup>C-urea breath test and histological examination, or positive *H. pylori* by culture were identified as *H. pylori* infection, and if these four tests were all negative, the patient was identified as being without *H. pylori* infection. Four pieces of lesioned or normal gastric mucosa biopsies were taken by gastroscopy, and mRNA and DNA were extracted for *Cx32* and *Cx43* mRNA expression and methylation detection. According to the quantity of mRNA and DNA, as well as no significant difference in sex, age and disease duration, there were 25 cases of NGM, 24 of NAG, 25 of CAG, 28 of IM, 24 of DYS, and 30 of GC, which were screened for *Cx32* and *Cx43* expression and methylation. Table 1 shows the clinical characteristics of the study population.

### Reagents

Total RNA extraction and reverse transcription reagents were purchased from Toyobo (Osaka, Japan); Wizard Genomic DNA purification kit was purchased from Promega (Madison, WI, United States); the EpiTect Bisulfite Kit was purchased from Qiagen (Germany); and methylase (M.S.s.I) was purchased from New England Biotech (United States).

**Table 1** Sex, age and disease duration of cases of each stage (mean  $\pm$  SD)

Stage	n	M	F	Age, yr	Disease duration (yr)
NGM without <i>H. pylori</i> infection	25	14	11	54.12 $\pm$ 8.21 (45-60)	0.85 $\pm$ 0.64 (0.12-1.23)
NAG with <i>H. pylori</i> infection	24	13	11	56.44 $\pm$ 11.29 (47-65)	0.85 $\pm$ 0.35 (0.37-1.03)
CAG with <i>H. pylori</i> infection	25	11	14	55.90 $\pm$ 7.80 (45-66)	0.73 $\pm$ 0.24 (0.13-0.90)
IM with <i>H. pylori</i> infection	28	12	16	52.16 $\pm$ 8.59 (48-68)	0.90 $\pm$ 0.65 (0.47-1.04)
DYS with <i>H. pylori</i> infection	24	12	12	54.05 $\pm$ 7.36 (48-69)	0.73 $\pm$ 0.57 (0.56-1.15)
GC with <i>H. pylori</i> infection	30	17	13	55.43 $\pm$ 10.33 (46-73)	0.60 $\pm$ 0.46 (0.10-1.04)

NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

**Table 2** *Cx32* and *Cx43* primer sequences, amplified fragment size and annealing temperature

Method	Gene	Primer sequence (5'→3')	Amplified fragment size (bp)	Annealing temperature (°C)
Real-time RT-PCR	<i>Cx32</i>	F: ATGAACTGGACAGGTTTGTAC	302	56
		R: ATGTGTTGCTGGTGAGCCA		
	<i>Cx43</i>	F: TGCAGCAGTCTGCCTTTCGTTG	219	56
		R: CCATCAGTTTGGGCAACCTTG		
	$\beta$ -actin	F: TGGACTTCGAGCACAGCAGATGG	289	56
		R: ATCTCCTTCTGCATCCCTGTCG		
MSP	<i>Cx32</i>	M F: GGGGCGGGTGC GCGGAT	245	64
		R: CTCCGCGCCTACGTCCC		
	U	F: GGGGTGGGTG GGTGAT	245	64
		R: CTCCACACCTACATCCCAA		
	<i>Cx43</i>	M F: AAATGTAAATATTTGGGTTTCAGCGC	156	58
		R: AATAACGCCATCTCTACTCACCG		
U	F: TTTTAAAATGTAAATATTTGGGTTTCAGTGT	161	56	
	R: AATAACACCATCTCTACTCACCACA			
BSP	<i>Cx32</i>	F: GGTTATTTTTTGGTGGGTTATG	313	58
		R: ACCCAAACAAATCCCCTATAATCTC		
	<i>Cx43</i>	F: TGTTTTTAAAATGTAAATATTTGGGTTTA	377	56
		R: AAAAAACAACTCATCTAACCTTCCTATTC		
MassArray	<i>Cx32</i>	F: CAGTTT CAGCAGTTTTGGGTTTTTGG	484	60
		R: TAACTCCCTATCCCCTAACCTCTTA		
	<i>Cx43</i>	F: ATGTTTTTGCAGGTGGATCAGGAAAT	447	60
		R: ACCAACAAAATAAAAAACAAATATTCC		

### Primers

The primers for detection of *Cx32* and *Cx43* expression and promoter methylation were designed with Primer5 [for real-time reverse transcription (RT)-polymerase chain reaction (PCR), including internal reference  $\beta$ -actin], MethPrimer [for methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)], and EpiDesigner (for MassArray). The primers were synthesized by Shanghai Sangon Biotech (China) (Figure 1, Table 2).

### Detection of *Cx32* and *Cx43* mRNA expression by real-time RT-PCR

The biopsy tissues of gastric mucosa were ground in liquid nitrogen, TRIzol was added to extract total RNA, and 1  $\mu$ g total RNA was reversely transcribed to cDNA. With 2  $\mu$ L cDNA as a template, real-time RT-PCR was carried out using SYBR qPCR Mix under the following conditions: 94 °C 4 min; 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, for 45 cycles; and 72 °C for 5 min.  $\beta$ -Actin was used as an internal reference, and nuclease-free water as a negative control. The relative mRNA expression was calculated according to the  $2^{-\Delta Ct}$  formula.

### Detection of *Cx32* and *Cx43* promoter methylation

DNA was extracted from the tissue with Wizard DNA purification kit, and then bisulfite-modified according to the steps in EpiTect Bisulfite Kit. DNA of normal human peripheral blood lymphocytes was methylated by *M.SssI* methylation enzyme (thus all the GC-sites were methylated), bisulfite-modified and acted as an all-site methylation positive control. The following three methods were used to detect *Cx32* and *Cx43* promoter methylation.

**MSP method:** After the tissue DNA was bisulfite modified, PCR was carried out using MSP primers under the following conditions: 95 °C for 10 min; 94 °C for 15 s, annealing temperature for 30 s, 72 °C for 30 s, for 38 cycles; and 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gel and imaged. From the gray values of methylation and unmethylation bands, the methylation level was calculated by the formula  $[M/(M + U) \times 100\%]$ .

**BSP sequencing method:** The PCR reaction mixture

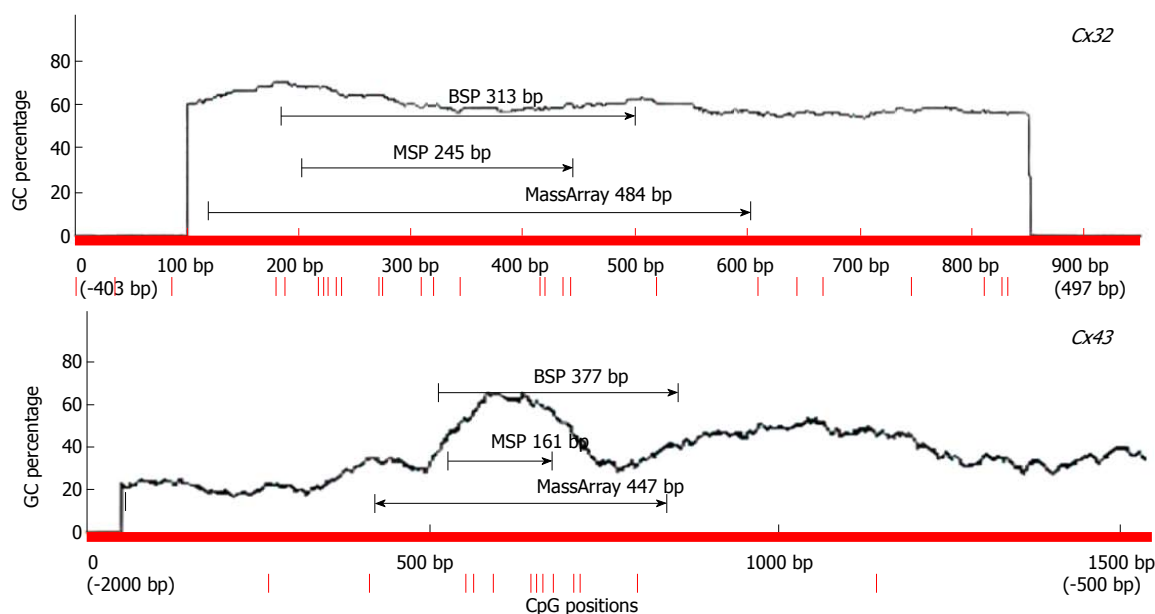


Figure 1 Location of primers for polymerase chain reaction for methylation detection.

(25  $\mu$ L) contained 2  $\mu$ L bisulfite-modified DNA template, 12.5  $\mu$ L TaKaRa Premix Taq HS, 1  $\mu$ L each 10  $\mu$ mol/L forward and reverse primers, and 8.5  $\mu$ L deionized distilled water. The PCR products were identified by electrophoresis, and sent to Huada Biotechnology (Shenzhen, China) for sequencing. The peak height ratio of the sulfonated methyl-CpG site still as C to the sum of C and the sulfonated non-methylated CpG site changed as T was calculated as the degree of methylation [*i.e.*,  $C/(C+T) \times 100\%$ ].

**MassArray method:** Genomic DNA after bisulfite modification was amplified with MassArray primers. The PCR products were introduced with T7 promoter sequence in the Beijing Bio-Miao Biotech Company, then *in vitro* transcribed to RNA products, processed by T-base-specific cleavage, and small RNA fragments were obtained. Flight mass spectrometry (MALDI-TOF) was used to detect the molecular weight of each fragment, and the methylation data were outputted with EpiTyper software.

### Statistical analysis

All data were processed with SPSS 16.0 software and shown as mean  $\pm$  SD. Averages of multiple samples were compared using univariate analysis of variance, and correlation analysis of ranked data was tested by the Spearman rank correlation method.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Cx32 and Cx43 mRNA expression profiles at different gastric carcinogenesis stages with *H. pylori* infection

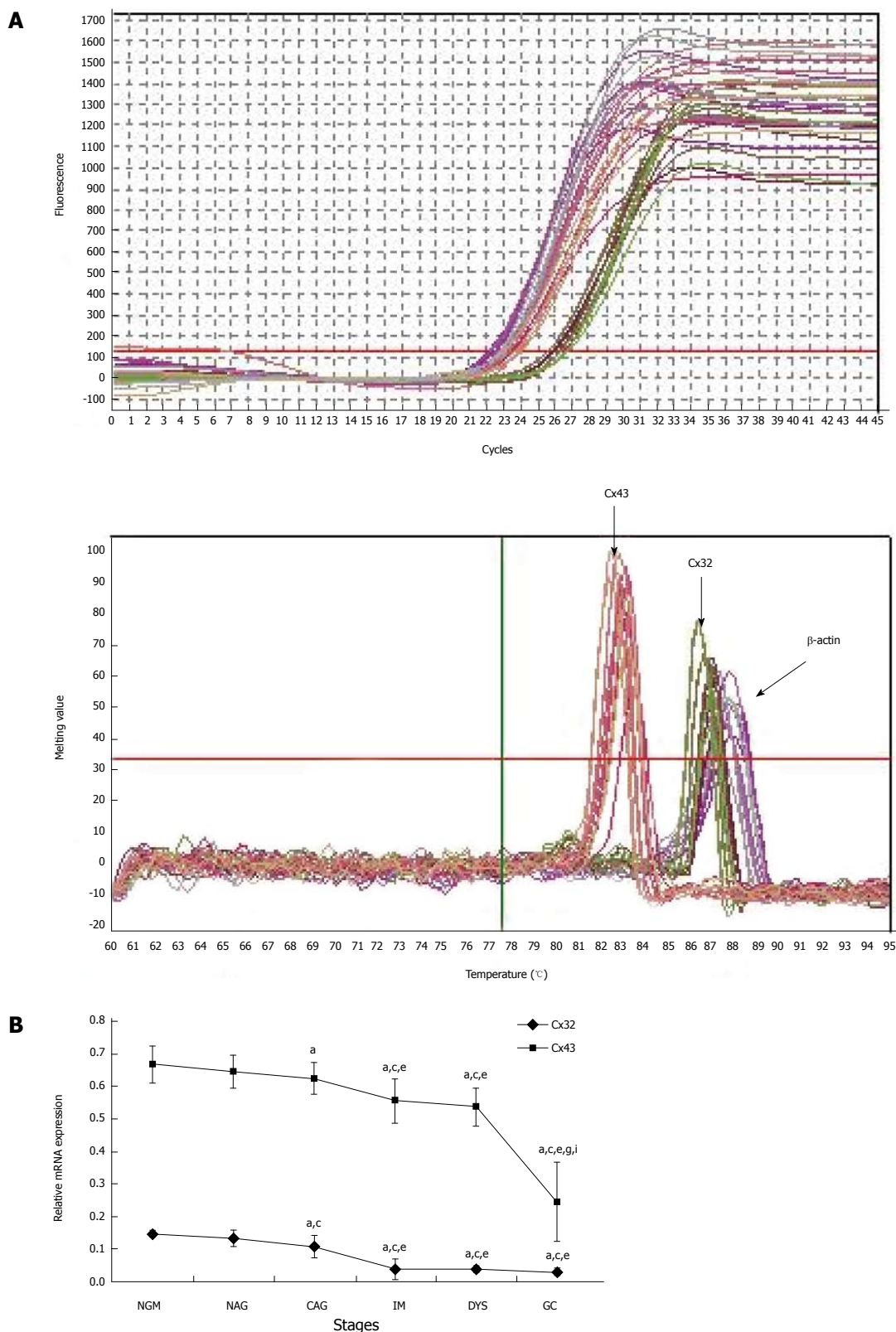
For cases with endoscopic and pathological confirmation and high mRNA quality (25 NGM, 24 NAG, 25 CAG, 28 IM, 24 DYS and 30 GC), the relative mRNA

expression in the gastric mucosa was  $0.146 \pm 0.011$ ,  $0.133 \pm 0.026$ ,  $0.107 \pm 0.035$ ,  $0.039 \pm 0.032$ ,  $0.037 \pm 0.01$  and  $0.03 \pm 0.011$  for *Cx32*; and  $0.667 \pm 0.057$ ,  $0.644 \pm 0.051$ ,  $0.624 \pm 0.049$ ,  $0.555 \pm 0.067$ ,  $0.536 \pm 0.058$  and  $0.245 \pm 0.121$  for *Cx43* (Figure 2). *Cx32* and *Cx43* mRNA expression decreased from NAG to GC stages with *H. pylori* infection ( $P < 0.001$ ), and that at CAG, IM, DYS and GC stages was lower than that at NGM ( $P < 0.008$ ; the largest in these comparisons), and that at IM, DYS and GC stages was lower than that at NAG and CAG stages ( $P < 0.036$ ). Specially, *Cx43* mRNA expression at GC stage was lower than that at IM and DYS stages ( $P < 0.001$ ).

### Cx32 and Cx43 promoter methylation at different gastric carcinogenesis stages with *H. pylori* infection

**MSP:** Eight DNA samples of good quality were selected from 24 cases in each group for MSP detection, and the actual number of samples whose methylated and unmethylated bands were significant was six for NGM, six for NAG, seven for CAG, six for IM, seven for DYS and eight for GC. Their promoter methylation levels were  $38.8\% \pm 9.0\%$ ,  $43.1\% \pm 9.4\%$ ,  $56.5\% \pm 3.1\%$ ,  $64.4\% \pm 9.7\%$ ,  $72.5\% \pm 4.2\%$  and  $79.6\% \pm 6.8\%$  for *Cx32*; and  $49.0\% \pm 3.9\%$ ,  $58.1\% \pm 5.0\%$ ,  $66.5\% \pm 7.9\%$ ,  $74.0\% \pm 8.8\%$ ,  $78.3\% \pm 3.6\%$  and  $88.7\% \pm 6.2\%$  for *Cx43* (Figure 3). *Cx32* and *Cx43* promoter methylation levels gradually increased from NAG to GC stages with *H. pylori* infection ( $P = 0.039$ ,  $P = 0.019$ ), and those at CAG, IM, DYS and GC stages were higher than that at NGM ( $P = 0.018$ ,  $P = 0.013$ ), with the highest at GC stage. *Cx32* methylation level at GC stage was higher than those at NAG, CAG and IM stages ( $P < 0.031$ ), and *Cx43* methylation level at GC stage was higher than those at NAG and CAG stages ( $P < 0.027$ ).

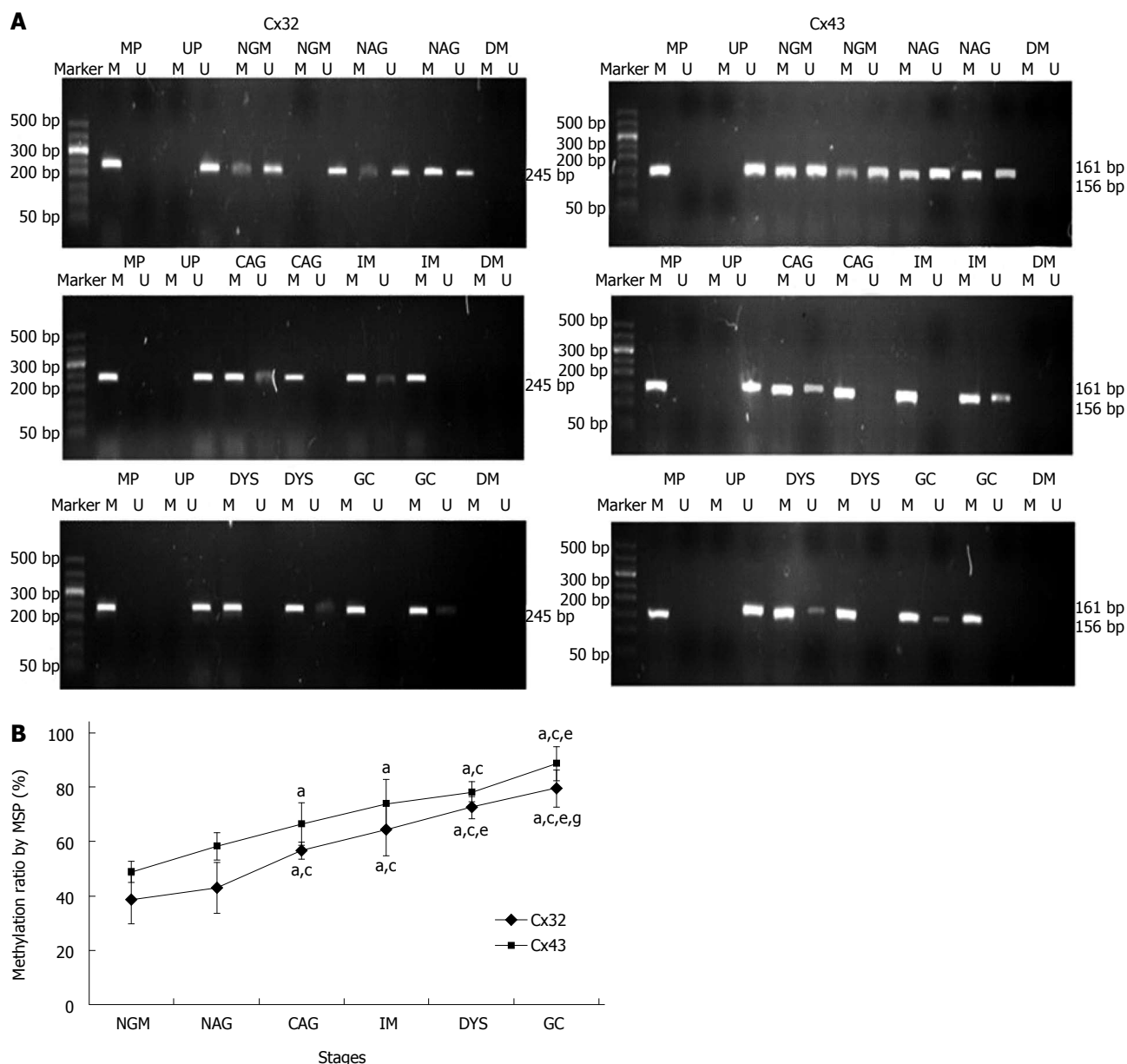
**BSP sequencing:** The samples with good MSP bands



**Figure 2** Real-time polymerase chain reaction results for *Cx32* and *Cx43* mRNAs. A: The amplification curve and melting curve of the real-time polymerase chain reaction; B: The relative expression of *Cx32* and *Cx43* mRNAs at different stages. <sup>a</sup>*P* < 0.05 vs NGM; <sup>b</sup>*P* < 0.05 vs NAG; <sup>c</sup>*P* < 0.05 vs CAG; <sup>d</sup>*P* < 0.05 vs IM; <sup>e</sup>*P* < 0.05 vs DYS. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

were selected for BSP reaction and sequencing. The BSP-amplified fragment from the *Cx32* gene CpG island had a length of 313 bp, containing 15 CpG sites, and the 5-15<sup>th</sup> CpG sites were detected by sequencing. The BSP

amplification fragment from the *Cx43* gene CpG island had a length of 377 bp, containing 12 CpG sites, and the 5-12<sup>th</sup> CpG sites were detected by sequencing (Table 3). The peak height ratio of the sulfonated methyl-CpG site



**Figure 3** Methylation-specific polymerase chain reaction results for *Cx32* and *Cx43* promoters at different gastric carcinogenesis stages with *Helicobacter pylori* infection. A: The agarose gel electrophoresis of the methylation-specific polymerase chain reaction (MSP) bands. Marker: 50bp ladder; M: Methylated; U: Unmethylated; MP: Methylation positive control; UP: Unmethylated positive control; DW: Negative control. B: The methylation levels of *Cx32* and *Cx43* promoters at different stages by MSP method. <sup>a</sup> $P < 0.05$  vs NGM; <sup>c</sup> $P < 0.05$  vs NAG; <sup>e</sup> $P < 0.05$  vs CAG; <sup>g</sup> $P < 0.05$  vs IM; <sup>i</sup> $P < 0.05$  vs DYS. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

still as C to the sum of this C and the sulfonated non-methylated CpG site changed as T was calculated as the degree of methylation. *Cx32* and *Cx43* methylation levels showed an increasing trend from NAG to GC stages with *H. pylori* infection ( $P = 0.031$ ,  $P = 0.040$ ), and those at CAG, IM, DYS and GC stages were higher than that at NGM ( $P < 0.029$ ,  $P < 0.03$ ), with the highest at GC. *Cx32* methylation level at GC stage was higher than those at NAG and CAG stages ( $P < 0.018$ ), and *Cx43* methylation level at GC stage was higher than that at NAG stage ( $P = 0.032$ ) (Figure 4, Table 4).

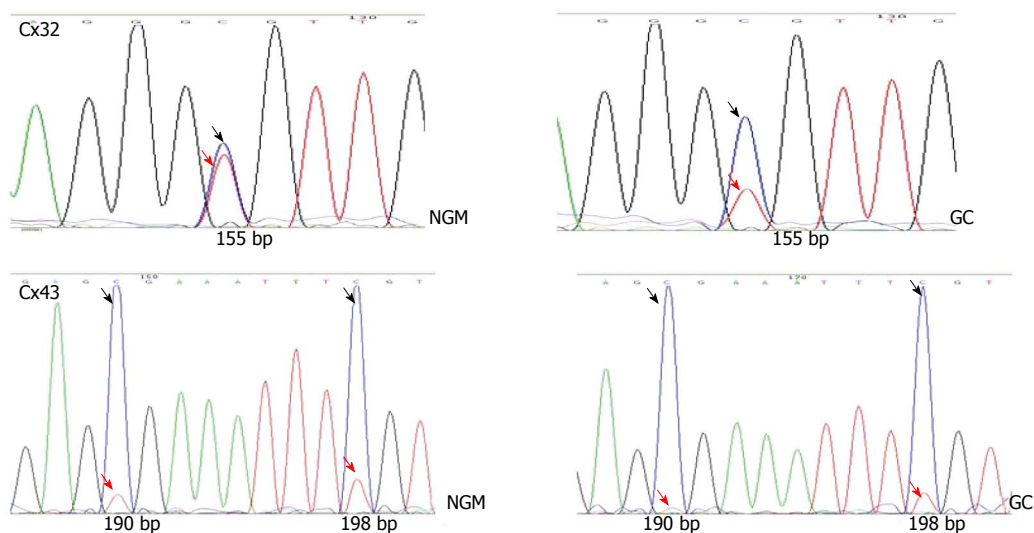
**MassArray detection:** The methylation of the CpG island was validated by MassArray method using one

sample from each group. As shown in Figure 5, the amplified fragment with MassArray *Cx32* primers contained 18 CpG sites, the first, second and 15<sup>th</sup> CpG sites were not detected by the MassArray method, and the 3-4-5, 6-7, 8-9, 13-14, and 16-17 loci were in close proximity and only measured on average, so a total of nine data were obtained. For the 12 CpG sites in the *Cx43* amplified fragment, the second site was not detected, and the 6-7 and 10-11-12 loci were in close proximity and only measured on average, so a total of eight data were obtained. The average of the methylation levels of these loci is shown in Table 5. The average of the methylation levels at all *Cx32* loci showed an increasing trend from NAG to GC stages with *H. pylori* infection ( $P = 0.037$ ), and that at IM, DYS

**Table 3** *Cx32* and *Cx43* CpG loci detected by bisulfite polymerase chain reaction sequencing

Cx32	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	bp	28	34	37	45	49	83	86	120	132	155	227	231	247	253	255
Cx43	CpG	1	2	3	4	5	6	7	8	9	10	11	12			
	bp	32	34	37	44	71	127	134	143	159	190	198	280			
		○	○	○	○	●	●	●	●	●	●	●	●	●	●	●
		○	○	○	○	●	●	●	●	●	●	●	●			

“●” denotes the detected CpG site; “○” denotes the undetected CpG site.



**Figure 4** Screenshots of bisulfite polymerase chain reaction sequencing of the *Cx32* and *Cx43* promoter CpG islands. The upper two show the 10<sup>th</sup> *Cx32* CpG site (155bp), with the C/(C+T) ratio of 60% in NM and 80% at GC stage. The lower two shows the 10<sup>th</sup> and 11<sup>th</sup> *Cx43* CpG sites (190bp, 198bp). Dark arrow indicates the peak of the sulfonated methyl-CpG site still as C; red arrow indicates the sulfonated non-methylated CpG site changed as T.

**Table 4** *Cx32* and *Cx43* promoter methylation status at different gastric carcinogenesis stages with *Helicobacter pylori* infection by bisulfite polymerase chain reaction sequencing method

	n	Cx32 (%)	Cx43 (%)
NGM without <i>H. pylori</i> infection	7	60.1 ± 5.9	75.5 ± 4.3
NAG with <i>H. pylori</i> infection	6	67.3 ± 4.9	82.9 ± 6.3
CAG with <i>H. pylori</i> infection	7	74.5 ± 7.5 <sup>a</sup>	87.1 ± 5.4 <sup>a</sup>
IM with <i>H. pylori</i> infection	7	84.2 ± 6.8 <sup>ac</sup>	90.5 ± 9.3 <sup>ac</sup>
DYS with <i>H. pylori</i> infection	6	82.3 ± 6.0 <sup>ac</sup>	91.6 ± 8.3 <sup>ac</sup>
GC with <i>H. pylori</i> infection	9	85.3 ± 9.7 <sup>ac</sup>	92.0 ± 7.1 <sup>ac</sup>

<sup>a</sup>P < 0.05 vs NM; <sup>c</sup>P < 0.05 vs NAG; <sup>a</sup>P < 0.05 vs CAG. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

and GC stages was higher than that at NGM ( $P < 0.028$ ); the methylation level at the 10-12<sup>th</sup> loci had an increasing trend from NAG to GC stages, while the methylation level of the remaining CpG sites did not change significantly. The average methylation levels at all *Cx43* loci showed an increasing trend from NAG to GC stages with *H. pylori* infection ( $P = 0.045$ ), and those at DYS and GC stages were higher than that at NGM ( $P < 0.041$ ). The methylation levels at the 3-5<sup>th</sup> loci tended to increase from NAG to GC stages, while the methylation levels of the remaining CpG sites did not change significantly.

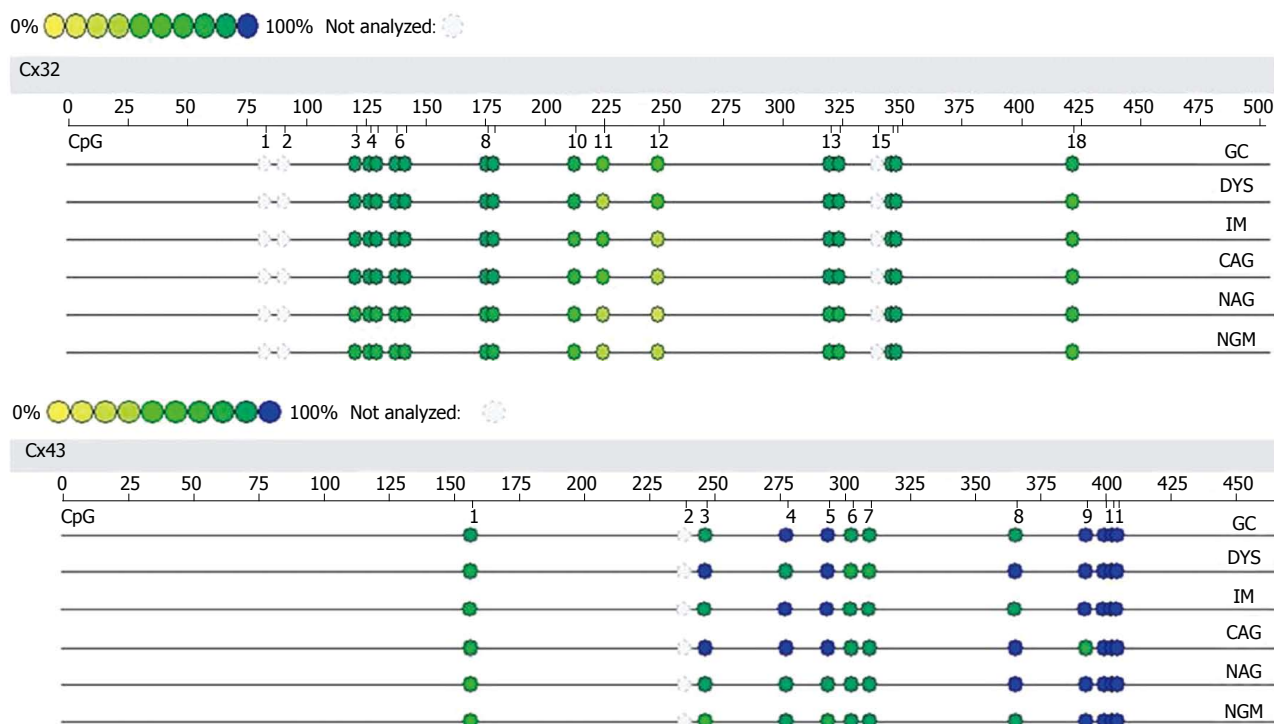
**Relationship between GC stages and *Cx32* and *Cx43* expression and methylation levels**

Spearman rank correlation analysis showed that *Cx32* and *Cx43* mRNA expression at different stages of gastric carcinogenesis with *H. pylori* infection was negatively correlated with the methylation level of their promoters ( $r = -0.653, P < 0.001; r = -0.367, P = 0.016$ , respectively), and negatively correlated with gastric carcinogenesis stage ( $r = -0.796, -0.852$ , respectively,  $P < 0.001$  for both).

**DISCUSSION**

Many studies have shown that expression of *Cx32* and *Cx43* shows a gradual downward trend from normal gastric mucosa to precancerous lesions and GC. *Cx32* and *Cx43* expression progresses from a high to a low level or no expression in the development of GC, and *Cx32* and *Cx43* abnormalities are an important molecular mechanism in the inhibition of gastric gap junction intercellular communication (GJIC)<sup>[4,14-16]</sup>.

The relationship between *Cx32* and *Cx43* expression and *H. pylori* infection is less reported. The results of our previous clinical studies<sup>[5-7]</sup> have suggested that eradication of *H. pylori* infection may improve *Cx32* and *Cx43* expression, promote recovery of cell GJIC function, and delay or prevent development of precancerous lesions.



**Figure 5** Methylation levels of *Cx32* and *Cx43* promoters at different gastric carcinogenesis stages with *Helicobacter pylori* infection by MassArray method. The validated length for *Cx32* gene was 484 bp, containing a total of 18 CpG sites (15 detected); the validated length for *Cx43* gene was 447 bp, containing a total of 12 CpG sites (11 detected).

**Table 5** Methylation levels (%) of *Cx32* and *Cx43* CpG islands at gastric different stages with *Helicobacter pylori* infection detected by MassArray method

	<i>Cx32</i> (9 loci)	<i>Cx43</i> (8 loci)
NGM without <i>H. pylori</i> infection	55.0 ± 14.9	77.9 ± 16.8
NAG with <i>H. pylori</i> infection	61.2 ± 17.9	86.0 ± 12.5
CAG with <i>H. pylori</i> infection	69.9 ± 16.2	88.6 ± 9.9
IM with <i>H. pylori</i> infection	72.4 ± 19.2 <sup>a</sup>	89.1 ± 9.6
DYS with <i>H. pylori</i> infection	72.6 ± 19.2 <sup>a</sup>	90.0 ± 9.3 <sup>a</sup>
GC with <i>H. pylori</i> infection	76.0 ± 20.1 <sup>a</sup>	91.6 ± 8.2 <sup>a</sup>

<sup>a</sup>*P* < 0.05 vs NGM. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

However, the pattern and mechanism by which *H. pylori* infection causes the change of *Cx32* and *Cx43* expression in gastric epithelial cells in the development of the inflammation-carcinoma chain are unclear.

In this study, we found that gastric *Cx32* and *Cx43* mRNA expression was downregulated from the initial CAG stage of *H. pylori* infection to latter carcinogenesis stages, which may have caused the decline in GJIC function, leading to the development of GC. *H. pylori* is deemed to be the first category of carcinogen for gastric cancer<sup>[17]</sup>, and current studies suggest that eradication therapy of *H. pylori* should be carried out at early stages of GC. Treatment before the occurrence of precancerous lesions can reduce the risk of GC, and treatment at the precancerous stage significantly decreases its role in prevention of GC<sup>[18-20]</sup>. From the profiles of *Cx32* and

*Cx43* mRNA expression, we provide a rationale that *H. pylori* eradication therapy should be carried out before the occurrence of CAG, that is, before the decline in *Cx32* and *Cx43* expression and GJIC function (NAG stage), thus the effect of preventing the occurrence and development of precancerous lesions and GC may be improved.

*H. pylori* infection can cause promoter CpG island hypermethylation of a variety of genes, such as *CDH1*, *p14*, *p16*, *APC* and *COX2*, and the methylation can be reversed after *H. pylori* eradication<sup>[21,22]</sup>, suggesting that gene hypermethylation is associated with *H. pylori* infection<sup>[23-25]</sup>, or *H. pylori* infection may be an inducer for some gene hypermethylation<sup>[26,27]</sup>. However, it has not been reported whether *H. pylori* infection causes *Cx32* and *Cx43* methylation.

Our research showed that *Cx32* and *Cx43* promoter methylation exhibited an increasing trend from NAG to GC stages with *H. pylori* infection, with the highest at the GC stage. Data from the MSP method were more significant than from those BSP or MassArray methods, mainly because MSP used direct PCR of several CpG sites in the primer regions, while different fluorescence or mass spectrum quantification and many CpG sites were considered in BSP sequencing or MassArray method. *Cx32* and *Cx43* mRNA expression was negatively correlated with methylation and gastric carcinogenesis stage, suggesting that *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism for the reduction of *Cx32* and *Cx43* expression and occurrence of GC. Hypermethylated promoter binds specific chromosome



remodeling proteins and suppresses the transcription of genes<sup>[8,28-30]</sup>.

Currently, many molecular technologies are being developed and applied for cancer epigenetics<sup>[31]</sup>. In GC patients, *TGF-β1* promoter is methylated<sup>[32]</sup>, and in colorectal cancer, underexpression of *LATS1* is associated with promoter hypermethylation<sup>[33]</sup>. Intervention with demethylation drugs has been reported to reactivate the expression of gastric tumor suppressor genes such as *CDX2*, *RASS-F1A* and *P16 (INK4A)*, and restore their functions<sup>[8-10]</sup>. *H. pylori* infection may cause *Cx32* and *Cx43* promoter hypermethylation to decrease their expression, then inhibit the GJIC function, and induce GC. Promoter methylation can be reversed, thus, it is expected that we can treat against promoter methylation to restore GJIC function, providing new therapies for *H. pylori* infection-related GC. Based on our research, the treatment of GC may include eradication of *H. pylori*, adding DNA-demethylation agents (*e.g.*, 5-azacytidine), and overexpression of *Cx32* and *Cx43* to compensate the decrease of *Cx32* and *Cx43* in the carcinogenesis.

In summary, *Cx32* and *Cx43* expression at the CAG stage of *H. pylori* infection began to decrease, suggesting that *H. pylori* eradication therapy before the CAG stage could effectively prevent the occurrence of precancerous lesions and GC. *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism of the decrease of *Cx32* and *Cx43* expression after *H. pylori* infection, and provides a new target for the demethylation treatment of GC.

## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) infection is an important risk factor for gastric cancer (GC), yet its carcinogenic mechanism is not yet fully understood. Connexin (Cx) 32 and Cx 43 are key members of gap junctions between gastric epithelial cells. The authors have found that the decrease of *Cx32* and *Cx43* expression in precancerous lesions and GC is associated with *H. pylori* infection, but the mechanisms by which *H. pylori* infection leads to the decrease of *Cx32* and *Cx43* expression are unclear.

### Research frontiers

It is important to understand the epigenetic mechanism of occurrence of GC. Many studies have demonstrated that the expression of *Cx32* and *Cx43* shows a gradual downward trend from normal gastric mucosa to precancerous lesions and GC. *Cx32* and *Cx43* abnormalities are the important molecular mechanism of the inhibition of gastric gap junction intercellular communication (GJIC) which may then lead to gastric carcinogenesis. *H. pylori* infection can cause promoter CpG island hypermethylation of a variety of genes, such as *CDH1*, *p14*, *p16*, *APC* and *COX2*, and methylation can be reversed after *H. pylori* eradication.

### Innovations and breakthroughs

Following previous work showing that the decrease of *Cx32* and *Cx43* expression in precancerous lesions and GC was associated with *H. pylori* infection, the present study found that *Cx32* and *Cx43* mRNA expression was negatively correlated with promoter methylation and gastric carcinogenesis stage. This suggests that *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism in the reduction of *Cx32* and *Cx43* expression and occurrence of GC. This can help find new targets for applying appropriate means to control the incidence of GC.

### Applications

This study shows that *Cx32* and *Cx43* promoter CpG islands are gradually methylated at gastric carcinogenesis stages with *H. pylori* infection. Promoter methylation can be reversed, therefore, it is expected that we can treat against

promoter methylation to restore GJIC function, providing new therapies for *H. pylori* infection-related GC.

### Terminology

CpG islands: CpG rich areas located in the promoter regions of many genes; CpG island methylation: the addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. *Cx32* and *43*: key members of gap junctions between gastric epithelial cells. Gastric carcinogenesis stages: graded according to the diagnoses by endoscopy and pathology, including non-atrophic gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, and GC.

### Peer review

The hypothesis was sound, the experiments were well designed and the results supported the conclusions. This is a well written manuscript about new therapeutic targets for treatment of GC and its different carcinogenesis stages with *H. pylori* infection. The analyses were performed well and are clearly described in the manuscript.

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