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의학박사 학위논문

위암에서 헬리코박터 파일로리 감염에 의한 Wnt 길항체 유전자들의 조절 변화

Helicobacter pylori–induced modulation of the promoter methylation of Wnt antagonist genes in gastric carcinogenesis

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양 효 준

A thesis of the Degree of Doctor of Philosophy

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Helicobacter pylori–induced modulation of the promoter methylation of Wnt antagonist genes in gastric carcinogenesis

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이 논문을 의학박사 학위논문으로 제출함

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ABSTRACT

Introduction: This study aimed to investigate the changes in the promoter methylation and gene expression of multiple Wnt antagonists between the chronic infection and eradication of *Helicobacter pylori* (*H. pylori*) in the gastric carcinogenesis.

Methods: The levels of methylation and corresponding mRNA expression of seven Wnt antagonist genes (SFRP1, 2, 5, DKK1, 2, 3, and WIF1) were compared among the patients with *H. pylori*–positive gastric cancers (GCs), *H. pylori*–positive and –negative controls by quantitative MethyLight assay and real–time RT PCR, respectively. The changes of the methylation and expression levels of the genes were also compared between the *H. pylori* eradication and persistent groups 1 year after endoscopic resection of GCs.

Results: The methylation levels of SFRP and DKK family genes were significantly increased in the patients with *H. pylori*–positive GCs and followed by *H. pylori*–positive controls compared with *H. pylori*–negative controls ($P < 0.001$). The SFRP1, 2, and DKK3 gene expression were stepwise down–regulated from *H. pylori*–negative controls, *H. pylori*–positive controls and to *H. pylori*–

posiiitive GCs ($P < 0.05$). Among the Wnt antagonists, only the degrees of methylation and down-regulation of DKK3 were significantly reduced after *H. pylori* eradication ($P < 0.05$).

Conclusions: Epigenetic silencing of SFRP and DKK family genes may facilitate the formation of epigenetic field during *H. pylori*-associated gastric carcinogenesis. The epigenetic field may not be reversed even after *H. pylori* eradication except for DKK3 methylation.

Keywords: *Helicobacter pylori*; Wnt Signaling Pathway; Intercellular Signaling Peptides and Proteins; DNA Methylation; Stomach Neoplasms.

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LIST OF ABBREVIATIONS

EGD: early gastric cancer

GC: gastric cancer

IHC: immunohistochemistry

IQR: interquartile range

PMR: percentage of methylated reference

INTRODUCTION

Worldwide gastric cancer (GC) ranks fifth in incidence and third in the mortality with estimated 0.9 million cases and 0.7 million deaths in 2012.[1] Especially, in eastern Asia, half of the world's cases occur and the morality rates are highest. In Korea and Japan, majority of GC is detected early by nationwide cancer screening,[2,3] and endoscopic resection has become an effective alternative to surgery for indicated cases of early gastric cancers (EGCs).[4,5] However, the risk of development of metachronous GC in the remnant stomach after endoscopic resection is 6.7-times higher than in surgical resection.[6] This suggests that the remnant gastric mucosa has already been affected by certain carcinogenic changes in patients with GC, forming a field cancerization.[7]

Aberrant DNA methylation is deeply involved in the gastric carcinogenesis as a major mechanism of epigenetic alteration.[8] Inactivation of tumor suppressor genes by promoter hypermethylation was observed in GC,[9] which was also induced by *Helicobacter pylori* (*H. pylori*) infection.[10] The levels of methylation were increased in non-cancerous gastric mucosae of patients with GC and higher in cases with multiple GCs,[11]

which suggested that *H. pylori* infection could induce epigenetic field for cancerization.[12] However, it has been little known how the epigenetic field for GC is affected by the eradication of *H. pylori.*[13,14]

The Wnt/β-catenin pathway has been known to play a crucial role in the development of GC.[15,16] The activation of Wnt/β-catenin pathway was enhanced by *H. pylori* infection[17] and partly attenuated by *H. pylori* eradication.[18] Several studies have shown that Wnt/β-catenin pathway was activated by epigenetic inactivation of various Wnt antagonists such as secreted frizzled-related protein (SFRP) family,[19,20] DICKKOPF (DKK) family,[21] and Wnt inhibitory factor-1 (WIF1)[22] in human GCs. However, there has not been no comprehensive study on promoter methylation and subsequent down-regulation of multiple Wnt antagonists in non-cancerous gastric mucosae of GC patients. Furthermore, it has not been either explored whether the epigenetic field of Wnt antagonists is influenced by *H. pylori* infection and eradication.

In this study, we aimed to evaluate whether epigenetic field of Wnt antagonists are formed during gastric carcinogenesis and affected by *H. pylori* infection and eradication. First, we investigated the levels of promoter methylation and

corresponding mRNA expression of seven Wnt antagonist genes (SFRP1, 2, 5, DKK1, 2, 3, and WIF1) in *H. pylori*–associated gastric carcinogenesis represented as *H. pylori*–negative controls, *H. pylori*–positive controls, and *H. pylori*–positive patients with GCs. To assess the effect of *H. pylori* eradication as well as *H. pylori* infection, we modified the design by Maekita et al.[10] and focused on *H. pylori*–positive GCs. Then, to evaluate the effect of *H. pylori* eradication on the possible regression of the filed, we compared changes in the gene methylation and mRNA expression levels between *H. pylori* eradication and persistent patients one year after endoscopic resection of EGC.

MATERIALS AND METHODS

1. Subjects and tissue samples

This study included 60 patients with *H. pylori*–positive GCs, 36 *H. pylori*–positive controls, and 36 *H. pylori*–negative controls. Among the patients with GCs, 30 patients were eradication group, and remaining 30 were persistent group. Outpatients or screening recipients who were underwent upper endoscopy and were diagnosed as normal or gastritis were consisted control group.

During endoscopy, two pieces of gastric mucosa were taken respectively from the antrum and the corpus for histological evaluation of inflammatory cell infiltration, atrophy, and intestinal metaplasia according to the updated Sydney scoring system.[23] The status of *H. pylori* was evaluated both by histology with modified Giemsa staining and a rapid urease test (CLO test; Delta West, Bently, Australia). *H. pylori* infection was considered as positive when at least one of the two results was positive.[24]

Two biopsy samples for promoter methylation and mRNA expression analyses were obtained from antral mucosa of the control subjects. In the GC patients, two pieces of non–

cancerous antral mucosa were taken respectively in baseline and after 1 year of endoscopic resection and subsequent *H. pylori* eradication or follow-up. This study was approved by Institutional Review Boards of Seoul National University Hospital (H-1405-003-574), and written informed consent was obtained from all subjects.

2. DNA extraction, bisulfite modification, and MethyLight assay

DNA was extracted from gastric mucosa and modified with sodium bisulfite as previously described.[25] In brief, specimens were dissolved in proteinase K solution (20 mmol/L Tris-hydrochloride [pH 8.0], 10 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and 20 mg/mL proteinase K) followed by DNA isolation using phenol/chloroform extraction and ethanol precipitation. Genomic DNA of 1 μg was bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) following the manufacturer's instruction.

The methylation status of seven Wnt antagonist genes from bisulfite-modified DNA samples was quantified using real-time PCR-based MethyLight technology as previously reported.[25-27] MethyLight, as a sensitive, high-throughput methylation assay,

provides highly specific detection of methylation by using probes which cover methylation sites as well as methylation-specific primers.[27] The primer and probe sequences used in the reaction are shown in Table 1.[28-31] Quantified level of each gene was reported as a percentage of methylated reference (PMR), which is relative methylation ratio of target gene to ALU gene of a sample divided by a ratio of target gene to ALU gene of *M.Sss*I-treated sperm DNA and multiplied by 100.[27] All samples were tested in triplicate.

Table 1. Primer and probe sequences used in MethyLight reaction.

Gene	Primer and probe sequences	Ref
SFRP1	Primer (Forward): 5'-GAATTCGTTCGCGAGGGA-3'	29
	Primer (Reverse): 5'-AAACGAACCGCACTCGTTACC-3'	
	Probe: 6FAM-5'-CCGTCACCGACGCGAAAACCAAT-3'-BHQ-1	
SFRP2	Primer (Forward): 5'-GCGTTTTAGTCGTCGGTTGTTAGT-3'	29
	Primer (Reverse): 5'-AAACGACCGAAATTCGAACTTATC-3'	
	Probe: 6FAM-5'-CGAACCCGCTCTCTTCGCTAAATACGA-3'-BHQ-1	
SFRP5	Primer (Forward): 5'-GCGTTTTAGTCGTCGGTTGTTAGT-3'	29
	Primer (Reverse): 5'-AAACGACCGAAATTCGAACTTATC-3'	
	Probe: 6FAM-5'-CGAACCCGCTCTCTTCGCTAAATACGA-3'-BHQ-1	
DKK1	Primer (Forward): 5'-TTTGGGATCGTAGGGGGTTTTC-3'	30
	Primer (Reverse): 5'-AACCTAAATCCCCACGAAACCG-3'	
	Probe: 6FAM-5'-TGATTTTGTAGTCGAATCGGT-3'-MGBNFQ	
DKK2	Primer (Forward): 5'-GCGTAAGTTCGTTTTTTAGGTATCG-3'	31
	Primer (Reverse): 5'-GTTATCCCCTAACTCACAAAAAACAAC-3'	
	Probe: 6FAM-5'-TGCGTTGGTAGCGATT-3'- BHQ-1	
DKK3	Primer (Forward): 5'-GCGTCGTTTTCGTATTTGTATTCG-3'	32
	Primer (Reverse): 5'-CGACTAAACCGAATTACGCTACGA-3'	
	Probe: 6FAM-5'-CGAACTAAATCTACTCGCTCCCGCCGAAA-3'-BHQ-1	
WIF1	Primer (Forward): 5'-GGTTGAGGGAGTTGTAGCGTAGTAG-3'	31
	Primer (Reverse): 5'-AAAACTCCTCGTACCGCACCTA-3'	
	Probe: 6FAM-5'-CGGCGTTAGGTTGC-3'- BHQ-1	

3. RNA extraction and quantitative RT-PCR

Gastric mucosa were homogenized followed by RNA isolation using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Complementary DNA was synthesized from 1 μg of total RNA with M-MLV reverse transcription reagent (Invitrogen), and 4 μl of the resulting cDNA was amplified in 10 μl of 2X SYBR Green master mix (Takara Bio, Shiga, Japan) using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA, USA). The primer sequences and reaction conditions are presented in Table 2.[32-37] Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method, and the human GAPDH gene was used as an endogenous reference.[38] All samples were tested in duplicate.

Table 2. Primer sequences used in the quantitative RT-PCR assay

Genes	Primer sequences	Cycles	T_A (°C)	Product size (bp)	Ref
SFRP1	F: 5'-ACGTGGGCTA CAAGAAGATGG-3' R: 5'-CAGCGACACG GGTAGATGG-3'	45	60	184	33
SFRP2	F: 5'-ACGGCATCGA ATACCAGAAC-3' R: 5'-GCTGGATGGT CTCGTCTAGG-3'	45	55	186	34
SFRP5	F: 5'-GTGCTGCACAT GAAGAATGG-3' R: 5'-GCAGGGGTAG GAGAACATGA-3'	45	62	180	34
DKK1	F: 5'-GAGTCCTTCTG AGATGATGG-3' R: 5'-TTGATAGCGTT GGAATTGAG-3'	45	62	141	35
DKK2	F: 5'-CCCAGTACCCG CTGCAATAATGGC-3' R: 5'-CTGTGCCGAGT ACCATCCAG-3'	35	48	92	36
DKK3	F: 5'-TGGTCACCAGG GCTGCTT-3' R: 5'-GTCTTCTGGGT GGCAGTGAT-3'	45	60	182	37
WIF1	F: 5'-TGAATTTTACC TGGCAAGCTG-3' R: 5'-GGACATTGAC GGTTGGATCT-3'	45	55	106	38
β-actin	F: 5'-TTCGAGCAAGA GATGGCCAC-3' R: 5'-CGGATGTCCA CGTCACACTT-3'	45	60		

T_A = annealing temperature

4. Immunohistochemistry

To evaluate cytoplasmic and nuclear stain of β-catenin which suggests activation of Wnt/β-catenin pathway, immunohistochemistry (IHC) was performed. Because of limited availability of formalin-fixed, paraffin-embedded tissues, three cases for each group were stained with a monoclonal antibody for β-catenin (BD biosciences, San Diego, CA, USA; dilution 1:800) using Ventana BenchMark XT staining systems (Ventana Medical Systems Inc., Tucson, AZ, USA) as previously described.[39]

The expression of β-catenin was evaluated semiquantitatively based on previous reports.[17,18,40] For the evaluation of membranous stain, *H. pylori*-negative controls were set as reference. Membranous expression of β-catenin in the *H. pylori*-positive controls or GCs was considered as 'maintained' when both the intensity and the frequency were similar to those in the *H. pylori*-negative controls, 'reduced' when the staining was decreased, and 'absent' when the membranous staining are not observed. In the evaluation of cytoplasmic and nuclear stain, low and high level expressions were defined as <10% and >10% of cells with positive cytoplasmic or nuclear β-catenin staining.

5. Statistical analysis

For the comparison between GCs and controls, Kruskal-Wallis or Mann-Whitney U test for continuous variables, and Pearson's chi-squared test or Fisher's exact test for the categorical variables were used. The gene methylation or expression levels at baseline and at one year follow-up were compared using Wilcoxon signed rank test for continuous variables and McNemar test for categorical variables. All statistical analyses were performed with SPSS (version 21.0; SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered significant.

RESULTS

Subject characteristics

Clinicopathological characteristics of study subjects are summarized in Table 3. There were significant differences in demographics among the *H. pylori*–negative and –positive controls, and the patients with *H. pylori*–positive GCs. The patients with *H. pylori*–positive GCs were oldest (median, 60.2 years; interquartile range [IQR], 53.5–68.1) and most likely to be male (65%, 39/60), followed by *H. pylori*–positive controls, and then *H. pylori*–negative controls (both $P < 0.001$). Histological characteristics were also different among the groups. The infiltration of neutrophil and monocyte were more prominent in the gastric mucosae of the patients with *H. pylori*–positive GCs and controls than in those of *H. pylori*–negative controls (both $P < 0.001$). In addition, intestinal metaplasia was more severe in the patients with GCs than the controls ($P < 0.001$).

Table 3 Clinicopathological characteristics of study subjects

	Hp negative controls (*n* = 36)	*Hp* positive controls (*n* = 36)	*Hp* positive GCs (*n* = 60)	*P* value
Age, years, median (IQR),	45.5 (31.8–61.5)	49.5 (40.0–57.8)	60.2 (53.5–68.1)	<0.001
Sex, *n* (%)				<0.001
Female	27 (75.0)	25 (69.4)	21 (35.0)	
Male	9 (25.0)	11 (30.6)	39 (65.0)	
Neutrophil, *n* (%)				<0.001
None to mild	33 (91.7)	3 (8.3)	2 (3.3)	
Moderate to severe	3 (8.3)	33 (91.7)	58 (96.7)	
Monocyte, *n* (%)				<0.001
None to mild	28 (72.2)	1 (2.8)	6 (10.0)	
Moderate to severe	10 (27.8)	35 (97.2)	54 (90.0)	
Atrophy, *n* (%)				0.135
None to mild	20/22 (90.0)	22/24 (91.7)	37/47 (78.7)	
Moderate to severe	2/22(9.1)	2/24 (8.3)	10/47 (21.3)	
Intestinal metaplasia, *n* (%)				<0.001
None to mild	36 (100)	36 (100)	28 (46.7)	
Moderate to severe	0 (0)	0 (0)	32 (53.3)	

Hp H. pylori, *GC* gastric cancer, *IQR* interquartile range

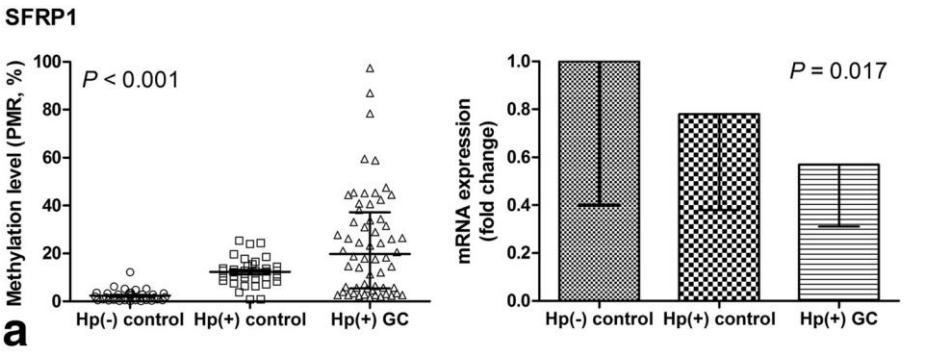
Aberrant DNA methylation and reduced mRNA expression of Wnt antagonists in *H. pylori*-associated gastric carcinogenesis

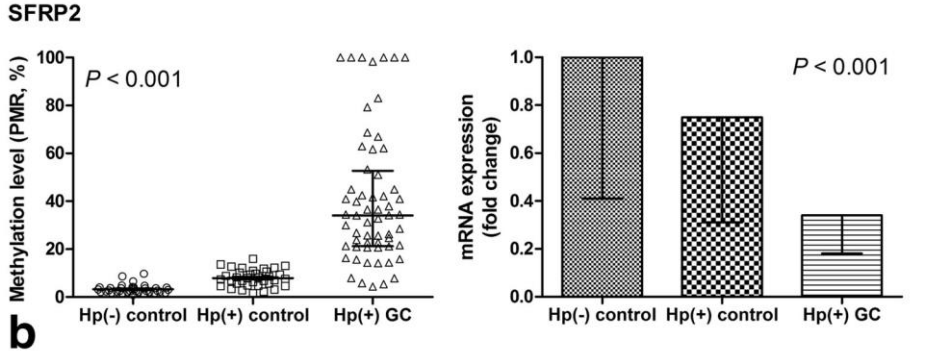
The methylation levels of seven Wnt antagonist genes in gastric samples of the three groups were quantified. In the MethyLight assay, methylation levels of SFRP and DKK family genes in the gastric mucosae were very low in *H. pylori*-negative controls with the median PMR values < 5 (Fig. 1a-f, Table 4). They were increased in *H. pylori*-positive controls with the median PMR values up to 12.4 and became highest in the non-cancerous mucosae of *H. pylori*-positive GCs although the methylation levels were quite variable among the cases and genes with median PMR values ranged between 8.2 and 34.1. The stepwise increment in the methylation levels of the genes from *H. pylori*-negative controls, *H. pylori*-positive controls, and *H. pylori*-positive GCs were statistically significant (all $P < 0.001$). Although methylation level of WIF1 was increased with *H. pylori* infection, it was rather decreased to lower level in patients with GCs (Fig. 1g).

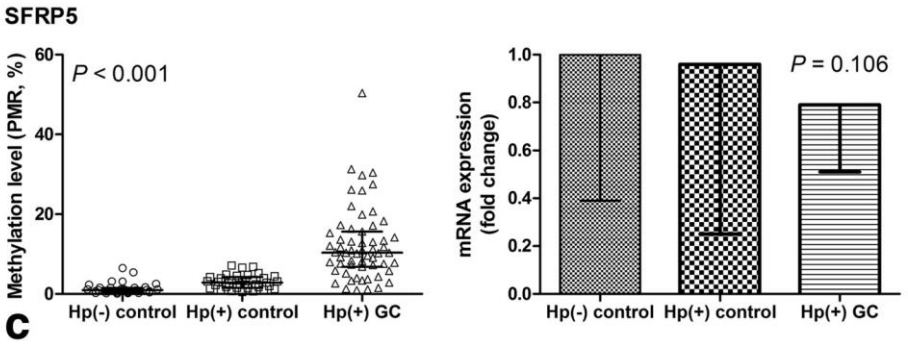
Next, mRNA expression levels of Wnt antagonists were compared among the three groups. In the real-time RT-PCR, the levels of mRNA expression of SFRP and DKK family genes were down-regulated in *H. pylori*-positive controls than in *H.*

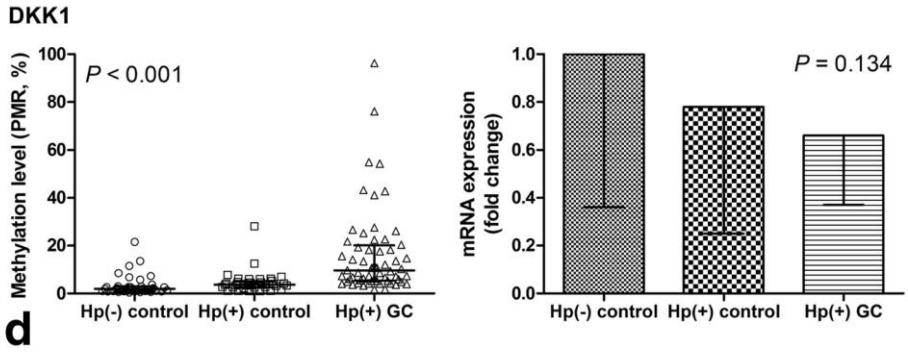
pylori-negative controls and decreased further in non-cancerous mucosae of patients with *H. pylori*-positive GCs (Fig. 1a-f). In particular, reduction of gene expression was statistically significant in SFRP1, 2, and DKK3 genes (all $P < 0.05$).

In IHC staining for β-catenin, all three cases in the *H. pylori*-negative controls showed almost exclusively membranous stain and negative for cytoplasmic or nuclear stain (Fig 2a). All three cases of *H. pylori*-positive controls showed low level expression of cytoplasmic and nuclear β-catenin while membranous staining was maintained (Fig 2b). In *H. pylori*-positive patients with GCs, two cases showed high level expression and the remaining one case showed low level expression of cytoplasmic and nuclear β-catenin, but membranous staining was also maintained in all cases compared to *H. pylori*-negative controls (Fig 2c, d).


SFRP1
Methylation level (PMR, %)
P < 0.001
Hp(-) control
Hp(+) control
Hp(+) GC
a
mRNA expression (fold change)
P = 0.017
Hp(-) control
Hp(+) control
Hp(+) GC


SFRP2
Methylation level (PMR, %)
P < 0.001
Hp(-) control
Hp(+) control
Hp(+) GC
b
mRNA expression (fold change)
P < 0.001
Hp(-) control
Hp(+) control
Hp(+) GC


SFRP5
Methylation level (PMR, %)
P < 0.001
Hp(-) control
Hp(+) control
Hp(+) GC
c
mRNA expression (fold change)
P = 0.106
Hp(-) control
Hp(+) control
Hp(+) GC


DKK1
Methylation level (PMR, %)
P < 0.001
Hp(-) control
Hp(+) control
Hp(+) GC
d
mRNA expression (fold change)
P = 0.134
Hp(-) control
Hp(+) control
Hp(+) GC

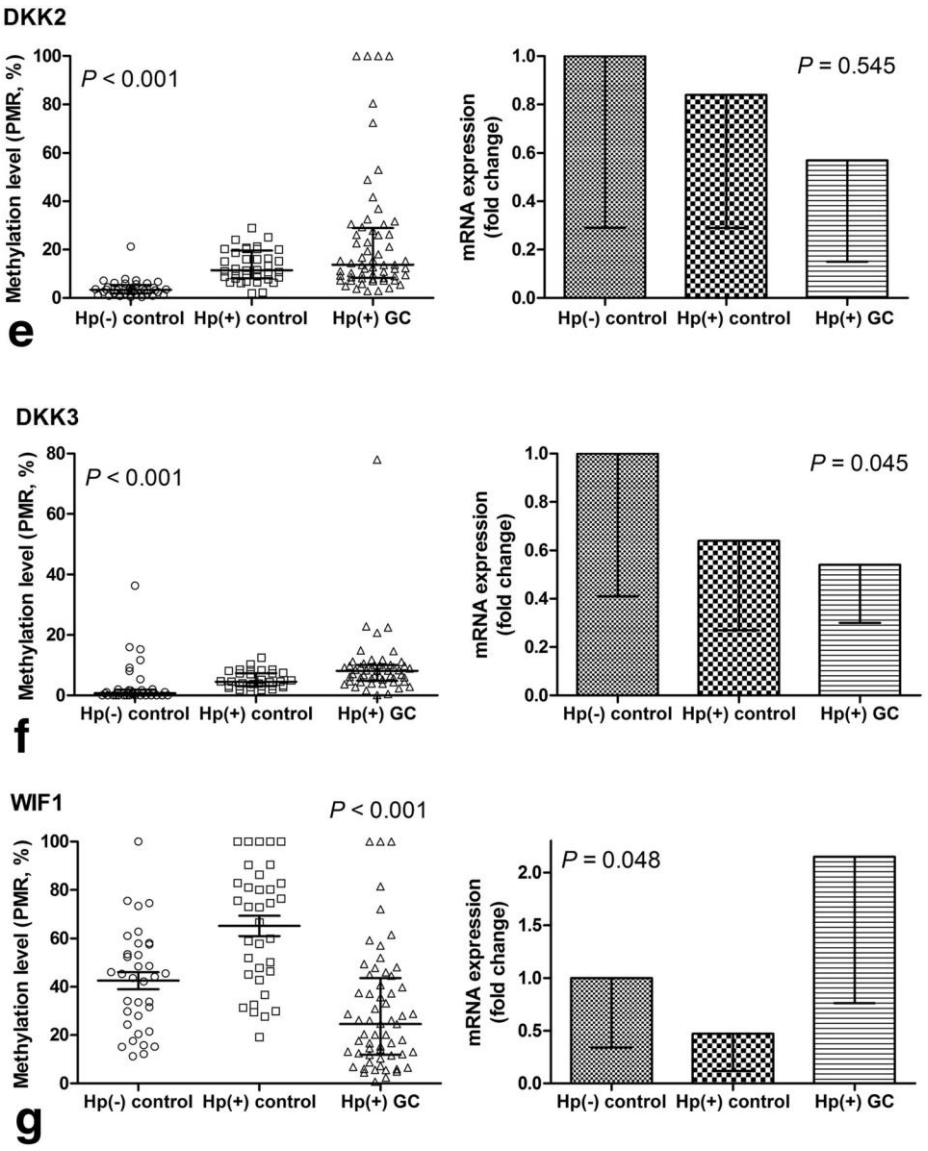


Fig. 1 DNA methylation levels and corresponding mRNA expression levels in the seven Wnt antagonist genes: (a) SFRP1, (b) SFRP2, (c) SFRP5, (d) DKK1, (e) DKK2, (f) DKK3, and (g) WIF1 among *H. pylori*−negative controls, *H. pylori*−positive controls, and *H. pylori*−positive patients with gastric cancers. Results of DNA methylations are given as medians and interquartile ranges. Results of mRNA expression are

presented as fold change compared to *H. pylori*−negative controls and standard deviations. Along with the *H. pylori*−associated gastric carcinogenesis, methylation levels in SFRP1, 2, 5, DKK1, 2, and 3 were significantly increased, and mRNA expression levels in SFRP 1, 2, and DKK3 were significantly reduced.

PMR percentage of methylated reference, *Hp H. pylori, GC* gastric cancer

Table 4. DNA methylation levels in the seven Wnt antagonist genes among *H. pylori*-negative controls, *H. pylori*-positive controls, and *H. pylori*-positive patients with gastric cancer.

	H. pylori(-) control (n = 36)	*H. pylori*(+) control (n = 36),	*H. pylori*(+) GC (n = 60)
SFRP1	1.7 (0.1, 12.2)	12.4 (0.8, 25.3)	19.7 (1.5, 97.4)
SFRP2	2.7 (0.9, 9.7)	7.8 (1.6, 16.0)	34.1 (4.4, 100.0)
SFRP5	1.0 (0.1, 6.5)	2.9 (0.5, 7.2)	10.4 (1.0, 50.4)
DKK1	2.0 (0.5, 21.6)	3.7 (1.1, 28.0)	9.6 (1.7, 96.3)
DKK2	3.3 (0.4, 21.3)	11.4 (2.0, 28.8)	13.8 (2.9, 100.0)
DKK3	0.8 (0.1, 36.3)	4.6 (1.5, 12.5)	8.2 (0.1, 78.0)
WIF1	44.1 (11.3, 100.0)	72.8 (19.2, 100.0)	7.1 (0.2, 44.8)

Values are presented in medians (ranges) of percentage of methylated reference (PMR).

GC gastric cancer

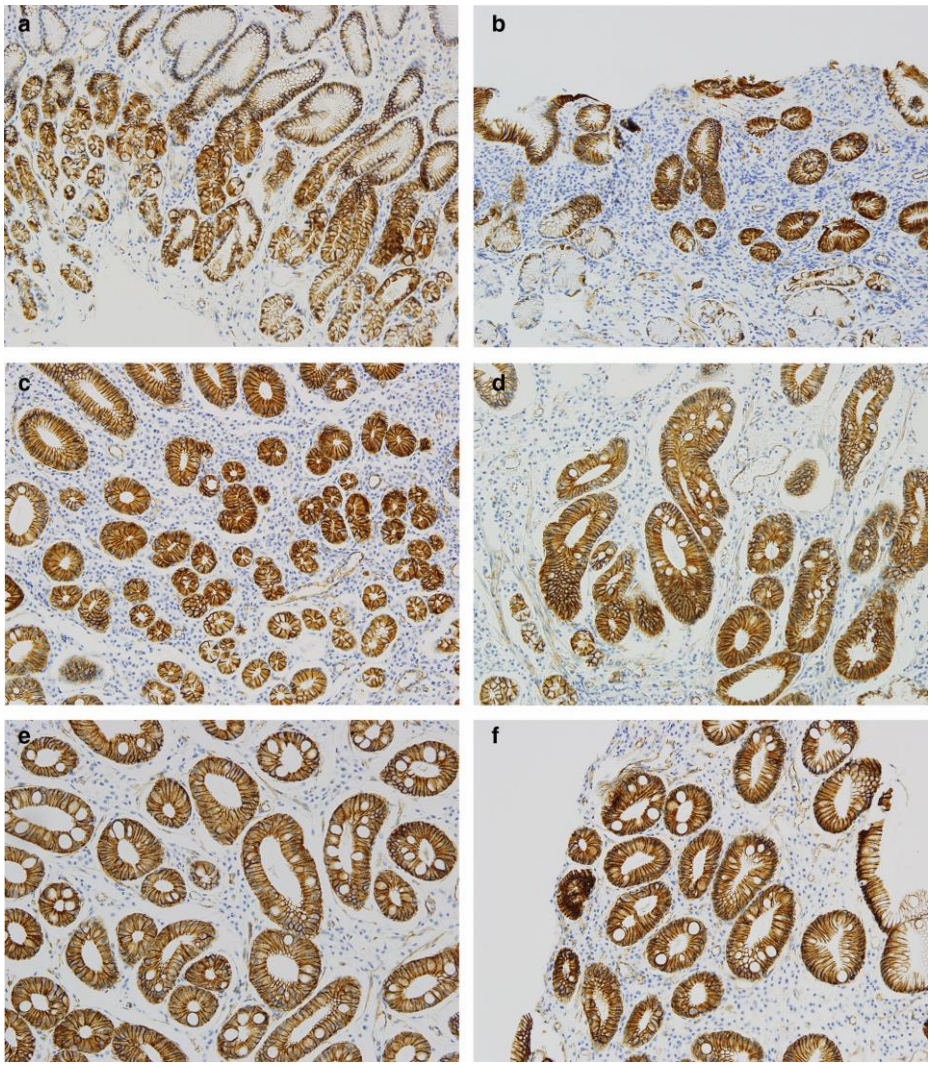


Fig. 2 Representative immunohistochemical staining for β-catenin (original magnification, x200). (a) Gastric mucosa of *H. pylori*-negative control showed continuous membranous staining without cytoplasmic or nuclear expression of β-catenin. (b) Gastric mucosa of *H. pylori*-positive control demonstrated both maintained membranous staining and low level expression of cytoplasmic and nuclear stating of β-catenin. (c and d) Non-cancerous mucosa of *H. pylori*-positive

patients with gastric cancers revealed high level expression of cytoplasmic and nuclear β-catenin as well as maintained membranous staining. (e) One-year follow-up sample of the case (c) after *H. pylori* eradication. (f) One-year follow-up sample of the case (d) with persistent *H. pylori* infection. There were no apparent changes in cytoplasmic and nuclear β-catenin staining and membranous staining between baseline and follow-up irrespective of *H. pylori* eradication.

Effect of *H. pylori* eradication on DNA methylation and mRNA expression of Wnt antagonists

The patients with *H. pylori*-positive GCs were divided into eradication and persistent groups. Although baseline characteristics were not different between the two groups (all $P > 0.05$), the difference in the pathological characteristics in the gastric mucosae became prominent at one year follow-up (Table 5). Neutrophil and monocyte infiltration were less prominent (both $P < 0.001$) and atrophy were less severe ($P = 0.045$) in the eradication group than in the persistent group, though severity of intestinal metaplasia were similar between the groups.

Methylation levels of Wnt antagonists were not reduced at one year after *H. pylori* eradication compared to the baseline value (Fig. 3a-e, g), except for the methylation level of DKK3 which was significantly decreased in the eradication group ($P = 0.017$) while it did not in the persistent group (Fig 3f). Rather, there were tendencies of increasing levels of DNA methylation of SFRP1, 2, 5, DKK1, and 2 genes irrespective of *H. pylori* eradication (Table 6). The levels of mRNA expression of these genes significantly reduced both in the eradication and persistent groups (Fig. 3 a-3). However, the expression of

DKK3 at one year after *H. pylori* eradication were similar to the baseline value and significantly less reduced in the eradication group than in the persistent group ($P = 0.023$) (Fig. 3f).

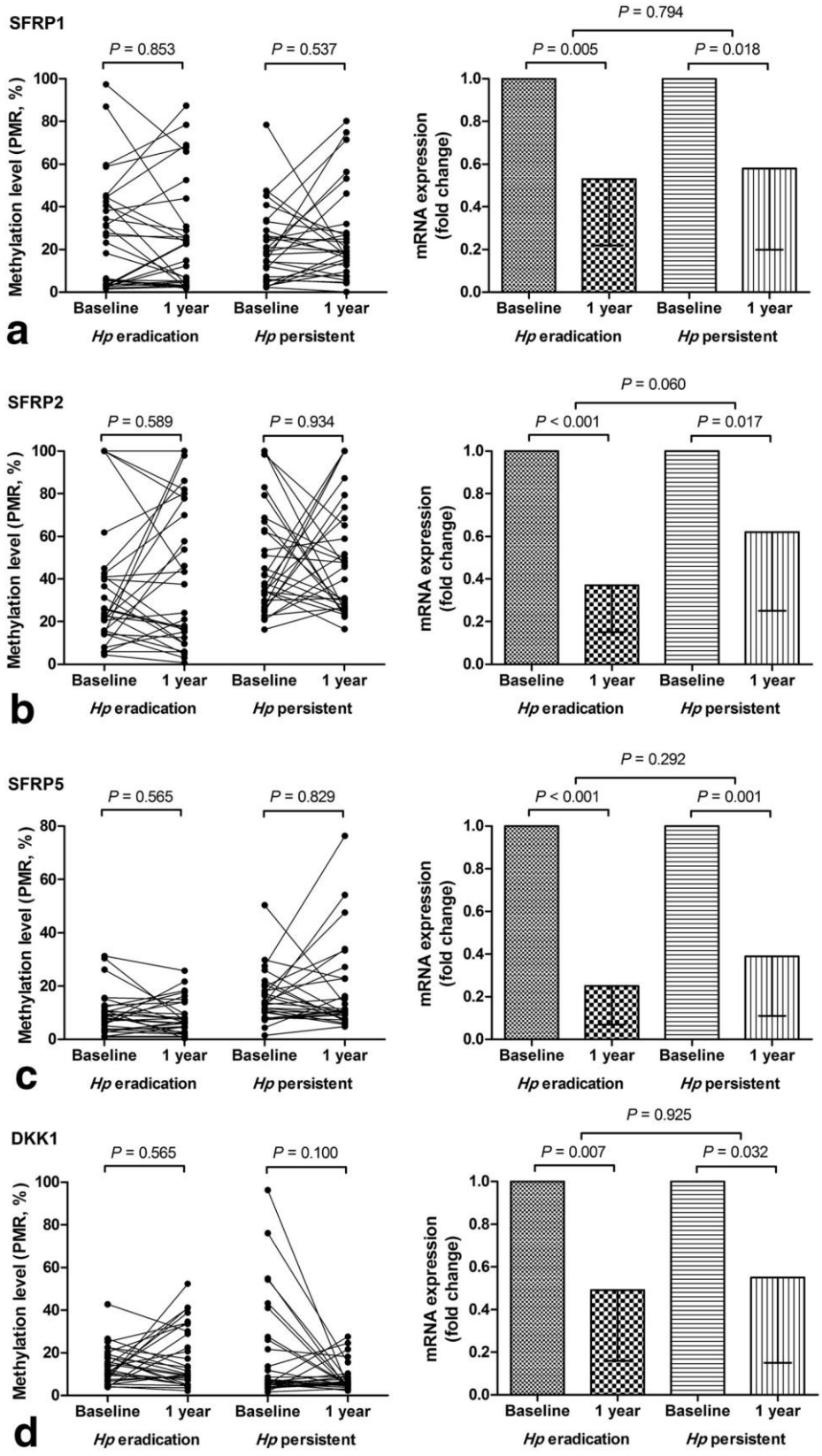
There were no apparent changes in the expression of cytoplasmic and nuclear β-catenin between baseline and follow-up both in the eradication (Fig. 2c and 2e) and persistent (Fig. 2d and 2f) groups.

Table 5 Clinicopathological difference between *H. pylori* eradication and persistent groups

	Hp eradication group (*n* = 30)		*Hp* persistent group (*n* = 30)		*P* value*
	Baseline	1 year f/u	Baseline	1 year f/u	
Age, years median (IQR),	61.7 (51.7-68.4)		59.5 (54.7-66.1)		
Sex, *n* (%)					
Female	9 (30.0)		12 (40.0)		
Male	21 (70.0)		18 (60.0)		
Neutrophil, *n* (%)					<0.001
None to mild	1 (3.3)	30 (100.0)	1 (3.3)	0 (0.0)	
Moderate to severe	29 (96.7)	0 (0.0)	29 (96.7)	30 (100.0)	
Monocyte, *n* (%)					<0.001
None to mild	3 (10.0)	14 (46.7)	3 (10.0)	0 (0.0)	
Moderate to severe	27 (90.0)	16 (53.3)	27 (90.0)	30 (100.0)	
Atrophy, *n* (%)					0.045
None to mild	19/24 (79.2)	18/25 (72.0)	18/23 (78.3)	10/23 (43.5)	
Moderate to severe	5/24 (20.8)	7/25 (28.0)	5/23 (21.7)	13/23 (56.5)	
IM, *n* (%)					0.184
None to mild	13 (43.3)	14 (46.7)	15 (50.0)	9 (30.0)	
Moderate to severe	17 (56.7)	16 (53.3)	15 (50.0)	21 (70.0)	

Hp H. pylori, *f/u* follow-up, *IM* intestinal metaplasia, *IQR* interquartile range

*One year follow-up results between *H. pylori* eradication and persistent groups were compared.


SFRP1
P = 0.853
P = 0.537
Methylation level (PMR, %)
Baseline
1 year
Baseline
1 year
Hp eradication
Hp persistent
a
P = 0.794
P = 0.005
P = 0.018
mRNA expression (fold change)
SFRP2
P = 0.589
P = 0.934
b
P = 0.060
P < 0.001
P = 0.017
SFRP5
P = 0.565
P = 0.829
c
P = 0.292
P < 0.001
P = 0.001
DKK1
P = 0.565
P = 0.100
d
P = 0.925
P = 0.007
P = 0.032

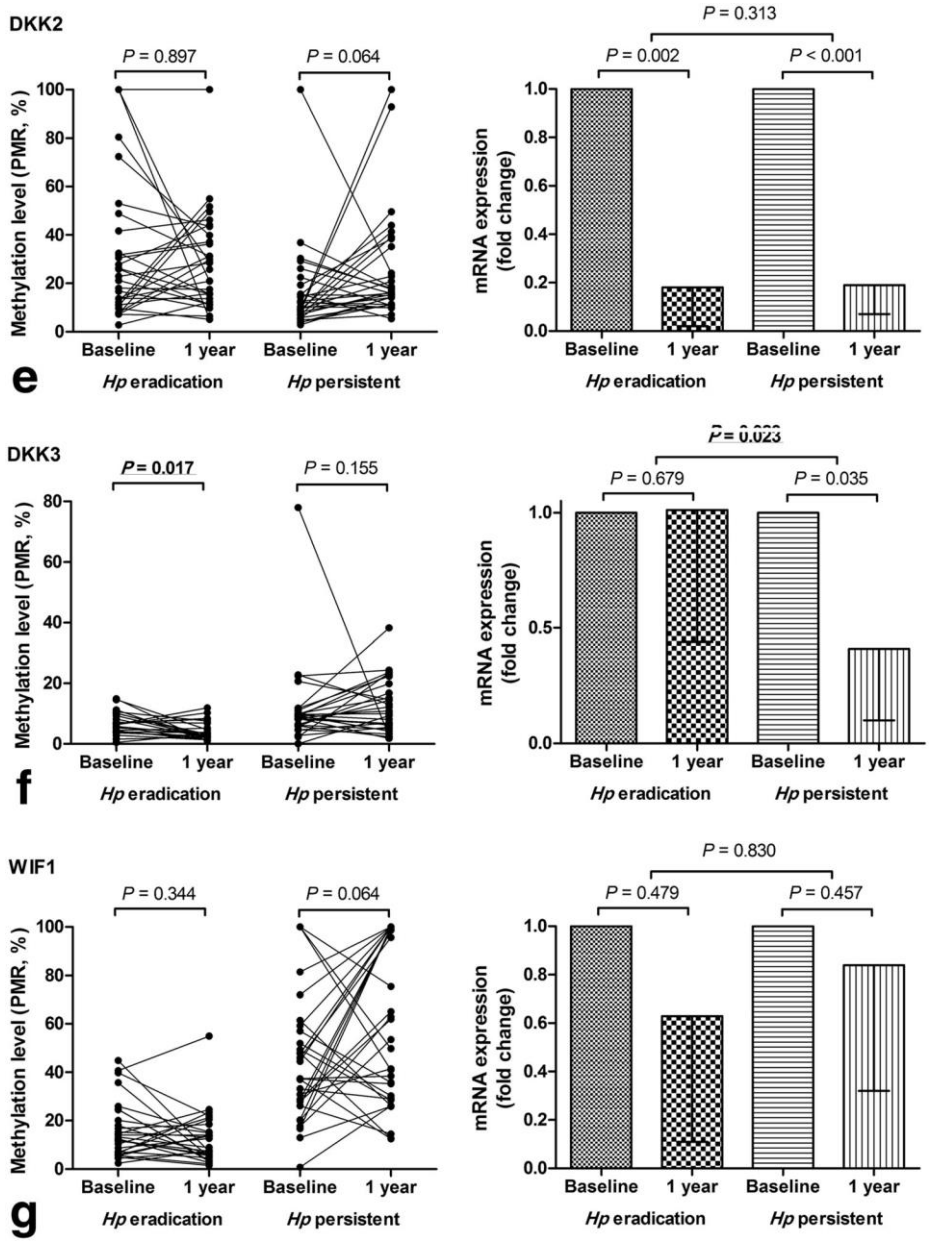


Fig. 3 DNA methylation levels and corresponding mRNA expression levels in the seven Wnt antagonist genes: (a) SFRP1, (b) SFRP2, (c) SFRP5, (d) DKK1, (e) DKK2, (f) DKK3, and (g) WIF1 in *H. pylori*-positive patients with gastric cancers at baseline and one-year follow-up in whom *H. pylori*

was eradicated (*Hp* eradication) or not (*Hp* persistent). After *H. pylori* eradication, methylation level only in DKK3 was significantly reduced (P = 0.017). Accordingly, mRNA expression level of DKK3 was significantly less reduced in the eradicated group (*Hp* eradication) than in the persistent group (*Hp* persistent) (P = 0.023).

PMR, percentage of methylated reference, *Hp H. pylori*

Table 6. Change of the methylation levels in *H. pylori* eradication and persistent groups

	H. pylori eradication group (*n* = 30)		
	Baseline	Follow-up	Change
SFRP1	18.7 (1.5, 97.4)	24.8 (1.8, 87.4)	+0.5 (-42.0, +56.1)
SFRP2	23.2 (4.4, 100.0)	30.8 (0.7, 100.0)	+0.8 (-56.6, +83.4)
SFRP5	6.8 (1.0, 31.3)	8.2 (0.5, 25.7)	+0.8 (-15.8, +23.5)
DKK1	10.7 (4.0, 42.8)	11.4 (2.2, 52.4)	+1.0 (-14.4, +45.0)
DKK2	22.1 (2.9, 100.0)	27.1 (5.1, 100.0)	+5.7 (-94.8, +42.8)
DKK3	6.5 (0.5, 14.9)	3.0 (1.0, 11.9)	-1.7 (-13.9, +5.4)
WIF1	12.4 (2.5, 44.8)	10.9 (1.5, 54.9)	-3.4 (-37.4, +16.9)
	H. pylori persistent group (*n* = 30)		
	Baseline	Follow-up	Change
SFRP1	18.4 (2.3, 78.4)	19.1 (0.2, 80.2)	+0.3 (-60.2, +62.3)
SFRP2	37.3 (16.3, 100.0)	42.7 (16.5, 100.0)	+3.6 (-73.2, +74.6)
SFRP5	10.8 (1.5, 50.4)	13.1 (4.8, 76.3)	+0.2 (-37.1, +48.8)
DKK1	5.2 (1.7, 96.3)	6.5 (2.3, 27.7)	+1.8 (-92.4, +22.4)
DKK2	10.1 (2.9, 100.0)	16.2 (5.5, 100.0)	+5.2 (-75.7, +91.0)
DKK3	9.1 (0.1, 78.0)	10.5 (1.8, 38.2)	+1.7 (-75.6, +26.4)
WIF1	2.7 (0.2, 30.7)	3.7 (0.3, 38.7)	+0.9 (-28.2, +31.6)

Values are presented in medians (ranges) of percentage of methylated reference (PMR).

DISCUSSION

In the present study, we evaluated the formation of epigenetic field for cancerization of Wnt antagonists during *H. pylori*-associated gastric carcinogenesis. MethyLight assay, quantitative methylation-specific PCR revealed that methylation levels of SFRP and DKK family gene promoters were increased in the non-cancerous mucosae of the patients with *H. pylori*-positive GCs, which were higher than *H. pylori*-positive controls and even higher than *H. pylori*-negative controls. Real-time RT-PCR showed corresponding stepwise down-regulation of SFRP1, 2, and DKK3 gene expression from *H. pylori*-negative controls to *H. pylori*-positive controls and then, to *H. pylori*-positive GCs. Furthermore, the effect of *H. pylori* eradication on the field were also explored. Among the Wnt antagonists, the degree of methylation and down-regulation were significantly reduced only in DKK3.

The epigenetic field in the gastric carcinogenesis is becoming an important issue as endoscopic resection has become widely performed for the treatment of EGC.[4,5] This is because not only the risk of metachronous recurrence is highly increased after endoscopic resection,[6] but the recurrence also has an important effect on the prognosis of the patients.[41]

Epigenetic alteration has been suggested as a possible mechanism for field cancerization. Previous studies showed that methylation-dependent silencing of tumor suppressor genes such as p16, CDH1, and THBD was present and associated with *H. pylori* infection in non-cancerous gastric mucosae of GC patients.[8,12,42] Recently, relationship has emerged on the Wnt/β-catenin and *H. pylori* infection in gastric carcinogenesis.[43] The Wnt/β-catenin pathway was also implicated in GC by epigenetic silencing of multiple Wnt antagonists.[19-22] However, the formation of epigenetic field of Wnt antagonists in the *H. pylori* infection and gastric carcinogenesis and the effect of *H. pylori* eradication on the field have not been investigated. Moreover, most of previous studies were limited in that they utilized non-quantitative method to detect promoter methylation.

In our study, *H. pylori* infection was associated with promoter methylation and down-regulation of all Wnt antagonist genes. IHC staining showed increased expression of cytoplasmic and nuclear β-catenin. These findings are consistent with a previous study[17] which showed *H. pylori* infection leaded to β-catenin nuclear accumulation, and we additionally found that *H. pylori*-induced dysregulation of β-

catenin might be mediated by methylation-dependent silencing of Wnt antagonists. We also showed that the levels of promoter methylation of SFRP and DKK family genes was higher and their mRNA expression was lower in non-cancerous mucosa of *H. pylori*-positive GC patients than in *H. pylori*-positive controls. These corroborate prior studies of SFRP and DKK family genes on GC cell lines and tissues.[19-21] In addition to them, our study further demonstrated that these epigenetic inactivation were also observed in non-cancerous gastric mucosae of GC cases. This is also supported by IHC staining which demonstrated more prominent and frequent nuclear β-catenin staining in GC cases. This may explain the observation in IHC staining which demonstrated low to high level cytoplasmic and nuclear β-catenin staining in non-cancerous mucosa of *H. pylori*-positive patients with GCs although the number of samples was too small and the expression was evaluated semiquantitatively. Taken together, our results suggest that SFRP and DKK family genes may be involved in the formation of epigenetic field in *H. pylori*-associated gastric carcinogenesis.

However, there are two points that should be considered in the interpretation of the aberrant DNA methylation of SFRP and

DKK family genes in non-cancerous mucosae of *H. pylori*-positive GCs. First, methylation levels of those genes were highly variable. Similar findings were observed in a previous study.[10] This seems to be because aberrant methylation develops only in a fraction of cells as suggested. Second, DNA methylation is closely associated with the presence of intestinal metaplasia of gastric mucosa. It was reported that hypermethylation of THBD, HAND1, and APC was associated with intestinal metaplasia.[42] In our study, intestinal metaplasia was more severe in the mucosae of GC patients than in the controls. Thus, increased level of methylation in Wnt antagonist genes may also be associated with the severity of metaplasia. Further study is required whether DNA methylation of Wnt antagonist genes are associated with intestinal metaplasia in patients without GCs.

It was noteworthy that methylation levels of WIF1 in patients with GCs were lower than those in *H. pylori*-positive and negative controls, while they were higher in the *H. pylori*-positive controls then in the *H. pylori*-negative controls. In a prior study, frequent hypermethylation and down-regulation of WIF1 were observed in GC cell lines and tissues.[22] This may be interpreted that epigenetic silencing of WIF1 may emerge after

the development of GC rather than before the cancer development or during the formation of epigenetic field.

Few studies conducted controlled comparison between eradication and persistent groups in the evaluation of the effect of *H. pylori* eradication on promoter hypermethylation as we performed in the present study.[25] In our study, increased methylation levels of DKK3 was decreased while those of SFPR and other DKK family genes were not at one year after *H. pylori* eradication. This is consistent with a prior longitudinal study which showed that *H. pylori* eradication reduced methylation levels in LOX but not in APC,[14] suggesting that the effect of *H. pylori* eradication on the reduction of aberrant DNA methylation may be gene-specific. It is interesting that mRNA levels of SFRP1, 2, 3, DKK1 and 2 were significantly down-regulated at one year follow-up from the baseline in both eradication and persistent groups. These change may be explained by the tendencies of increasing methylation levels of these genes observed both in the eradication and persistent groups though the extent of increased methylation levels were not significantly high (Table 6). Another possible explanation could be that epigenetic mechanism other than DNA methylation such as histone modification might have also involved in the

reduced mRNA expression.[44] Nevertheless, these may suggest that *H. pylori* eradication could not lead to the reduced DNA methylation and increased mRNA expression in those genes at least one year of follow-up. Because aberrant cytoplasmic and nuclear β-catenin was not decreased, it may be suggested that, once the epigenetic field of Wnt antagonists had been generated, it may persist even after *H. pylori* eradication at least for one year. In the follow-up sample, mucosal atrophy as well as inflammatory cell infiltration were more severe in the persistent group than in the eradication group. Ongoing inflammation induced by persistent *H. pylori* infection could have resulted in aggravated mucosal atrophy. Thus, further study may be warranted to elucidate these difference may lead to the difference in the DNA methylation levels of SFRP and DKK family genes other than DKK3 in the longer-term follow-up.

The strengths of this study include adoption of quantitative methods in the evaluation of DNA methylation and mRNA expression and controlled design comparing *H. pylori* eradication with persistent groups. However, there are also some limitations. First, we did not include the patients with *H. pylori*-negative GCs. It is known that non-cancerous gastric

mucosae of *H. pylori*–negative patients with GCs are at higher risk of developing metachronous cancer than those of *H. pylori*–positive patients.[45] Although our study has advantage in involving *H. pylori* eradication group, presence of *H. pylori*–negative patients with GCs could have provided additional insights in the gastric carcinogenesis as in the previous studies.[10,42] Second, IHC staining was conducted on only three cases and assessed semiquantitatively. For more exact evaluation, quantitative assessment using the ratio of β–catenin expression for the entire cases should have been done. In addition, immunofluorescence is necessary to evaluate accurately the changes of β–catenin expression pattern. Thus, caution is required when interpreting the results of IHC of the present study.

In conclusion, epigenetic silencing of SFRP and DKK family genes may mediate the formation of epigenetic field during *H. pylori*–associated gastric carcinogenesis. Although most of them may persistent even after *H. pylori* eradication once the field has been developed, methylation of DKK3 may be reversed by *H. pylori* eradication in GC patients.

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국문 초록

서론: 본 연구에서는 위암 발생 과정에서 헬리코박터 파일로리 감염 및 제균 치료에 따른 Wnt 길항체 유전자들의 DNA 메틸화와 mRNA 발현의 변화를 살펴보고자 하였다.

방법: 헬리코박터 파일로리 양성 위암 환자군에서 암이 아닌 위점막 조직을 채취하고 헬리코박터 파일로리 양성 및 음성 대조군에서 위 점막 조직을 채취한 뒤, 7 개의 Wnt 길항체 유전자들 (SFRP1, 2, 5, DKK1, 2, 3, WIF1)의 DNA 메틸화와 그에 따른 mRNA 발현 정도를 각각 quantitative MethyLight assay 와 real-time RT PCR 로 정량하여, 그 결과를 세 군에서 서로 비교하였다. 또한, 위암에 대한 내시경적 절제술 후 헬리코박터 파일로리가 제균된 군과 지속적으로 감염된 군에서 1 년 추적 검사 때 위 점막 조직을 채취한 뒤, 7 개 유전자들의 메틸화와 발현 정도의 변화를 비교하였다.

결과: SFRP 와 DKK family gene 들의 DNA 메틸화 정도가 헬리코박터 파일로리 음성 대조군에 비해서 헬리코박터 파일로리 양성 대조군 그리고 양성 위암군의 순서로 유의하게 증가하였다 ($P < 0.05$). 또한, SFRP1, 2, 및 DKK3 의 mRNA 발현 정도가 헬리코박터 파일로리 음성 대조군, 양성 대조군 및 양성 위암군의 순서로 단계적으로 하향 조절되었다 ($P < 0.05$). 헬리코박터 파일로리 제균 치료 후 Wnt 길항체 중에서 오직 DKK3 에서만 DNA 메틸화 및 mRNA 발현의 하향 조절 정도가 감소하는 것을 확인할 수 있었다 ($P < 0.05$).

결론: 헬리코박터 파일로리 감염과 연관된 위암 발생 과정에서 SFRP와 DKK family gene 들의 후성유전학적 비활성화가 필드 종양화 형성을 촉진할 가능성이 있다. 이렇게 형성된 후성유전학적 필드 종양화는 헬리코박터 파일로리 제균 치료 후에도 호전되지 않을 수 있으나, DKK3는 예외가 될 수 있다.

주요어 : 헬리코박터 파일로리, Wnt 신호 체계, DNA 메틸화, 위암

학 번 : 2013-30555