ORIGINAL ARTICLE

Eradication of *H. pylori* Did Not Improve Abnormal Sonic Hedgehog Expression in the High Risk Group for Gastric Cancer

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Abstract

Background   Sonic hedgehog (SHH) acts as a proliferation factor in both the normal mucosa and in malignant lesions. *Helicobacter pylori*-associated atrophic gastritis is characterized by loss of SHH.

Aim   The purpose of this study was to investigate the effects of *H. pylori* eradication on *SHH* mRNA and methylation levels in the patients at high risk for gastric cancer comparing to those in the controls.

Methods   Gastric corpus biopsies taken from 20 patients with endoscopic resection for early gastric cancer and 14 sex- and age-matched controls before and 1 year after eradication were examined for *SHH* and downstream regulators mRNA expression using whole biopsy specimens and microdissected gastric glands. Methylation of *SHH* promoter was evaluated using quantitative methylation-specific PCR.

Results   *SHH* mRNA levels eradication were significantly lower (2.75×10^{-2} vs. 7.37×10^{-2}, $P = 0.004$) in the cancer group than in the controls. *PTCH* and *BMP4* mRNA levels as well as *MUC5AC* were significantly increased only in the control group and were significantly higher in the controls than those in the cancer group after eradication. After eradication, *SHH* methylation levels in the non-metaplastic glands were significantly higher (86.4% vs. 22.2%, $P < 0.001$) in the cancer group than in the controls.

Conclusions   *H. pylori* eradication can enhance *SHH* and its downstream regulators expression diminishing SHH methylation and reverse gastric phenotype, but not in the patients with high risk for gastric cancer.

Keywords   MUC5AC · Intestinal metaplasia · Gastric cancer · Methylation

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Introduction

Sonic hedgehog (SHH) is a regulator of patterning processes throughout development [1–3], and abundantly expressed in the normal gastric funduic mucosa but normally not expressed in either the esophagus or intestine [4]. SHH is particularly important in development of the stomach, for example, SHH null mice exhibit gastrointestinal (GI) malformations and replacement of the normal gastric epithelium with intestinal metaplastic cells. SHH binds to its receptor "Patched" (Ptc-Ptch1, Ptch2), which reduces the inhibitory effect of Ptch on another transmembrane protein, smoothened (Smo). Binding results in de-repression of Smo, activating a cascade that leads to the translocation of the active form of the transcription factor glioma-associated oncogene homolog (Gli) to the nucleus [5–7]. SHH signaling activation leads to cell proliferation through cell cycle regulation. Nuclear Gli activates expression of a variety of target genes including Bmp4 in the mesenchym and plays an important role in gastric organogenesis and maintenance of organ structure [8, 9]. Bmp4 is known to be a potent inducer of *ATP4a* gene

expression and promotes the induction and maintenance of a differentiated parietal cell phenotype [10].

Previously, we used immunohistochemistry to examine the stomach in *Helicobacter pylori*-associated atrophic gastritis and showed that SHH expression was reduced and that CDX2, which is a key event in the pathogenesis of intestinal metaplasia (IM) in the stomach [11, 12], was aberrantly expressed. The loss of SHH correlated with the presence and type of IM. *H. pylori* eradication resulted in an improvement in gastritis and this was associated with an increase in SHH expression and a decrease in CDX2 expression in the gastric mucosa ($P < 0.01$). However, gastric mucosa with incomplete IM regained the abnormal pattern of expression of SHH and CDX2, while *CDX2* aberrant gene expression was detected at the glands without goblet cells (non-metaplastic) in the corpus and disappeared after *H. pylori* eradication [13–15].

Abnormal activation of the *SHH* pathway is associated with tumorigenesis and has been reported in the GI tract malignant lesions [9, 16–18]. Epigenetic changes, particularly DNA methylation, are susceptible to change and are excellent candidates to explain how certain environmental factors may increase the risk of cancer. Recent study indicated that hypomethylation of a CpG locus within the *SHH* promoter correlated with increased gene expression and was significantly associated with colorectal cancers [19, 20].

In order to explore the potential roles of *SHH* in gastric carcinoma, we compared *SHH* and its downstream regulators (*PTCH1, GLI1,* and *BMP4*) mRNA expressions in gastric glands among patients at high risk for gastric cancer versus controls. We also investigated methylation levels of the *SHH* promoter region in the fundic glands.

Materials and Methods

This was a case–control study of patients with a history of endoscopic submucosal dissection (ESD) for early gastric cancer. The study was performed at the Osaka Medical Center for Cancer and Cardiovascular Diseases and Kawasaki Medical School in Japan. Patients were enrolled for the study between April 2007 and April 2010.

Patients

Patients with a history of ESD for early stage, non-cardia intestinal-type gastric cancer without lymph node metastasis were enrolled. Controls were patients who underwent endoscopy for follow-up to peptic ulcer or health screening; none had gastric cancer. Patients were excluded if they had undergone *H. pylori* eradication, used anti-secretory or non-steroidal anti-inflammatory drugs (NSAIDs), or had

hemorrhagic diseases, insulin-dependent diabetes mellitus, cirrhosis, or renal failure. Demographic data collected at study entry included age, gender, smoking habit, alcohol consumption and drug treatments. Drinking and smoking were defined as "regular" when consumption was more than 35 g for ethanol or 5 cigarettes per day, respectively.

The study was approved by the Osaka Medical Center for Cancer and Cardiovascular Diseases Ethical Committee and Kawasaki Medical School Ethical Committee, and written informed consent was obtained from each patient.

Biopsy Samples

Endoscopies were performed by experienced endoscopists after patients had fasted for 12 h. At endoscopy before and 1 year after eradication, two specimens from each sample site, the greater and lesser curves of the corpus, were taken using the endoscopic forceps (FB231K (A) Olympus, Tokyo, Japan). The biopsy samples were immediately frozen with liquid nitrogen and stored at −80°C until use.

Laser-Captured Microdissection

The frozen samples obtained at endoscopy were embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and cut into serial 8-μm sections. Before microdissection, up to eight sections from each block were mounted on slides and one slide was stained with Alcian blue and the others were stained using HistGene LCM Frozen Section Staining Kit (Arcturus Bioscience, Mountain View, CA, USA). The cryostat sections were laser-microdissected with a PixCell II lase-microdisecction system (Arcturus Engineering, Mountain View, CA, USA). Both glands with goblets cells (metaplastic glands) and without were isolated. Goblet cells were identified by their characteristic shape with an expanded apical portion and a nucleus at the base of it referring to the slide stained with Alcian blue.

RNA Extraction and Quantitative Polymerase Chain Reaction

Total RNA was extracted from whole biopsy samples using RNeasy Mini kit (Qiagen, Hilden, Germany) and microdissected tissue using a Pico Pure RNA Isolation kit (Arcturus Bioscience), and cDNA synthesis were performed using the SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *SHH, PTCH1, GLI1, BMP4, MUC5ac,* and *β-actin* mRNA expression was performed using the 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) employing TaqMan gene expression assay according to the

manufacturer's instruction (Applied Biosystem). Real-time PCR was performed with cDNA for both target genes and the endogenous control using TaqMan Universal PCR Master Mix (Applied Biosystems). Each amplification reaction was performed in triplicate and the average of the threshold cycles was used. The amount of target was obtained by normalization to an endogenous reference (β-actin) and relative to a calibrator.

DNA Extraction and Quantification of SHH Methylation

DNA was extracted from the microdissected tissue using the QIAamp DNA Micro kit (Qiagen). A bisulfate modification on a DNA sample using the CpGenome Fast DNA Modification kit (Chemicon, Temecula, CA, USA) was performed. MSP involves PCR amplification with specific primer designed to distinguish methylated from unmethylated DNA. The primer sequences are specified below: primary PCR, forward primer: 5'-GGG AGT GGT GGA GAG TTTTTT G-3', reverse primer: 5'-AAC TAA TAA CTTCCA AAC TAT CCC CA-3' for unmethylated DNA sequences; primary PCR, forward primer: 5'-GAG CGG TGG AGA GTT TTT CG-3', reverse primer: 5'-ACT AAT AAC TTC CGA ACT ATC CCCG-3' for methylated DNA sequences [21]. Real-time PCR was done using SYBR Premix Ex Taq GC (Perfect Real Time) (TaKaRa) and 7500 real-time PCR System (Applied Biosystems). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI, USA). The number of molecules methylated and unmethylated for a genomic region in a sample was measured separately, and the methylation level was calculated as the fraction of methylated molecules in the total number of methylated and unmethylated molecules, as previously reported [22]. Plasmid DNA was extracted from individual clones using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using ABI PRISM3130 Genetic Analyzer (Applied Biosystems).

Diagnosis of H. pylori

Venous blood samples were analyzed for specific IgG H. pylori antibodies with an enzyme-linked immunosorbent assay (ELISA) kit using the E plate test (Eiken Kagaku, Inc., Tokyo, Japan). Patients were considered to be infected with H. pylori if the serum test was positive combined with evidence of chronic gastritis or atrophy with H. pylori on histopathological examination. Eradication was confirmed by negative histological examination of gastric biopsies, together with a negative ^{13}C-urea breath test (^{13}C-UBT) following the completion of eradication therapy for 6–8 weeks.

Eradication of H. pylori

Patients were treated with a 7-day regimen consisting of amoxicillin (500 mg three times a day), clarithromycin (200 mg three times a day) and a proton pump inhibitor twice daily (total dose = omeprazole 40 mg, lansoprazole 60 mg, or rabeprazole 20 mg), which was the standard approved first line regimen in Japan. The patients with unsuccessful eradication were retreated with the regimen of changing clarithromycin to metronidazole (250 mg three times a day).

Statistical Analyses

Values are expressed as the mean $\pm$ SD or the median with a 25–75% range, whichever was appropriate depending on whether the data were normally distributed. Mantel–Haenszel Chi square analysis and the unpaired t test were performed to measure differences in demographic and clinical characteristics and *SHH* methylation levels. Statistical analyses for significant differences of mRNA levels were performed using the nonparametric Mann–Whitney U test between two groups and the Wilcoxon's signed rank test for paired data. Spearman's correlation coefficient was calculated to examine correlations. A two-sided P value of less than 0.05 was considered statistically significant. All statistical computations were performed using SPSS (SPSS Inc. Chicago, IL, USA).

Results

Twenty-five *H. pylori*-positive patients with ESD for early gastric cancer (cancer group) and 30 controls who were *H. pylori*-positive and matched to the cancer group by gender and age were enrolled in the study. Three patients in the cancer group and eight patients in the control were lost to follow-up. Eradication failure occurred in six patients in the cancer group and seven in the control group; four cancer patients and three controls of them were successfully retreated with the second regimen. Four control patients were excluded to match to the cancer group by gender and age. The final study groups consisted of 20 *H. pylori*-positive patients with ESD for early gastric cancer (cancer group) and 14 *H. pylori*-positive controls matched to the cancer group by gender and age. Demographic and clinical characteristics of the study groups are shown in Table 1. The locations of the early gastric cancers were: seven in the lower stomach, seven in the middle stomach, and four in the upper stomach. The remaining two patients had malignant lesions located in two different locations. A subpopulation of these patients (28 patients) were also involved in another study regarding *H. pylori*

Table 1 Demographic and clinical characteristics of the study groups

Demographic	Controls ($n = 14$)	Cancer ($n = 20$)	P values
Age mean (SD)	64.3 (5.7)	65.5 (8.9)	0.66[a]
Gender, male/female	8/6	12/8	1.0
Current smokers	0 (0%)	3 (15%)	0.25[b]
Regular alcohol intake	5 (35.7%)	9 (45%)	0.73[b]

P values calculated using [a] unpaired t test or [b] Mantel–Haenszel chi square analysis
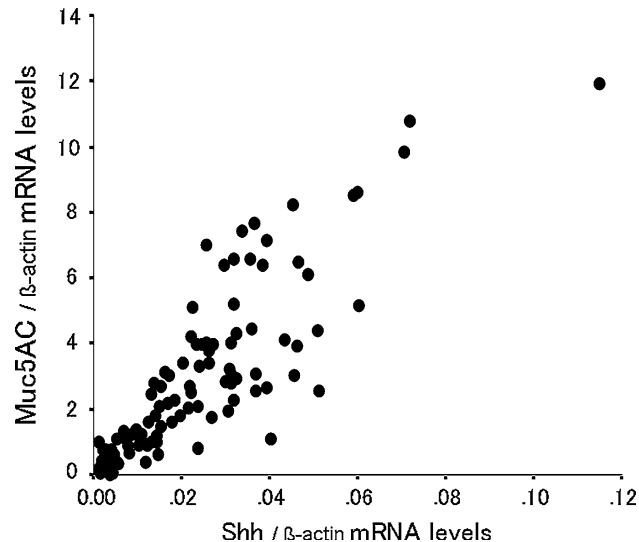
Fig. 1 Correlation between the sonic hedgehog (*SHH*) and *MUC5AC* mRNA levels in biopsy tissue from the lesser and greater curve of the corpus. Data are expressed relative to the control gene β-actin

eradication preventing extension of intestinalization even in the high-risk group for gastric cancer [23].

Whole Biopsy Samples

Using the entire biopsy samples, *SHH* mRNA levels in the gastric mucosa positively correlated with the downstream regulators (*PTCH1, GLI1, BMP4*) mRNA levels, and especially *MUC5AC* ($r = 0.91$, $P < 0.001$, Fig. 1).

MUC5AC mRNA levels significantly increased (4.18–10.1, $P = 0.01$) only in the control group and were significantly higher (10.1 vs. 4.4, $P = 0.004$) in the controls than those in the cancer group after eradication (Fig. 2).

Although *SHH* mRNA levels in the gastric mucosa significantly increased after eradication in both groups ($P = 0.02$), *SHH* mRNA levels after eradication were significantly lower (2.75×10^{-2} vs. 7.37×10^{-2}, $P = 0.004$) in the cancer group than in the controls (Fig. 3a). *PTCH* (7.29×10^{-2} to 18.6×10^{-2}, $P = 0.03$) and *BMP4*
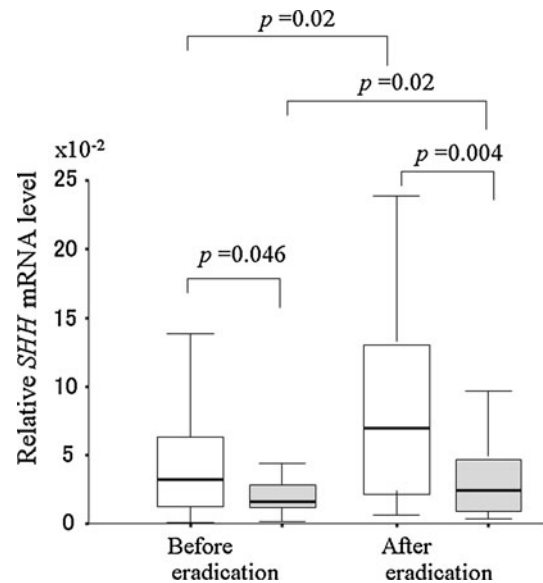
Fig. 2 Comparisons of *MUC5AC* mRNA expressions between the control group and the cancer group before and after eradication. *Horizontal bars* are medians. *Boxes* are the 25th–75th interquartile ranges. *Vertical lines* are range of values. mRNA levels are expressed relative to the control gene β-actin. P values were calculated using the nonparametric Mann–Whitney U test between the two groups and the Wilcoxon's signed rank test for paired data

(2.15×10^{-3} to 5.95×10^{-3}, $P = 0.006$) mRNA levels as well as *MUC5AC* were significantly increased only in the control group and were significantly higher in the controls than those in the cancer group after eradication. *GLI1* mRNA levels only in the cancer group significantly decreased (2.10×10^{-3} to 1.45×10^{-3}, $P = 0.02$) after eradication and were significantly lower (1.45×10^{-3} vs. 6.20×10^{-3}, $P = 0.001$) in the cancer group than those in the controls after eradication (Fig. 3).

Microdissected Gastric Glands

Using the microdissected gastric glands, the mRNA levels of *SHH* and *PTCH* as well as *MUC5AC* were significantly higher in the non-metaplastic glands than in the metaplastic glands (Table 2). However, there was no significant difference in the mRNA levels between the two groups both before and after eradication (Table 3).

Methylation levels within the *SHH* promoter region in the fundic glands with and without goblet cells were not significantly different between the two groups before eradication. After eradication methylation levels in the non-metaplastic glands were decreased in the controls and increased in the cancer group, and were significantly higher (86.4% vs. 22.2%, $P < 0.001$) in the cancer group than in the controls. However, methylation levels in the metaplastic glands were not significantly different between the two groups even after eradication (Table 4).

Fig. 3 Comparisons of *SHH* (**a**) and the downstream regulators (*PTCH*). **b** *BMP4*. **c** *GLI1*. **d** mRNA expressions between the control group and the cancer group before and after eradication. *Horizontal bars* are medians. *Boxes* are 25th–75th interquartile ranges. *Vertical lines* are the range of values. mRNA levels are expressed relative to the control gene *β*-actin. *P* values were calculated using the nonparametric Mann–Whitney *U* test between the two groups and the Wilcoxon's signed rank test for paired data
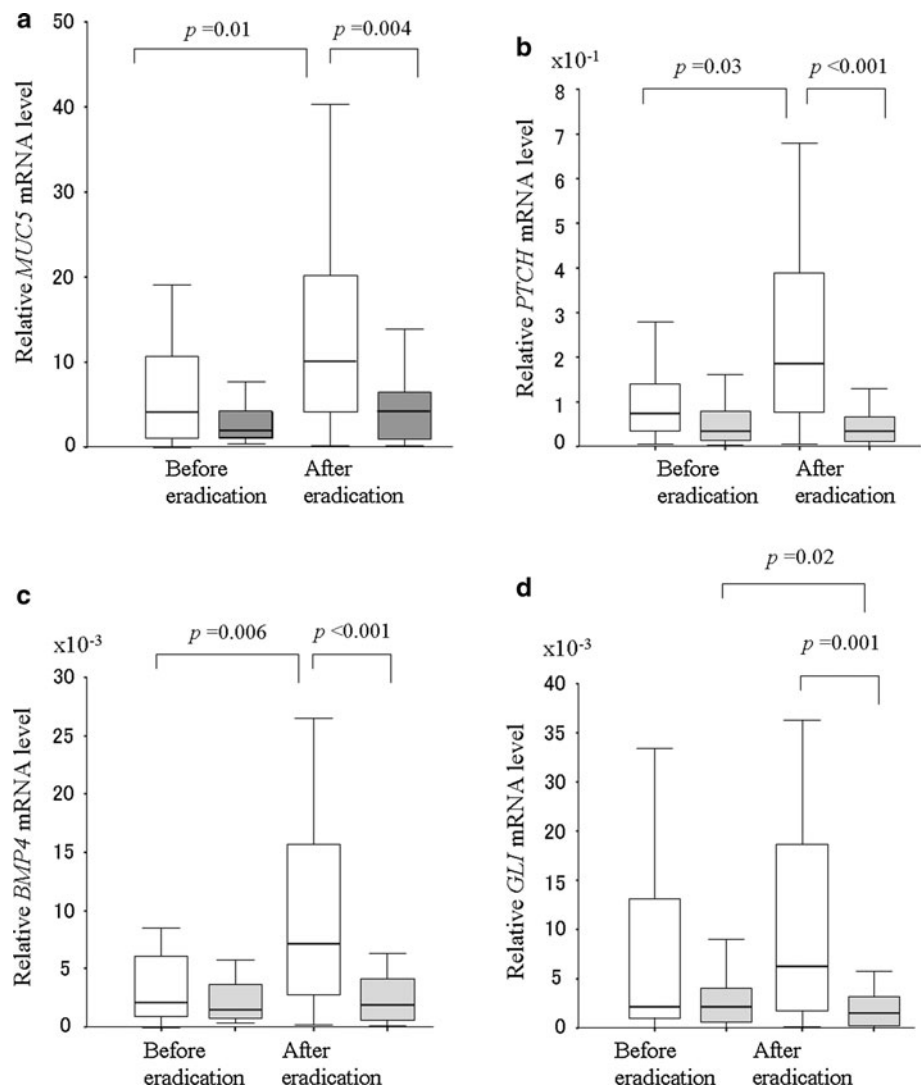
Table 2 Comparison of mRNA levels between the metaplastic glands and non-metaplastic glands

mRNA	Non-metaplastic glands	Metaplastic glands	*P* values
MUC5AC	15,500 (5,250–28,000)	1,040 (154–4,140)	<0.001
SHH	80 (43–234)	11 (4.6–49)	<0.001
PTCH	168 (47–747)	39 (21–262)	0.008
GLI	4.5 (0–8.9)	2.1 (0.2–8.8)	0.88
BMP4	1.6 (0–4.2)	0.8 (0.5–1.6)	0.70

Values are expressed relative to the control gene *β*-actin as the median with a 25–75% range. *P* values were calculated using the nonparametric Mann–Whitney *U* test

Discussion

The gastric phenotypic gene expressions including those *SHH* downstream regulator gene expressions were lower in the cancer group than those in the controls, and the difference between the two groups became more remarkable

after eradication. These results confirm and extend our previous results using immunostainings [14–18]. *SHH* expression correlated with the atrophy score and its expression decreased in *H. pylori*-negative and -positive controls, and in the cancer group, in that order. The difference between atrophy in the control and the cancer group became significant after eradication. *H. pylori* eradication was associated with an increase in *SHH* and its downstream regulators expressions, however, the beneficial effect of eradication on *SHH* diminished in the patients with high risk of gastric cancer.

Aberrant DNA methylation in noncancerous mucosa was shown to be related with chronic inflammation such as ulcerative colitis and chronic gastritis, and Maekita et al. [22] previously indicated that high levels of methylation of multiple CpG islands in noncancerous mucosa induced by *H. pylori* infection was associated with gastric cancer risk. In the present study, *SHH* methylation after eradication was decreased in the controls and detected more frequently in

Table 3 Comparisons of mRNA levels in the non-metaplastic glands and metaplastic glands between the cancer group and the controls

Glands	Control	Cancer	P values
Non-metaplastic glands			
SHH			
Pre	103 (63–166)	64 (21–459)	0.65
Post	52 (44–174)	102 (71–216)	0.07
PTCH			
Pre	147 (38–652)	252 (89–1,942)	0.07
Post	334 (69–532)	307(107–490)	0.60
GLI			
Pre	6.3 (0–8.7)	3.0 (0–9.4)	0.79
Post	5.1 (1.2–15.4)	5.9 (0–13.7)	0.50
Metaplastic glands			
SHH			
Pre	7.3 (3.1–46.6)	18.8 (10.5–123)	0.47
Post	5.9 (1.8–15.4)	26.4 (2.2–55.4)	0.57
PTCH			
Pre	24.3 (9.8–375)	73.6 (24.4–577)	0.47
Post	38.5 (17.2–66.7)	32.6 (0.2–203)	0.15
GLI			
Pre	1.4 (0–14.4)	2.0 (0–2.5)	0.81
Post	2.1 (0.4–5.0)	0 (0–8.1)	0.36

Values are expressed relative to the control gene β-actin as the median with a 25–75% range. P values were calculated using the non-parametric Mann–Whitney U test

Table 4 Comparison of frequency of SHH methylation in the metaplastic and non-metaplastic glands between the cancer group and the controls

Measurement	Non-metaplastic glands	Metaplastic glands
Before		
Control	53.1 (49.1)	71.2 (48.6)
Cancer	52.2 (51.0)	58.2 (51.4)
P	0.96	0.60
After		
Control	22.2 (42.7)	28.6 (48.8)
Cancer	86.4 (31.8)	44.3 (52.6)
P	<0.001	0.55

Values are expressed as the mean (SD). P values were calculated using the unpaired t test

the cancer group, especially within the non-metaplastic glands. In contrast, Wang et al. [21] previously reported that methylation of the SHH promoter region was frequent (61%) in normal mucosa but rare (0–1.6%) in chronic active gastritis, IM and gastric carcinoma in human. The results differ from ours possibly because we used different methods to detect methylation levels. In both studies, the target promoter lesions and use of dissected tissues were the same, however, we quantified the methylation levels using real time PCR instead of nested PCR. The recent study using Cdx2-transgenic mice indicated that the SHH promoter was unmethylated in both the normal gastric mucosa and the intestinal metaplastic mucosa and loss of SHH expression in the intestinal metaplasia is not associated with promoter hypermethylation [24]. The different results in this animal model form the others in humans is probably caused by no inflammation in this animal model.

Recent studies indicated that methylation levels within the SHH promoter are associated with SHH expressions and hypomethylation of a CpG locus within the SHH promoter correlated with increased gene expression [20]. However, SHH mRNA levels were not significantly different between the methylated group and the non-methylated group of our subjects, although those tended to be lower in the methylated group. Therefore, other molecular

mechanisms instead of SHH promoter methylation seem to play a more important role in the regulation of SHH expression, and it still remains unclear whether methylation of the SHH promoter region plays a role in the regulation of phenotype during development of intestinalization. Interestingly, methylation levels in the non-metaplastic glands of the controls tended to be decreased after eradication, while that increased in the cancer group. The decrease of methylation after eradication in the controls seems to enhance SHH signals and recover gastric phenotypes. The effects of eradication on SHH methylation may induce the difference in the gastric phenotypes between the two groups after eradication.

The type, location, and extent of IM related with atrophic gastritis are also thought to provide important information regarding cancer risk [15, 25, 26]. The grade of corpus atrophy was found to closely relate to the risk of development of gastric cancer [27, 28]. Our previous study suggested that the reversibility of atrophic gastritis after eradication depended upon the severity of the changes prior to H. pylori eradication and the persistence of corpus gastritis after eradication might be a marker for cancer risk [13]. The reversibility of changes of H. pylori associated with atrophic gastritis is related with the recovery of SHH expression and might depend upon diminishing of SHH promoter methylation by eradication. We propose that prevention of cancer likely requires H. pylori eradication prior to the development of atrophic gastritis with IM [23, 29].

In summary, the gastric phenotypic genes expressions including SHH downstream regulators were lower in the cancer group than those in the controls, and the difference between the two groups became more remarkable after eradication. After eradication, SHH methylation was detected more frequently in the cancer group than in the controls, especially within the non-metaplastic glands.

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