Host Wnt/ β -catenin pathway triggered by *Helicobacter pylori* correlates with regression of gastric intestinal metaplasia after *H. pylori* eradication

Kuei-Hsiang Hung,¹ Jiunn-Jong Wu,^{1,2} Hsiao-Bai Yang,^{3,4} Li-Ju Su⁵ and Bor-Shyang Sheu^{1,6,7}

Correspondence
Bor-Shyang Sheu
sheubs@mail.ncku.edu.tw

¹Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

²Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

³Department of Pathology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

⁴Department of Pathology, Ton-Yen General Hospital, Hsinchu, Taiwan, ROC

⁵Institute of Clinical Pharmacy, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

⁶Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

⁷Department of Internal Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

Helicobacter pylori eradication can reverse gastric intestinal metaplasia (IM) in some but not all patients. *H. pylori* induces high levels of nuclear β -catenin staining in IM tissues, as well as overexpression of cyclooxygenase-2 (COX-2). This study investigated whether the Wnt/ β -catenin pathway plays a role in IM regression following *H. pylori* eradication. Sixty-five *H. pylori*-infected patients with IM who had achieved successful *H. pylori* eradication provided paired gastric samples before and after eradication to analyse the persistence of IM, and to assess COX-2 and nuclear β -catenin expression. The host genotypes of single nucleotide polymorphisms (SNPs) of the COX-2, β -catenin (*CTNNB1*) and adenomatous polyposis coli (*APC*) genes were analysed. In addition, expression of β -catenin, E-cadherin and phosphorylated and unphosphorylated glycogen synthase kinase 3 β (GSK-3 β) in cell lines challenged with *H. pylori* isolates from patients with and without IM persistence was compared by immunoanalysis. After a mean 33.9-month follow-up after *H. pylori* eradication, 44 patients (67.7%) with IM persistence had a higher rate of high-level nuclear β -catenin expression in IM tissue than those without IM persistence ($P=0.008$). The patients with IM persistence had a higher rate of AA, GG and AA *APC* SNP genotypes at positions 4479, 5268 and 5465, respectively, than the patients without IM persistence ($P=0.022$). The *H. pylori* isolates from the patients with IM regression after *H. pylori* eradication induced more phospho-GSK-3 β in AGS cells than isolates from patients with IM persistence ($P=0.011$). It is likely that interactions with *H. pylori* and the patient's Wnt/ β -catenin genetic predisposition determine the outcome of IM persistence following *H. pylori* eradication.

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INTRODUCTION

Helicobacter pylori has been designated a type I carcinogen of gastric cancer by the WHO (Correa & Houghton, 2007). Gastric intestinal metaplasia (IM) can be induced after

chronic *H. pylori* infection, and has been validated as a precancerous lesion of gastric carcinogenesis (Sheu *et al.*, 2003; Walker, 2003; Correa & Houghton, 2007). Several clinical trials have analysed whether *H. pylori* eradication can lead to regression of such precancerous lesions (Cheung *et al.*, 2007), but the results have been diverse, which may be due to the different follow-up durations and case numbers (Uemura *et al.*, 1997; van der Hulst *et al.*,

Abbreviations: CI, confidence interval; COX-2, cyclooxygenase-2; GSK-3 β , glycogen synthase kinase 3 β ; IM, intestinal metaplasia; SNP, single nucleotide polymorphism.

1997; Correa *et al.*, 2000; Sung *et al.*, 2000b; Ohkusa *et al.*, 2001; Leung *et al.*, 2004). However, focusing on trials conducted in a high cancer endemic area with a longer follow-up duration of greater than 1 year, *H. pylori* eradication seems to have a positive effect on the improvement of IM regression (Uemura *et al.*, 1997; Correa *et al.*, 2000; Ohkusa *et al.*, 2001; Leung *et al.*, 2004; Yang *et al.*, 2007). Despite the fact that *H. pylori* eradication may improve regression of IM, such positive effects are limited to a certain proportion of patients: the positive effect is not uniform for all patients receiving the treatment. Therefore, there must be other factors that determine whether patients achieve regression of IM after *H. pylori* eradication.

Cyclooxygenase-2 (COX-2) is an inducible enzyme that is triggered by *H. pylori* infection, hormones, cytokines, growth factors and tumour promoters (Lim *et al.*, 2000; Sheu *et al.*, 2003; Walker, 2003). Previous studies have confirmed that COX-2 can be overexpressed in *H. pylori*-infected precancerous lesions such as IM (Sung *et al.*, 2000a; Sheu *et al.*, 2003). Successful clearance of *H. pylori* leads to downregulation of COX-2 expression in gastric mucosa (McCarthy *et al.*, 1999; Sung *et al.*, 2000a). However, it is not totally eliminated in some patients, such as those with IM (McCarthy *et al.*, 1999), suggesting the need to investigate whether the degree of COX-2 overexpression or the host's COX-2-related genetic predisposition may be a determinant of the persistence of IM after *H. pylori* eradication.

In addition to COX-2, the upstream molecule β -catenin, which is a component of the Wnt pathway, has been reported to exhibit high levels of nuclear accumulation in *H. pylori*-infected IM tissues (Franco *et al.*, 2005). This nuclear accumulation of β -catenin is a consequence of a complex process, triggered by *H. pylori* infection, which can be dominantly mediated by translocation of CagA into host epithelial cells (Franco *et al.*, 2005; Murata-Kamiya *et al.*, 2007; Kurashima *et al.*, 2008). β -Catenin binds to E-cadherin to maintain the stability of the epithelial architecture (Clevers, 2006; Nathke, 2006). However, this stability can be significantly degraded by *H. pylori* infection, especially by CagA-positive virulent isolates, to mobilize β -catenin into the cytosol and even into the nucleus (Murata-Kamiya *et al.*, 2007). In Taiwan, nearly all *H. pylori* isolates are CagA-positive; therefore, it is of interest to investigate whether there is differential host expression of factors involved in the Wnt/ β -catenin pathway in patients with and without IM persistence after *H. pylori* eradication.

This longitudinal follow-up study was carried out to assess the benefits of *H. pylori* eradication on IM regression and to analyse the presence of host single nucleotide polymorphisms (SNPs) related to IM regression after *H. pylori* eradication.

METHODS

Patients and study design. This study prospectively enrolled patients with *H. pylori* infection and IM from 1266 dyspeptic patients

who received a panendoscopy. Patients were not enrolled if they had malignancy or had been treated with non-steroidal anti-inflammatory drugs, proton pump inhibitors, bismuth salts or antibiotics within the 2 weeks prior to the panendoscopy. After obtaining informed consent from each patient and approval by the Ethical Committee of the National Cheng Kung University Hospital, each patient received a panendoscopy to obtain gastric biopsy samples to confirm the presence of *H. pylori* infection and any related gastric histological features, including IM. The presence of *H. pylori* infection was defined by a positive histology with or without a positive culture. Patients found to have gastric cancer on enrolment or during follow-up were excluded. Among the 611 *H. pylori*-infected patients, 130 were confirmed to have IM on enrolment. All patients with IM then received anti-*H. pylori* therapy, comprising 1 g amoxicillin, 500 mg clarithromycin, and 20 mg omeprazole or 30 mg lansoprazole twice daily for 1 week.

To address any bias that might arise by not allowing enough time for IM regression after *H. pylori* eradication, this study enrolled only patients who had achieved successful *H. pylori* eradication and had been followed for more than 24 months after successful *H. pylori* eradication. Based on this strict inclusion, only 65 cases of *H. pylori*-infected IM (32 men and 33 women; mean age 55.3 years) with successfully eradicated *H. pylori* were included in the analysis. The patients who were defined as having successful *H. pylori* eradication were also validated by follow-up histology and a urea breath test. These 65 patients provided paired gastric samples at enrolment and at the follow-up endoscopic session 30–36 months (mean 33.9 months) after successful *H. pylori* eradication. During each panendoscopy, five gastric biopsy samples were taken from the antrum (two samples), corpus (two samples) and cardia (one sample) for histological examination (Sheu *et al.*, 2003, 2006). *H. pylori*-related histology was scored using the updated Sydney System (Dixon *et al.*, 1996). Assessment of the presence and severity of IM has been described previously (Sheu *et al.*, 2003; Yang *et al.*, 2007). During post-eradication follow-up, for patients in whom there was uniform absence of IM in these five biopsy samples, an additional panendoscopy was carried out 3 months later. The definition of regression of IM was thus based on the absence of IM in all ten gastric biopsy samples, five from each of the two serial endoscopic sessions. This definition of IM regression was considered strict enough to prevent bias from the limited number of biopsies in each session, and also to fulfil the ethical concerns of carrying out too many biopsies, raised by the institute's ethical committee.

In each patient, the paired gastric specimens from the antrum and corpus on enrolment and from the long-term follow-up were also graded for COX-2 and β -catenin expression by immunohistochemistry. Some patients provided an additional two gastric biopsy specimens for *H. pylori* culture (Sheu *et al.*, 2003, 2006). The *H. pylori* isolate was tested for the *cagA* genotype by PCR and for the CagA phenotype by Western blotting. In addition, blood samples from each patient were tested for SNPs of the COX-2, β -catenin (*CTNNB1*) and adenomatous polyposis coli (*APC*) genes.

Gastric histology of *H. pylori*-infected patients. One pathologist, blinded to the clinical data, reviewed the histology of the collected samples. The acute inflammation score (range: 0–3), chronic inflammation score (range: 0–3), *H. pylori* density (range: 0–5), atrophic change (absence: 0; presence: score 1–3) and IM (absence: 0; presence: score 1–3) were graded using the updated Sydney System (Dixon *et al.*, 1996). In this study, the presence of IM was defined by at least one gastric biopsy showing metaplastic cells (goblet cells) involving at least 5% of the upper third of the gastric mucosa (Sheu *et al.*, 2003; Yang *et al.*, 2007), or by two or more biopsy samples containing goblet cells on <5% of the upper third of the mucosa. Total regression of IM in histological terms was defined as the absence of IM (score 0) in all gastric specimens (Yang *et al.*, 2007).

Expression of gastric COX-2 and β -catenin by immunohistochemistry. Paraffin sections (4 μ m) of the biopsy samples were deparaffinized and endogenous peroxidase activity was blocked with 3% H_2O_2 . Tissue sections were incubated overnight at 4 °C with primary antibodies against COX-2 (Cayman Chemical) and β -catenin (Santa Cruz Biotechnology) diluted 1:100 and 1:200, respectively. A SuperPicTure Polymer detection kit (Zymed Laboratories) using horseradish peroxidase/Fab polymer conjugate was adapted for staining according to the manufacturer's instructions. Sections were then counterstained with haematoxylin. Non-immune mouse IgG served as a negative control.

For each biopsy sample, the level of gastric COX-2 expression was graded in the epithelial cells and stromal cells of the mucosa into the following categories: 0 (negative), 1 (<5% of cells showing positive staining), 2 (5–30% of cells), 3 (30–60% of cells) or 4 (>60% of cells) (Sung *et al.*, 2000a; Sheu *et al.*, 2003). In addition, the expression of β -catenin was evaluated in epithelia with or without IM. Localization of epithelial cells with positive β -catenin staining was found at the membrane (Fig. 1a), in the cytosol (Fig. 1b) and in the nucleus (Fig. 1c). Nuclear localization was further categorized into high- or low-level subgroups, defined by the presence of >10% (Fig. 1c) or <10% (Fig. 1d) of cells with positive nuclear β -catenin staining (Romiti *et al.*, 2005).

Genotyping of the COX-2, CTNNB1 and APC genes. DNA was extracted from peripheral blood mononuclear cells using a Blood and Tissue Genomic DNA Miniprep System (Viogene) according to the manufacturer's instructions. A DNA fragment of 1343 bp for the 5'-flanking region of the COX-2 gene at positions –1519 to –177 was amplified by PCR with the primers described in Table 1. The A/G SNP at –1195 was determined using a sequence-specific oligonucleotide probe (Lu *et al.*, 2005; Table 1). The probes (2 pmol ml^{–1}) were

labelled at their 5' end with digoxigenin, and unlabelled competitor oligonucleotide (70 pmol ml^{–1}) was added to prevent non-specific binding (Table 1). The genotype of the promoter polymorphism was confirmed by DNA sequencing. The primers for the analysis of exons 2–4 of the CTNNB1 gene and exon 15 of the APC gene are listed in Table 1. Polymorphisms of the CTNNB1 and APC genes were determined by PCR and direct sequencing.

Co-culture of *H. pylori* and gastric epithelial cells. *H. pylori* was cultured on CDC anaerobic blood agar (BBL Microbiology Systems) and incubated for 2 days under microaerobic conditions (5% O_2 , 10% CO_2 , 85% N_2). The cell lines AGS (ATCC CRL-1739) and MKN45 (JCRB0254) were cultured in F12 medium and RPMI 1640 (Gibco-BRL), respectively, supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humid atmosphere at 37 °C with 5% CO_2 . For infection with *H. pylori*, AGS or MKN45 cells (3×10^5 cells per well of a six-well plate) were co-cultured with *H. pylori* strain HP27, *cagA*, *vacA* isogenic mutants, and with clinical strains isolated from patients with and without regression of IM (m.o.i. of 100) for 2 and 20 h at 37 °C in a microaerophilic incubator. Cell lysates were collected to detect the expression of phosphorylated glycogen synthase kinase 3 β (phospho-GSK-3 β), β -catenin and E-cadherin.

Western blot analysis and preparation of membrane fractions.

Following *H. pylori* infection, AGS and MKN45 cells were washed with PBS and lysed with ice-cold lysis buffer [Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF, 1 μ M leupeptin, 1 mM Na_3VO_4 , 1% Triton X-100]. The soluble extracts were subjected to 12% SDS-PAGE. The membrane fraction of the cells was obtained by centrifugation twice for 30 min at 45 000 g and the supernatant was collected and stored at –80 °C. Proteins were transferred to PVDF membrane (Millipore) and incubated overnight at 4 °C with primary

Fig. 1. Immunohistochemical localization of β -catenin in gastric mucosa. (a, b) Membrane (a) and cytosol (b) staining of β -catenin on superficial epithelium. Magnification $\times 200$. (c, d) In epithelium with IM, the nuclear β -catenin expression was determined as either high (>10% nuclear staining) (c) or low (<10% nuclear staining) (d). Magnification $\times 400$. Arrows indicate nuclear β -catenin staining.

Table 1. Oligonucleotide primers for PCR and PCR-sequence-specific oligonucleotide probe analysis

The probes and competitors were used to detect the A/G SNP of *COX-2* at nt -1195 by PCR-sequence-specific oligonucleotide probe analysis. The oligonucleotide probes were labelled with DIG at the 5' end of the sequence. The bold underlined letters indicate the position of the SNP at nt -1195 of the *COX-2* promoter.

Gene	Primer	Sequence (5'→3')	nt range	Reference
<i>COX-2</i>	Sense	GTAATGCTAAGGACTTAGGAC	-1499 to -1519	This study
	Antisense	CAAGCCCATGTGACGAAATG	-177 to -196	
<i>COX-2</i>	Probe	AAATTCCA G CTGTCAAA	-1187 to -1203	This study
	Probe	AAATTCCA A CTGTCAAA	-1187 to -1203	
	Competitor	AAATTCCA A CTGTCAAA	-1187 to -1203	This study
	Competitor	AAATTCCA G CTGTCAAA	-1187 to -1203	
<i>CTNNB1</i>	Sense	AAAATCCAGCGTGGACAATGG	344-364	Jeng <i>et al.</i> (2000)
	Antisense	TGTGGCAAGTTCTGCATCATC	1434-1454	
<i>APC</i>	Sense	AGACTTATTGTGTAGAAGATAC	101487-101508	Prall <i>et al.</i> (2007)
	Antisense	CATTTGATTCTTTAGGCTGC	102328-102347	
	Sense	CCCAAAGGGAAGTCACAA	102905-102924	Wei <i>et al.</i> (2004)
	Antisense	GGTTCTCCCTGTGAGTCAGG	103685-103704	

antibody against β -catenin (Santa Cruz Biotechnology), phospho-GSK-3 β and GSK-3 β (Cell Signaling Technology), E-cadherin (BD Biosciences) and β -actin (Chemicon International) at a dilution of 1:1000. Immunoreactive bands were detected using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Protein bands on the PVDF membrane were detected using a Las-3000 Imaging System (Fujifilm) and analysed using Quantity One software (Bio-Rad). Data from three independent experiments were used for the calculation of the results.

Statistical analysis. Student's *t*-test and a paired *t*-test were applied to the continuous data. Pearson's χ^2 test was used to assess the categorical data. Fisher's exact test was used to test the relative risk and the 95% confidence interval (CI) of the AGA genotype of the *APC* SNPs compared with that of the non-AGA genotype. Fisher's exact test was also used to test the relative risk and 95% CI of the AGA genotype of *APC* SNPs combined with high-level nuclear β -catenin staining compared with that of the non-AGA genotype with low-level nuclear β -catenin staining. All tests were two-tailed and a value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

H. pylori-related IM regresses only in some subjects after eradication therapy

The benefit of *H. pylori* eradication on IM regression remains controversial, with diverse outcomes ranging from 15 to 61% regression (Correa *et al.*, 2000; Ohkusa *et al.*, 2001; Wong *et al.*, 2004). A longer follow-up duration may give more positive results (Leung *et al.*, 2004). We thus conducted this study to analyse a long follow-up period with a mean duration of 33.9 months after *H. pylori* eradication. Moreover, we used a strict definition of IM regression based on at least ten gastric biopsy samples, including five samples from each of two serial endoscopic follow-ups that were at least 3 months apart. This was to avoid the bias of a relatively limited number of biopsy samples per endoscopic session and to fulfil the ethical

concerns of carrying out too many biopsies during each endoscopic session for the study subjects (Sheu *et al.*, 2003). Based on this long follow-up period, our results found that 32.3% of patients showed IM regression after *H. pylori* eradication, supporting the positive benefits of *H. pylori* eradication for IM regression. However, there were still almost 70% of patients with persistence of IM. Thus there must be other factors that determine whether patients achieve regression of IM or not after *H. pylori* eradication.

Levels of COX-2 prior to treatment and analysis of COX-2 genotypes with respect to IM regression after *H. pylori* eradication

The demographic characteristics, *H. pylori*-related histology, especially the severity of IM prior to treatment, and the levels of COX-2 prior to treatment were not significantly different between patients with or without IM persistence ($P > 0.05$) (Table 2). The mean post-treatment COX-2 levels were significantly higher in patients with IM persistence than in patients without ($P < 0.001$) (Table 2). There was a significant decrease in COX-2 expression in patients with IM regression; in contrast, in patients with IM persistence, COX-2 expression levels remained high after *H. pylori* eradication (Table 2). These data indicated that levels of COX-2 prior to treatment cannot determine the outcome of IM regression. We also determined the host genotype of the SNP -1195 locus of the *COX-2* promoter, which has a G→A change creating a c-Myb-binding site and thus displaying increased promoter activity to enhance COX-2 overexpression with a higher risk of gastric cancer (Zhang *et al.*, 2005). However, there was a similar distribution of genotypes of COX-2 -1195 SNPs between patients with and without IM persistence after *H. pylori* eradication ($P = 0.452$) (Table 3).

Table 2. Pre-treatment clinical and histological differences between *H. pylori*-infected patients with or without persistence of IM after *H. pylori* eradication

Parameter*	Persistence of IM		P value
	No (n=21)	Yes (n=44)	
Gender (female: male)	10:11	23:21	0.888
Age (years) (mean $\pm$ SD)	53.0 $\pm$ 13.2	56.5 $\pm$ 11.2	0.266
Endoscopic diagnosis (n)			
DU:GU:gastritis	3:3:15	7:7:30	0.916
Mean AIS $\pm$ SD	1.4 $\pm$ 1.0	1.5 $\pm$ 0.9	0.689
Mean CIS $\pm$ SD	2.8 $\pm$ 0.4	2.9 $\pm$ 0.4	0.564
Mean HPD $\pm$ SD	2.7 $\pm$ 1.5	2.7 $\pm$ 1.6	0.932
AT (%)	84.2	67.6	0.190
IM over antrum only (%)	85.7	78.9	0.523
IM over corpus only (%)	9.5	7.9	0.830
COX-2 before <i>H. pylori</i> eradication			
Superficial epithelium (mean score $\pm$ SD)	2.1 $\pm$ 0.6†	2.3 $\pm$ 1.0	0.595
Stroma (mean score $\pm$ SD)	3.5 $\pm$ 0.6†	3.6 $\pm$ 0.8	0.881
COX-2 after <i>H. pylori</i> eradication			
Superficial epithelium (mean score $\pm$ SD)	0.4 $\pm$ 0.8†	2.2 $\pm$ 0.7	<0.001
Stroma (mean score $\pm$ SD)	1.1 $\pm$ 1.5†	3.6 $\pm$ 0.8	<0.001

*DU, Duodenal ulcer; GU, gastric ulcer; AIS, acute inflammation score from antrum (range: 0–3); CIS, chronic inflammation score from antrum (range: 0–3); HPD, *H. pylori* density from antrum (range: 0–5); AT, antral mucosal atrophy.

†A significant decrease in COX-2 staining intensity on either superficial epithelium or stroma after therapy ($P < 0.001$, paired *t*-test).

Table 3. Comparisons of pre-treatment gastric expression of β -catenin and host genomic SNPs between patients with or without persistence of IM after *H. pylori* eradication

Parameter	Persistence of IM		P value
	No (n=15)	Yes (n=25)	
Immunohistochemistry			
β -Catenin distribution (%)			
Membranous: cytoplasmic	21.4: 78.6	33.3: 66.7	0.474
High levels of nuclear β -catenin staining (%)			
In non-IM tissue	14.3	53.3	0.027*
In IM tissue	54.5	100	0.008*
Host SNP [n (%)]†			
COX-2 –1195 locus (GG: GA: AA)	3(23): 7(54): 3(23)	8(40): 10(50): 2(10)	0.452
APC nt 4479 locus (GG: GA: AA)	1(7): 6(43): 7(50)	1(4): 5(20): 19(76)	0.253
APC nt 5268 locus (GG: GT: TT)	7(50): 6(43): 1(7)	17(77): 5(23): 0(0)	0.160
APC nt 5465 locus (AA: AT)	9(64): 5(36)	19(86): 3(14)	0.120
APC nt 4479, 5268 and 5465 loci (AGA: non-AGA)	5(36): 9(64)	17(74): 6(26)	0.022*

* $P < 0.05$.

†AGA was defined as the haplotype of APC SNPs with genotypes of AA, GG and AA at the 4479, 5268 and 5465 loci, respectively. Non-AGA indicates genotypes of APC SNPs other than those of AA, GG and AA at these loci. The number of subjects was smaller than in Table 2 as there was not enough genomic DNA for PCR amplification of the COX-2 and APC genes in all subjects.

The Wnt/ β -catenin pathway regulates IM regression after *H. pylori* eradication

The Wnt/ β -catenin pathway is known to be involved in colorectal cancer (Clevers, 2006; Nathke, 2006), and mutations in certain components of this pathway generally occur early in colon cancer progression (Nathke, 2006). Gastric pre-malignant and malignant lesions have been found to have translocation of β -catenin from the membrane to the cytoplasm and even to have high levels of accumulation in the nucleus (Franco *et al.*, 2005; Romiti *et al.*, 2005). Moreover, this translocation of β -catenin into the nucleus can be enhanced by *H. pylori* infection, especially in *H. pylori* with CagA-positive status (Murata-Kamiya *et al.*, 2007). These data support the suggestion that *H. pylori* mediates its effect through interactions with the Wnt/ β -catenin pathway to induce carcinogenesis.

β -Catenin staining of the gastric mucosa was found over the membrane and cytoplasm, and even in the nucleus (Fig. 1a–c). Prior to *H. pylori* eradication, there was a similar distribution of β -catenin in patients with and without persistence of IM after *H. pylori* eradication ($P=0.474$) (Table 3). However, there was a significantly increased number of patients with high-level β -catenin nuclear staining in patients with IM persistence compared with those without IM persistence (53.3 vs 14.3 % in non-IM tissue, $P=0.027$; 100 vs 54.5 % in IM tissue, $P=0.008$). After *H. pylori* eradication, the relative proportions of membrane and cytoplasmic β -catenin in IM tissue were not significantly changed in patients with IM persistence (Fig. 2a). The 100 % high-level intensity of nuclear β -catenin staining in IM tissue in patients with persistence of IM after *H. pylori* eradication decreased after treatment but remained greater than 50 % (Fig. 2b). These data suggest indirectly that, in patients with IM persistence after *H. pylori* eradication, there exist other factors in addition to *H. pylori* that maintain the Wnt/ β -catenin pathway and thus yield the persistently high levels of nuclear β -catenin staining in IM tissue.

Mutations of the APC gene play a major role in the early development of colorectal neoplasms (Powell *et al.*, 1992). Moreover, mutations in the CTNNB1 (β -catenin) and APC genes have commonly been found in gastric cancer (Ogasawara *et al.*, 2006; Tajima *et al.*, 2006). Individuals with the aspartate/valine substitution at codon 1822 of APC were found to be at statistically significantly lower risk of colon cancer if they also ate a low-fat diet (Slattery *et al.*, 2001). Codon 1822 is located in the region involved in binding and downregulation of β -catenin; although the functional significance of the 1822 substitution is uncertain, the possible consequences may affect the function or structure of APC binding to β -catenin (Slattery *et al.*, 2001). We thus further tested the host genomic predisposition of CTNNB1 and APC in patients with and without IM persistence after *H. pylori* eradication. For patients both with and without IM persistence, there was no change in the nucleotide sequence of CTNNB1 within exons 2–4.

Fig. 2. Relative proportions of membrane (□) and cytoplasmic (■) localization of β -catenin in IM tissue before and after *H. pylori* eradication in patients with and without regression of IM. (a) The relative proportions of membrane and cytoplasmic β -catenin in IM tissue were not significantly changed in patients with IM persistence after *H. pylori* eradication. (b) The high intensity of nuclear β -catenin (100 %) in IM tissue decreased but remained greater than 50 % in patients with persistence of IM after *H. pylori* eradication. ■, High; □, low.

Within exon 15 of the APC gene, there were similar distributions of the APC SNPs between patients with and without IM persistence at the nt 4479, 5268 and 5465 loci (codon 1822), respectively ($P > 0.05$) (Table 3). However, when the three loci were combined, defined as the haplotype AGA genotype, comprising AA, GG and AA of the APC SNPs, patients with IM persistence had a higher rate of the AGA genotype than patients without IM persistence (74 vs 36 %, $P=0.022$, relative risk 2.64; 95 % CI 1.11–6.33). These data suggest that the host genomic predisposition of the APC gene is important in correlating the outcome of *H. pylori* eradication with IM regression.

In Table 4, when the two factors of host AGA genotype APC SNP and high-level nuclear β -catenin expression were combined, these patients had an increased relative risk of up to 4.5 (95 % CI 1.33–15.28) of IM persistence compared with hosts with the non-AGA genotype and low-level

Table 4. *APC* genotype and expression of nuclear β -catenin determine the relative risk of IM persistence after *H. pylori* eradication

The results show the grouping by *APC* SNP/level of nuclear β -catenin staining. AGA was defined as the genotypes AA, GG and AA of the *APC* SNP at the 4479, 5268 and 5465 loci, respectively, in the *H. pylori*-infected host. Non-AGA of the host indicates any genotype other than AA, GG and AA at these loci. Nuclear β -catenin staining was defined as low or high based on positive nuclear staining in the IM tissue prior to *H. pylori* eradication.

	Non-AGA/low	AGA/low	Non-AGA/high	AGA/high
Persistence of IM [% (n)]	0 (0/3)	0 (0/1)	60 (3/5)	77.8 (7/9)
<i>P</i> value*	—	1	0.196	0.045
Increased risk (95 % CI)	1	1	2.5 (0.86–7.31)	4.5 (1.33–15.28)

**P* value was analysed using Fisher's exact test. The increased risk of each group was compared with the reference index of patients with a non-AGA *APC* SNP genotype and with low-level nuclear β -catenin staining before treatment. The number of subjects was smaller than in Table 2 as there was not enough genomic DNA for PCR amplification of the *APC* gene in all subjects.

nuclear β -catenin expression ($P < 0.05$). These data support the suggestion that both host predisposition and the strong trigger by *H. pylori* isolates resulting in a more evident nuclear β -catenin accumulation are important during the maintenance of IM.

***H. pylori* CagA decreases cell membrane β -catenin in E-cadherin-expressing cells**

In general, an intact E-cadherin/ β -catenin complex maintains the localization of β -catenin on the cell membrane. We thus investigated whether *H. pylori* virulence factors such as CagA or VacA could interact with the E-cadherin/ β -catenin complex to mobilize the β -catenin from the cell membrane. In this study, we selected E-cadherin-negative AGS cells and E-cadherin-promoter-mutated MKN45 cells (Yokozaki, 2000) and challenged them with *H. pylori* for 20 h. Fig. 3(a) shows that *H. pylori* wild-type and the *vacA* mutant, but not the *cagA* mutant, could mobilize β -catenin from the membrane fraction in MKN45 cells. These data support the suggestion that *H. pylori* CagA can interact with E-cadherin and deregulate the β -catenin signal in gastric epithelial cells (Murata-Kamiya *et al.*, 2007). However, in E-cadherin-negative AGS cells, which have constitutively activated β -catenin signals (Ikenoue *et al.*, 2002), mobilization of β -catenin from the cell membrane was still related to *H. pylori* CagA status (Fig. 3a). This suggests indirectly that even in the absence of or with a weakened E-cadherin/ β -catenin complex on the cell membrane, *H. pylori* may still have an impact on the regulation of the β -catenin signal. Thus the downstream regulation process of the Wnt/ β -catenin pathway needs further investigation at the cytosol level.

Phospho-GSK-3 β induction and IM regression by *H. pylori* eradication

Cytosolic β -catenin can be further inactivated by GSK-3 β . Therefore, inhibition of GSK-3 β may be essential for the

subsequent increase in β -catenin nuclear translocation (Cohen & Frame, 2001; Clevers, 2006). Because of the higher levels of nuclear β -catenin in the IM epithelium, we speculated that cytosolic GSK-3 β might be inactivated by *H. pylori* infection. GSK-3 β inactivation can be achieved by two mechanisms: Wnt-dependent inhibition and phosphorylation of GSK-3 β at the Ser9 residue (Cohen & Frame, 2001). We found that *H. pylori* could inhibit GSK-3 β by Ser9 phosphorylation of GSK-3 β in AGS cells, and that this phosphorylation of GSK-3 β was independent of the *cagA* or *vacA* status of *H. pylori* (Fig. 3b). This finding is compatible with the results of recent studies that found that *H. pylori* induces phosphorylation of GSK-3 β independently of *cagA* and *virB7*, but that the combined activities of the *cag* pathogenicity island and OipA are involved in full-level phosphorylation of GSK-3 β (Sokolova *et al.*, 2008; Tabassam *et al.*, 2009). Our result was incompatible with those of Nakayama *et al.* (2009), who reported that *H. pylori vacA* is important for phosphorylation of GSK-3 β . These different findings may be due to the different cell lines used.

In Fig. 3(c), we found that the induction levels of phospho-GSK-3 β in AGS cells were significantly higher with *H. pylori* isolates from patients with IM regression than with *H. pylori* isolates from patients with IM persistence (1.5 vs 1.2, $P = 0.011$). These data are reported here for the first time and indicate that, if patients have more phospho-GSK-3 β induced by *H. pylori*, then there should be a major increase in the proportion of β -catenin nuclear distribution. Thus, after *H. pylori* eradication in these patients, reversal of the Wnt/ β -catenin pathway may be achieved leading to better IM regression. In contrast, for those patients with initial lower levels of phospho-GSK-3 β induced by *H. pylori*, the efficacy of *H. pylori* eradication will have a more limited effect on IM regression, as there will not be a major shift of nuclear β -catenin by the GSK-3 β -dependent process.

There remained several limitations of the current study. Our study case number was relatively limited as a result of

Fig. 3. The Wnt-associated pathway can be induced by *H. pylori* infection. (a) *H. pylori* wild-type and a *vacA* mutant, but not a *cagA* mutant, can mobilize β -catenin resulting in almost absent expression in the membrane fraction of AGS and MKN45 cells. MKN45 cells, but not AGS cells, express E-cadherin in the membrane fraction, but expression of E-cadherin was not enhanced by *H. pylori* challenges using wild-type, *cagA* and *vacA* isogenic mutants for 20 h. (b) GSK-3 β in the cytosol can bind with β -catenin mobilized from the membrane into the cytosol. This binding can thus interfere with consequent triggering of the Wnt-associated pathway. Phosphorylated GSK-3 β (p-GSK-3 β) can inhibit the activity of GSK-3 β as a result of *H. pylori* infection for 2 h and thus indirectly facilitate the Wnt-associated pathway. (c) The expression of p-GSK-3 β in AGS cells infected by *H. pylori* for 2 h was lower with *H. pylori* isolates from patients with IM persistence (IM-P) (HP449, HP609, HP632, HP662 and HP873) than with those from patients without IM persistence (HP517, HP533, HP673, HP762 and HP851). β -Actin was analysed in all experiments as a loading control.

the strict selection of cases with a longer follow-up period to prevent the bias of having too short a follow-up period, which might have masked the regression of IM after *H. pylori* eradication. Future studies need to be carried out on a larger scale and with a longer follow-up period. Secondly, our results disclosed certain APC SNP genotype types with correlation to regression of IM after *H. pylori* eradication. The exact biological function of such APC genotypes deserves further investigation with regard to the regulation of the maintenance of IM during *H. pylori* infection. Moreover, the current study did not conduct direct staining of phospho-GSK-3 β in the clinical samples, which would be interesting for future research.

In conclusion, the genetic predisposition of the host Wnt/ β -catenin pathway and the variable triggering of Wnt molecules by infecting *H. pylori* isolates may be important for determining the persistence of IM following *H. pylori* eradication. For those patients with host APC SNPs such as the AGA genotype and high levels of nuclear β -catenin, in

addition to *H. pylori* eradication, studies of additional factors targeting the regulation of this pathway may improve our understanding of ways to improve regression of IM.

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