Effect of *Helicobacter pylori* infection on the sonic hedgehog signaling pathway in gastric cancer cells

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Abstract. This study aimed to investigate the effect of *Helicobacter pylori (H. pylori)* infection on the sonic Hedgehog (Shh) signaling in gastric cancer. Shh, Patched (Ptch), and transcription factor Gli1 were overexpressed in *H. pylori*-infected gastric cancer cells. The oncoprotein, CagA positive *H. pylori* resulted in significantly higher Shh expression. Pretreatment with MG-132 or PDTC significantly lowered Shh expression. Significant overexpression of Shh and Gli1 were noted in *H. pylori*-infected compared to non-infected gastric cancer tissues. Conclusively, *H. pylori* activated the Shh signaling pathway in CagA-dependent manner partly through the NF-κB pathway in gastric cancer cells.

Introduction

The Hedgehog (Hh) signaling pathway plays a critical role in embryonic development, tissue polarity, and carcinogenesis (1,2). Secreted Hh molecules bind to the Patched (Ptch; Ptch-1, Ptch-2) receptor, alleviating Ptch-mediated suppression of smoothened (Smo), a putative seven-transmembrane protein. Smo signaling triggers a cascade of intracellular events, leading to activation of the pathway through glioma-associated oncogene (Gli)-dependent transcription (2). In the stomach, sonic hedgehog (Shh) is almost exclusively expressed in parietal cells, influencing fundic gland differentiation (3).

Gastric cancer is one of the most common malignancies in the world, and *Helicobacter pylori (H. pylori)* is a major risk factor linked to gastric carcinogenesis. *H. pylori* may participate in gastric carcinogenesis through the interaction between bacterial factors such as CagA and host signal transduction pathways (4). Shh expression is significantly lower in *H. pylori*-associated gastritis or with intestinal metaplasia (IM) changes (3,5-7). This reduction correlates with the loss of parietal cells on the morphogenic bases (3,5-7). However, some studies report overexpression of Shh in human gastric cancers (8-10). Therefore, we examined Shh signaling pathway changes in gastric cancer cells in response to *H. pylori* infection in order to elucidate the influence of Shh signaling activation in *H. pylori*-mediated gastric carcinogenesis.

Materials and methods

Gastric cancer cells and H. pylori infection. AGS (ATCC CRL 1739), MKN 28 (JCRB 0253), MKN45 (KCLB 80103), and KATO III (KCLB 30103) gastric cancer cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin sulfate (Gibco). All cultures were maintained in a 37˚C incubator supplemented with 5% CO$_2$. *H. pylori* strains were cultured on blood agar plates containing 5% sheep blood in a 37˚C incubator with a gas pak (BD, Sparks, MA). We cultured *H. pylori* strains 8822 (cagA-negative), 60190 (cagA-positive), and Δ*cagA* (isogenic mutant lacking *cag*A; a kind gift from Professor Richard M. Peek Jr, Vanderbit University). For *H. pylori* infection of gastric cancer cells, *H. pylori* strains were cultured for 24 to 36 h. Cultured *H. pylori* were harvested in phosphate-buffered saline (PBS, pH 7.4) and added to the serum-starved host cells at a multiplicity of infection (MOI) of 100.

Reagents. The Smo-specific inhibitor cyclopamine (Calbiochem, La Jolla, CA) was dissolved in DMSO. Proteasome inhibitor MG-132 (Sigma-Aldrich, St. Louis, MO) and pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich), a specific inhibitor of nuclear factor-κB (NF-κB), were used at previously described concentrations (11).

Tissue samples and immunohistochemistry. Surgical specimens were obtained from 20 patients with gastric cancer. Tissue sections in microslides were deparaffinized with xylene, hydrated in serial dilutions of alcohol, and immersed in 3% H$_2$O$_2$. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with protein blocking

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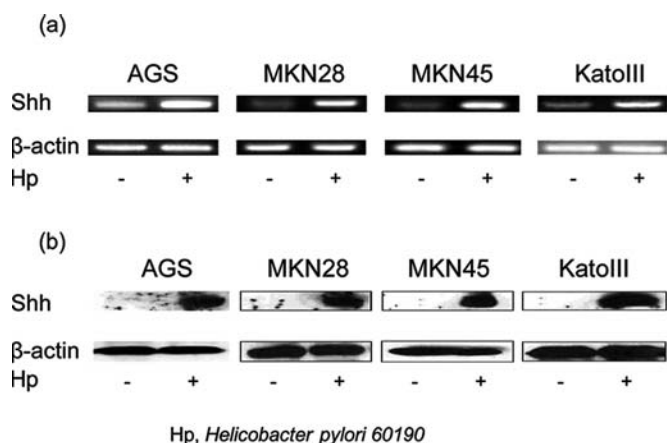E-mail: leeyc@yuhs.ac

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Figure 1. *Helicobacter pylori (H. pylori)* infection induced sonic hedgehog (Shh) overexpression in gastric cancer cells. (a) AGS, MKN-28, MKN-45, and KATO-III cells were infected by *cag A*-positive *H. pylori* (60190), and RT-PCR for Shh mRNA was performed. (b) Immunoblot of Shh expression in the gastric cancer cell lines.

agent (Immunotech, Coulter, Inc., Marseille, France) to block nonspecific antibody binding for 20 min at room temperature and then incubated overnight at 4˚C with respective primary polyclonal goat antibodies against human SHH (H-160, Santa Cruz, 1:50) and Gli1 (H-300, Santa Cruz, 1:50). After washing with PBS three times, the sections were incubated with a biotinylated secondary antibody (goat anti-rabbit IgG, Immunotech, Coulter, Inc.) and streptavidin conjugated to horseradish peroxidase (Immunotech) for 20 min at room temperature, followed by a PBS wash. The chromogen was developed for 5 min with liquid 3,3'-diaminobenzidine (Immunotech). Next, slides were counterstained with Meyer's hematoxylin. Expression status was quantified by scoring both the intensity and proportion of SHH or Gli1 staining. The intensity of cytoplasmic SHH staining was scored as 0, negative (weak or similar to background); 1, weak (less intense than normal cells); 2, moderate (similar intensity to normal cells); and 3, strong (stronger than normal cells). The intensity of GLI1 staining was scored as 0, no detectable nuclear staining; 1, weak nuclear staining; and 2, strong nuclear staining. The proportion of expression in each tissue was determined by counting positively stained cancer cells in relation to a total of three hundred cells, and expressing the result as a percentage.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses. Total RNA was extracted from cultured cells using an RNeasy mini kit (Qiagen, Tokyo, Japan). The RNA was reverse transcribed using oligo (dT) primers and Superscript™ II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed with a PCR Maxi kit (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. Amplification conditions included denaturation at 95˚C for 5 min, followed by 30 cycles of 30 sec each at 95, 57, and 72˚C for Shh, 30 cycles of 30 sec each at 95, 57, and 72˚C for Ptch1, 30 cycles of 30 sec each at 95, 59, and 72˚C for Gli1, and 18 cycles of 30 sec each at 95, 60, and 72˚C for ß-actin. The following primer pairs were used: ß-actin, 5'-TTG CCG ACA GGA TGC AGA AGA-3' and 5'-AGG TGG
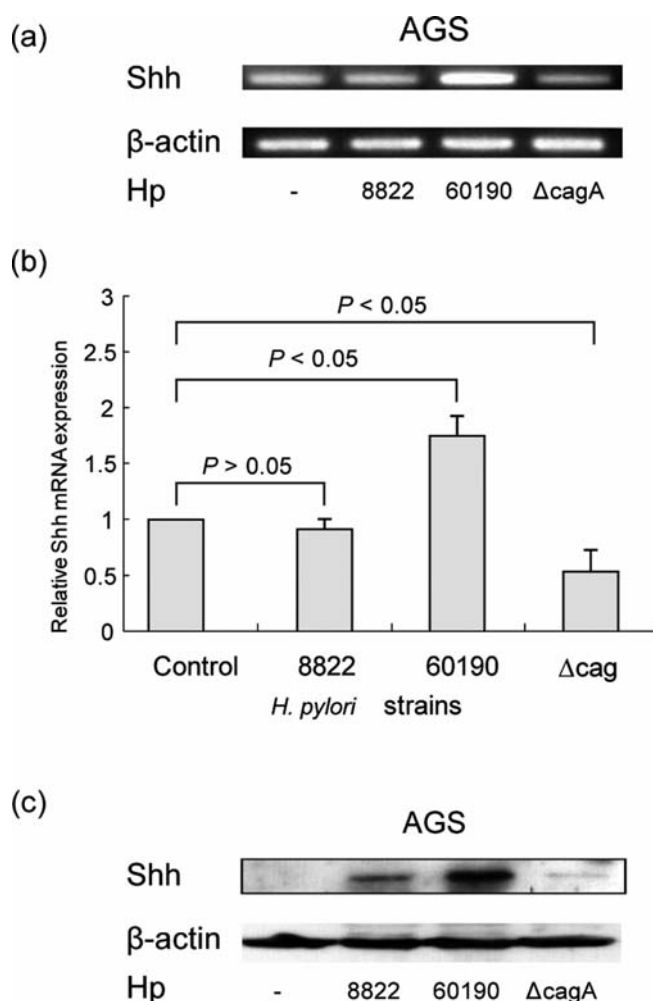

Figure 2. *H. pylori* infection induced sonic hedgehog (Shh) overexpression in gastric cancer cells in a *cagA*-dependent manner. (a) AGS cells were infected by *cagA*-positive (60190) and -negative (Δ*cag*, 8822) *H. pylori* strains, and RT-PCR for Shh mRNA was performed. (b) Shh mRNA expression was also examined by real-time RT-PCR. Relative Shh mRNA levels are shown after normalization to corresponding ß-actin mRNA levels. Columns, mean of three independent experiments; bars, SD. (c) Immunoblot of SHH expression.

ACA GCG AGG CCA GGA T-3'; Shh, 5'-GAG ATG TCT GCT GCT AGT CC-3' and 5'-GTT TCT GGA GAT CTT CCC TT-3'; Ptch1, 5'-CTC CCT ACA GCA GCC ACA GC-3' and 5'-AGG TCC CTT GTG GAG CTG GT-3'; and Gli1 5'-TAT GGA CTT CCC ACC TAC TG-3' and 5'-AAT GTT CAA GTC GAG GAC AC-3'. Real-time RT-PCR was carried out with a PCR mixture containing 1 μmol/l of each primer and SYBR Green master mix (Applied Biosystems, Foster City, CA). The amplifications were conducted at 95˚C for 10 sec and 60˚C for 60 sec using an ABI PRISM 7000 Quantitative PCR system (Applied Biosystems). Each sample was examined in triplicate and the amount of PCR product was normalized with respect to ß-actin as an internal control.

Immunoblotting. Prepared cells were harvested after washing with PBS. Collected cells were lysed with buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche, Molecular Biochemical, Indianapolis,
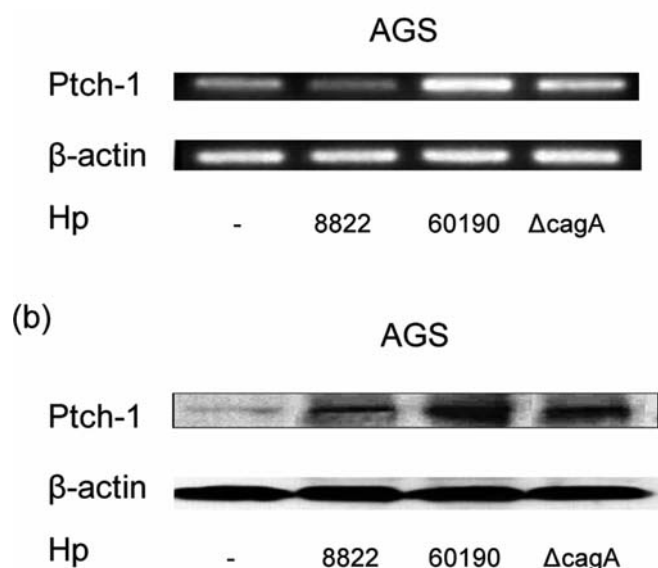
Figure 3. *H. pylori* infection induced patched-1 (Ptch-1) overexpression in gastric cancer cells. (a) RT-PCR analysis of the expression of Ptch-1 in AGS cells. (b) PTCH1 protein as shown by immunoblotting in AGS cells.

IN)]. The same amount of protein was boiled at 95˚C after adding SDS sample buffer [62.5 mM Tris-Cl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, ß-mercaptoethanol, and 0.002% bromophenol blue]. Samples were loaded in 12% SDS-PAGE gels for SHH and 8% SDS PAGE gels for PTCH1 and GLI1 and then transferred to PVDF membranes (Amersham Biosciences, Pisctaway NJ). Rabbit anti-SHH (Santa Cruz, 1:1000) and anti-GLI1 (Santa Cruz, 1:1000) were used as the primary labeling antibodies and the appropriate horseradish peroxidase-conjugated antibodies (Santa Cruz) were used as secondary antibodies. An enhanced chemiluminescence detection system (ECL-Plus, iNtRON, Seoul, Korea) was used for detection according to the manufacturer's protocol.

Statistical analyses. Statistical analyses were conducted using the Student t-test for real-time RT-PCR and Pearson's Chi-square test for immunohistochemistry. A two-sided p-value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (SPSS 12.0, Chicago, IL, USA).

Results

SHH expression correlated with H. pylori infection in gastric cancer cells. To examine whether *H. pylori* infection was associated with increased expression of Shh mRNA and protein, we performed *in vitro* experiments with four different types of human gastric cancer cell lines. We first examined whether *H. pylori* infection altered Shh mRNA transcription. We infected the cells with bacterial strains 8822 (cagA-negative), 60190 (cagA-positive), and Δ*cagA* (*cagA* knockout mutant) for 6 h in order to evaluate the effect of cagA status on Shh expression by gastric cancer cells. The

transcription of Shh mRNA was examined by real-time RT-PCR. *H. pylori* infection increased the transcription of Shh mRNA (Fig. 1a), and infection with strain 60190 enhanced the transcription of Shh mRNA more significantly than strain 8822 or Δ*cagA* (Fig. 2).

To confirm that the Shh production is related to *H. pylori* infection in gastric cancer cells, we performed immunoblotting analyses. In all four cell lines, *H. pylori* infection increased SHH expression (Fig. 1b). Furthermore, infection by the cagA-positive strain 60190 caused higher SHH expression than infection with cagA-negative 8822 or Δ*cagA* (Fig. 2c) bacteria. These findings suggest that *H. pylori* infection affected SHH mRNA transcription and enhanced Shh protein expression in gastric cancer cells in a cagA-dependent manner.

Overexpression of Shh induced by H. pylori led to enhanced Hh pathway activation in gastric cancer cells. We examined ligand-dependent Hh pathway activation in gastric cancer cells. As described above, we infected gastric cancer cells with *H. pylori* to promote overexpression of Shh, and then monitored expression of Ptch-1 and Gli-1, target genes of the Hh pathway, by RT-PCR and immunoblotting. Cells infected with *H. pylori* expressed higher levels of Ptch-1 and Gli-1 than uninfected controls. Furthermore, the 60190 strain significantly induced a greater magnitude expression of both genes in AGS cells (Figs. 3, and 4a and b). These data suggest that overexpression of Shh enhanced Hh signaling pathway activation in gastric cancer cells in a cagA-dependent manner.

To confirm this finding, we treated AGS cells infected by *H. pylori* with cyclopamine to suppress the Hh signaling pathway. Immunoblotting revealed that cyclopamine significantly suppressed GLI1 protein overexpression induced by *H. pylori* infection (Fig. 4c). These data further confirm that *H. pylori*-induced Shh expression activated the Hh pathway in gastric cancer cells.

H. pylori-induced Shh expression is related to the NF-κB pathway. *H. pylori* has been shown to induce chemokine expression and NF-κB activation through the ligation of toll-like receptor (TLR) 2 and TLR 5 (12). We questioned whether *H. pylori*-induced overexpression of Shh protein could be mediated through the NF-κB pathway. AGS cells were pretreated with a NF-κB inhibitor, MG-132 (2 μM), for 1 h before infection with *H. pylori*. The expression of Shh protein was analyzed by immunoblotting. MG-132 treatment resulted in a near complete inhibition of SHH protein expression by AGS cells (Fig. 5a). These results indicate that *H. pylori*-induced Shh expression in AGS cells is mediated through NF-κB activation. To confirm this result, AGS cells were pretreated with another specific NF-κB inhibitor, PDTC, for 6 h before *H. pylori* infection. When treated with *H. pylori* in the presence of PDTC, levels of Shh were also significantly lower than in controls (Fig. 5b).

SHH expression correlated positively with H. pylori infection in human gastric cancer tissues in vivo. To examine whether *H. pylori* infection was associated with Hh signal expression in gastric cancer *in vivo*, we immunostained a series of 20
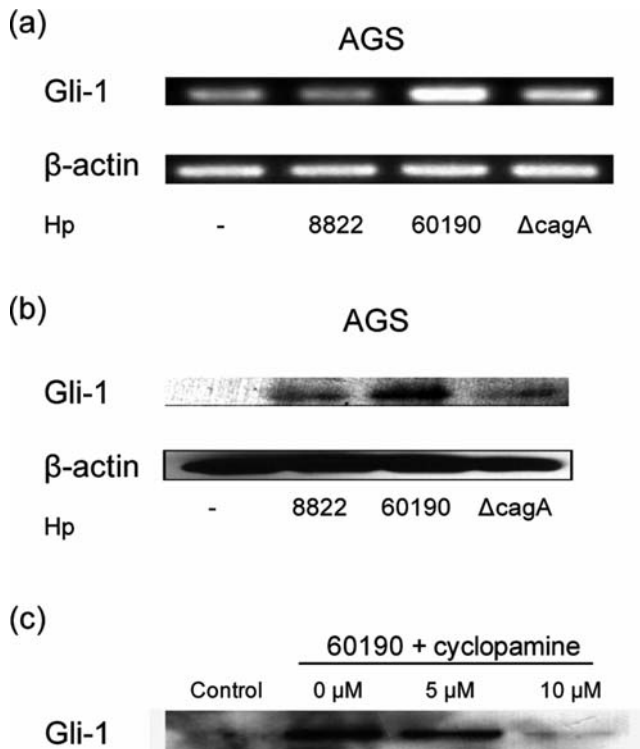
Figure 4. *H. pylori* infection induced glioma-associated oncogene (Gli)-1 overexpression in gastric cancer cells. (a) RT-PCR analysis of the expression of Gli-1 in AGS cells. (b) GLI1 protein as shown by immunoblotting in AGS cells. (c) AGS cells were infected with *H. pylori* after they were pretreated with cyclopamine for 6 h. Immunoblotting was done with anti-Gli-1 antibodies.

Figure 5. *H. pylori* induced sonic hedgehog (SHH) protein expression through the NF-κB pathway in AGS cells. (a) AGS cells were infected with *H. pylori* after they were pretreated with MG-132 at indicated doses for 1 h. Immunoblotting was done with anti-SHH antibodies. (b) AGS cells were infected with *H. pylori* after they were pretreated with pyrrolidine dithiocarbamate (PDTC) at indicated doses for 6 h. Immunoblotting was done with anti-SHH antibodies.

Table I. Relationship between sonic hedgehog (SHH) expression and *Helicobacter pylori* positivity.

	Expression intensity of SHH (no., %)				
	0	1	2	3	p-value
WD Hp+	11.0	6.4	23.8	58.8	<0.05
WD Hp-	20.0	7.4	40.6	32.0	
PD Hp+	0.0	31.6	50.4	18.0	<0.05
PD Hp-	20.0	22.0	38.6	19.4	

WD, well-differentiated; PD, poorly-differentiated; Hp+, *Helicobacter pylori*-positive; Hp-, *Helicobacter pylori*-negative.

Table II. Relationship between glioma-associated oncogene (GLI1) expression and *Helicobacter pylori* positivity.

	Expression intensity of GLI1 (no., %)			
	0	1	2	p-value
WD Hp+	30.8	43.5	25.7	<0.05
WD Hp-	51.5	35.2	13.3	
PD Hp+	53.2	32.5	14.3	<0.05
PD Hp-	57.2	33.1	9.7	

WD, well-differentiated; PD, poorly-differentiated; Hp+, *Helicobacter pylori*-positive; Hp-, *Helicobacter pylori*-negative.

paraffin-embedded surgical specimens, including 10 specimens of well-differentiated carcinomas (5 *H. pylori*-positive and 5 *H. pylori*-negative) and 10 specimens of poorly-differentiated carcinomas (5 *H. pylori*-positive and 5 *H. pylori*-negative). Expression of SHH and GLI1 proteins was analyzed in each specimen. SHH expression was mainly detected in the cytoplasm (Fig. 6) and GLI1 proteins were strongly expressed in both the cytoplasm and nuclei of gastric cancer cells (Fig. 7). *H. pylori*-positive samples displayed higher SHH staining ratios than the *H. pylori*-negative samples in each type of differentiated tissue (p<0.05) (Table I). The percentage of cells with nuclear staining of GLI1 showed a similar pattern (p<0.05) (Table II). These data suggest that *H. pylori* infection was associated with overexpression of SHH, resulting in activation of Hh signaling pathways in gastric cancer tissues *in vivo*.

Discussion

The evolution of *H. pylori*-associated gastric cancer begins with superficial gastritis that progresses to chronic gastritis, atrophy with islands of IM, development of dysplasia, and finally frank carcinoma (13,14). Since SHH is one of the peptide morphogens expressed in the parietal cells of normal human stomachs, the loss of fundic glands due to *H. pylori*-related atrophic changes or IM is accompanied by the loss of Shh expression (5,15,16). Thus, loss of Shh expression due to *H. pylori* may be an early change that occurs in the mucosa
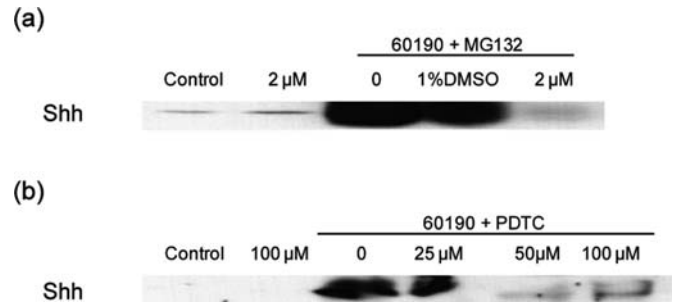
prior to neoplastic transformation. However, the level of Shh is also reportedly elevated in human gastric cancers, especially intestinal types of gastric cancer, which develop through a sequential precancerous cascade of chronic superficial gastritis, chronic atrophic gastritis, IM, and dysplasia (8-10). The discordance of Shh expression levels between preneoplastic lesions and gastric cancer may be a consequence of dual roles
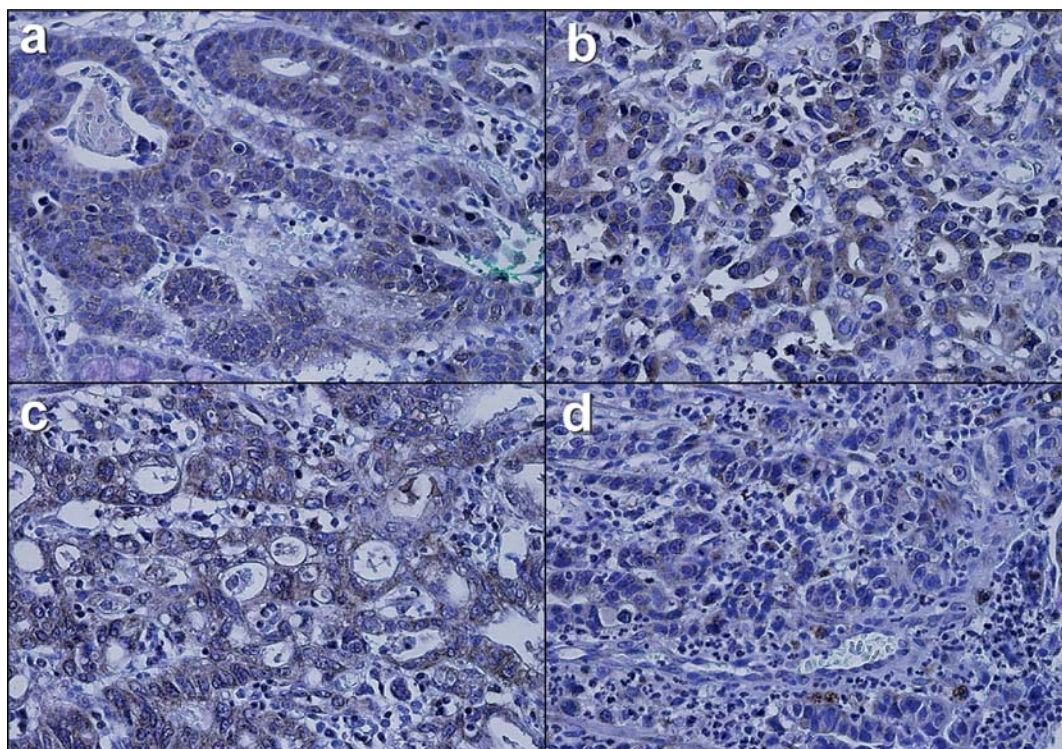
Figure 6. Expression of sonic hedgehog (SHH) in gastric carcinoma tissues. SHH expression was mainly seen in the cytoplasm. SHH staining revealed strong expression in *H. pylori*-positive tissues in each type of differentiated adenocarcinoma (immunohistochemical staining x400). (a) Well-differentiated *H. pylori*-positive tissue. (b) Well-differentiated *H. pylori*-negative tissue. (c) Poorly-differentiated *H. pylori*-positive tissue. (d) Poorly-differentiated *H. pylori*-negative tissue.
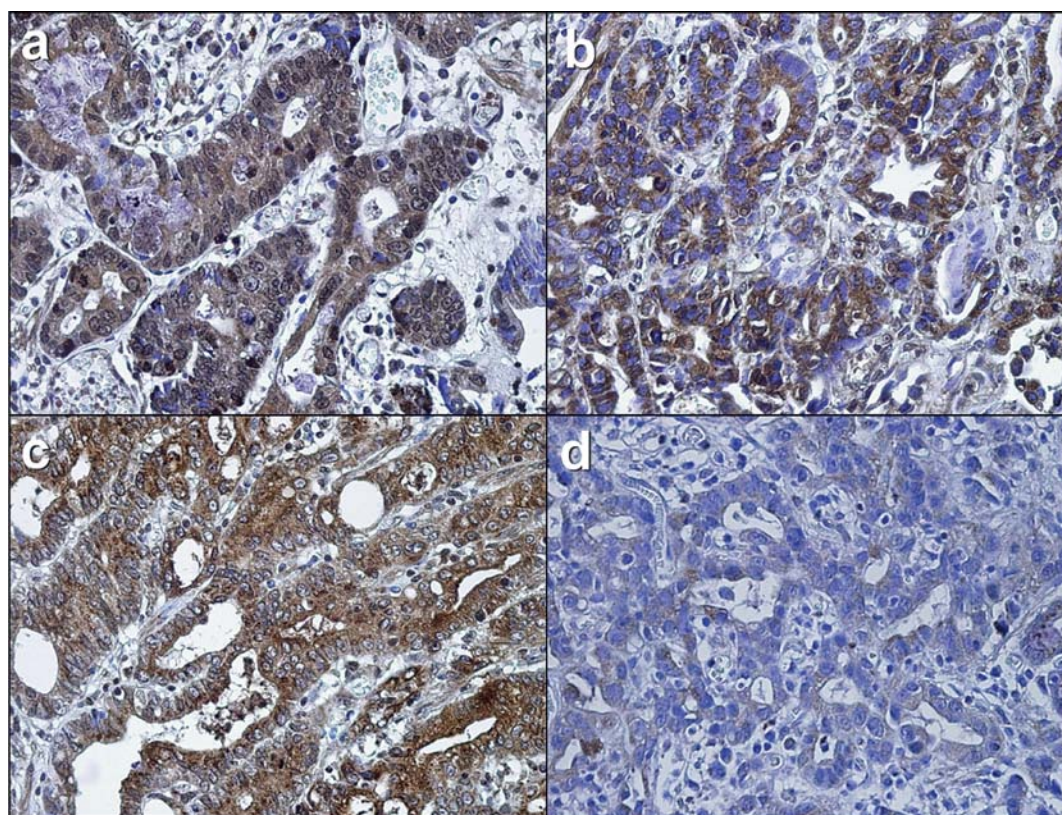

Figure 7. Expression of glioma-associated oncogene (GLI)-1 in gastric carcinoma tissues. The GLI1 protein was expressed in both the cytoplasm and nuclei of gastric carcinoma tissues. GLI1 staining revealed strong expression in *H. pylori*-positive tissues in each type of differentiated adenocarcinoma (immunohistochemical staining x400). (a) Well-differentiated *H. pylori*-positive tissue. (b) Well-differentiated *H. pylori*-negative tissue. (c) Poorly-differentiated *H. pylori*-positive tissue. (d) Poorly-differentiated *H. pylori*-negative tissue.

of Shh in morphogenesis and carcinogenesis. Accordingly, we examined Shh changes following *H. pylori* infection in gastric cancer cells to clarify the role of Hh signal activation in *H. pylori*-associated gastric carcinogenesis.

In the present study, *H. pylori* activated the Hh signaling pathway through the up-regulation of Shh expression in gastric cancer cells. This Shh overexpression was dependent on cagA, the pivotal virulence factor for gastric carcinogenesis in *H. pylori* infection. This finding suggests that the Hh signaling pathway is one of the host signal transduction pathways related to gastric carcinogenesis by *H. pylori*. Furthermore, Shh was detected in gastric cancer cells and overexpressed following *H. pylori* infection in gastric cancer cells. These findings suggest that *H. pylori* may be related to cancer growth/metastasis after cancer transformation by overexpression of Shh.

We further found that the Shh overexpression following *H. pylori* infection was mediated through the NF-κB pathway. NF-κB is a transcription factor that controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion and adhesion, angiogenesis, and apoptosis (17). More importantly, *H. pylori* induces chemokine expression and NF-κB activation through the ligation of TLR 2 and TLR 5 receptors (12). We blocked the NF-κB signal pathway using a specific inhibitor to evaluate whether NF-κB participates in Shh overexpression by *H. pylori* infection. Blockade of the NF-κB pathway suppressed constitutive Shh expression, in agreement with previous results in pancreatic cancer (18). These findings suggest a possible role of NF-κB inhibitors in the prevention of *H. pylori*-mediated gastric carcinogenesis. Also, as cagA-positive strains were associated with higher levels of Shh expression, our data raise the possibility that intracellularly translocated oncoprotein CagA may be implicated in Shh overexpression through the NF-κB pathway in gastric cancer cells.

Changes in the level of Shh expression in the gastric epithelium may provide a marker for chemopreventive measures. When the level of Shh declines in *H. pylori*-associated gastritis or IM, the interaction between Shh and *H. pylori* is based on the process of morphogenesis in the stomach. Eradication of *H. pylori*, resulting in the restoration of SHH protein expression, can lead to a morphogenetic drive in gastric epithelial homeostasis. However, if the level of Shh is higher in *H. pylori*-associated preneoplastic tissues or gastric cancer during follow-up, the interaction between Shh and *H. pylori* is converted into a process of cancer progression. Thus, the Hh signaling pathway may play a role in neoplastic transformation and progression in gastric carcinogenesis by *H. pylori*. This stage may be a sign of another 'point of no return' in gastric carcinogenesis caused by *H. pylori*. However, this hypothesis must be further examined by serial experiments involving the *H. pylori*-related precancerous cascade.

In conclusion, *H. pylori* infection activates the Hh signaling pathway through the up-regulation of Shh in gastric cancer cells. Shh activation is cagA-dependent and mediated through the NF-κB pathway. Accordingly, the Shh signaling pathway should be considered as a possible explanation for *H. pylori*-related gastric carcinogenesis.

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