The effect of *Helicobacter pylori* infection on sonic hedgehog signal pathways in gastric epithelial cells

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The effect of *Helicobacter pylori* infection on sonic hedgehog signal pathways in gastric epithelial cells

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Abstract

The effect of Helicobacter pylori infection on sonic hedgehog signal pathways in gastric epithelial cells

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Aberrant activation of hedgehog (Hh) signaling pathway leads to pathological consequences in a variety of human tumors. Sonic hedgehog (Shh) is implicated in stem/progenitor cell restitution of damaged gastric mucosa during chronic infection with *Helicobacter pylori* (*H. pylori*). *H. pylori* with various virulence factors cause mucosal damage and may participate in gastric carcinogenesis. The aim of this study was to examine the changes in Shh signal pathways in gastric epithelial cells in

response to *H. pylori* infection. We also assessed the influence of *cagA* and *cagE* genes of *H. pylori* on the Shh expression by gastric epithelial cells. Real time RT-PCR, immunoblot and immunohistochemical stains were performed to analyze the expression, localization and transcriptional regulation of Shh, Patched1 (Ptc1) and Glioma-associated oncogene (Gli1). *H. pylori* strains 60190 (*cag*PAI positive), 8822 (*cag*PAI negative), and gene knock-out mutants *H. pylori* strains of *cagA* and *cagE* were used. AGS and MKN45 cells were used as for gastric epithelial cells. Paraffin-embedded tissues from resected human gastric cancers were used to evaluate the expression of Shh and related signal proteins *in vivo*. Overexpression of Shh in gastric epithelial cells with *H. pylori* infection was confirmed by immunoblot and real time RT-PCR. Furthermore, *cagA* positive strains showed higher level of Shh expression. Gli1 mRNA, the transcriptional target of the Hh pathway, were overexpressed in cells infected with *H. pylori* compare to uninfected control, implying that *H. pylori* infection induced the Hh pathway activation in gastric epithelial cells. The Shh positive immunoexpression in *H. pylori*-infected gastric cancer tissue was higher than those of uninfected gastric cancer tissue while the immunostaining of Shh correlated with tumor differentiation apart from the status of *H. pylori* infection. In conclusion, Shh expression correlates with the status of *H. pylori* infection and *H. pylori* may activate the Shh signal pathways through the up-regulation of Shh in gastric epithelial cells.

Key words: Gastric cancer, Hedgehog signal pathway, *Helicobacter pylori*

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I. INTRODUCTION

The Hedgehog (Hh) signaling pathway is intimately linked to cell growth and differentiation, with physiological roles in embryonic pattern formation, adult tissue homeostasis and pathological roles in tumor initiation and growth.[1] In the adult, Hh signaling remains active

in some organs where it has been implicated in the regulation of stem-cell maintenance and proliferation.[2] The contribution of Hh pathway to the development and homeostasis of gastric gland has been reported.[3] Mammalian Hh proteins include sonic Hh (Shh), indian Hh (Ihh), and desert Hh (Dhh). Hh ligands undergo post-translational modifications, including autocatalytic cleavage and coupling of cholesterol to the amino-terminal peptide, which is the fragment that possesses all the signaling activity.[1] In the absence of Hh signaling, patched family receptors (Ptch1/Ptch and Ptch2) inhibit the smoothened (Smo) signal transducer.[4] Hh binding to Ptch removes the inhibitory effect and allows Gli to enter the nucleus and act as a transcriptional activator. Hh signaling activation leads to Gli-dependent transcriptional activation of target genes, such as Gli1, Ptch1, cyclin D2 (CCND2), Forkhead Box M1 (FoxM1) and Jagged-2 (Jag2).[5] Hh signaling targets include genes that are important for cell proliferation and growth. Shh is up-regulated in a variety of human tumors, such as gastric cancer, pancreatic cancer, esophageal cancer, and prostatic cancer. Shh up-regulation through the aberrant activation of Hh signaling pathway may leads to carcinogenesis.[6]

Gastric cancer is frequently associated with chronic *Helicobacter pylori* (*H. pylori*) infection and is one of the most common malignancies in the world.[7] Accumulation of genetic alterations or epigenetic changes caused by genetic predisposition, life style, and persistent mucosal damage associated with chronic *H. pylori* infection may eventually leads to gastric cancer.[4], [8] *H. pylori* strains can be divided into two major subpopulationes based on their ability to produce a 120-145 kDa immunodominant protein, cytotoxin-associated gene A (CagA) antigen.[9] Clinically, infection with the *cagA*-positive *H. pylori*

strain has been associated with higher degree of gastric mucosal inflammation as well as severe atrophic gastritis and has been suggested to play an important role in the development of gastric cancer.[10]

H. pylori induced nuclear factor κB (NF-κB) activation in MKN45 gastric epithelial cells through the ligation of toll-like receptor (TLR) 2 and TLR5, but not TLR4.[11] NF-κB, a transcriptional regulator, controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion and adhesion, angiogenesis, and apoptosis.[12] It has also been reported that NF-κB activation up-regulates expression of Shh, resulting in activation of Hh signaling in cancer.[13]

This study examined the changes in Shh signal pathways in gastric epithelial cells in response to *H. pylori* infection. We checked whether *H. pylori* infection could lead to enhanced Hh pathway activation in gastric cancer cells and this was associated with *cagA* positive strains. We then examined the role of NF-κB pathway activation on Shh expression in various gastric cancer cells infected with *H. pylori*.

II. MATERIALS AND METHODS

1. Cell and H. pylori culture and infection.

AGS (ATCC CRL 1739) and MKN45 (KCLB No. 80103) gactric epithelial cells, were maintained in RPMI 1640 medium (Gibco, Grand Island. NY) supplemented with 10% fetal bobine serum (Gibco) and 1% penicillin-streptomcin sulfate (Gibco). All cultures were maintained at 37°C incubator supplemented with 5% CO_2. *H. pylori* strains were cultured on blood agar plate containing 5% sheep blood at 37°C incubator with gas pak (BD, Sparks, MA). We cultured 8822 (PAI-negative strain) and 60190 (PAI-positive strain) along with △cag (*cagA* knock-out isogenic mutant) strains[14] (kind gift from Prof. Richard M. Peek. Jr., Vanderbit Univ.). For *H. pylori* infection with gastric epithelial cells, *H. pylori* strains were cultured for 24-36 hr. Cultured *H. pylori* were harvested in PBS (pH 7.4) and added to the serum-starved host cells at a multiplicity of infection (M.O.I) of 50.

2. 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 NF-κB, were used at the concentrations previously described .[15]

3. 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_2O_2 to quench endogenous peroxidase activity. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with protein blocking agent (Immunotech, Coulter, Inc., Marseille) to block nonspecific antibody binding for 20 min at room temperature and then incubated overnight at 4°C with an optimal dilution of a primary polyclonal goat antibody against human Shh (H-160, Santa Cruz), Ptc1 (G-19, Santa Cruz), Gli1 (H-300, Santa Cruz). After washing with PBS three times, the sections were incubated with the biotinylated secondary antibody (goat anti-rabbit IgG, Immunotech, Coulter, Inc., Marseille) and streptavidin conjugated to horseradish peroxidase (Immunotech) for 20 min at room temperature, followed by PBS wash. The chromogen was developed for 5 min with liquid 3, 3′-diaminobenzidine (Immunotech). Next, slides were counterstained with Meyer's hematoxylin, then dehydrated, and mounted with histochoice mounting media for examination. A total of 100-150 cancer cells were counted in each section. For immunostaining of Shh, cancer cells showing no detectable cytoplasm staining were given 'negative' and strong cytoplasm staining were given 'positive'. For immunostaining of Gli1, cancer cells showing no detectable nuclear staining were given 'negative' and strong nuclear staining were given 'positive'.

4. Immunoblotting.

Prepared cells were harvested after washing with phosphate buffered saline. 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, protease inhibitor cocktail (Roche, Molecular biochemical Indianapolis, IN)). 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, β-mercaptoethanol, 0.002% bromophenol blue). Samples were loaded in 12% SDS-PAGE for Shh and 8% SDS PAGE for Ptc1 and Gli1 and transferred to PVDF membrane (Amersham Biosciences, Pisctaway NJ). Rabbit anti-Shh, anti-Gli1 and goat anti-Ptc1 (Santa Cruz) 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.

5. Reverse transcription-PCR and real-time reverse transcription-PCR analyses.

Total RNA was extracted from cultured cells using a RNeasy mini kit (Qiagen, Tokyo, Japan). The RNA was reverse transcribed using oligo $(dT)_{12-18}$ primers and superscrip^{TM} II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was done with PCR Maxi kit (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. The amplifications were done by denaturation at 95°C for 5 min, followed by 30 cycles of 30 sec each at 95°C, 57°C, and 72°C for Shh and Ptc1, 30 cycles of 30

sec each at 95°C, 59°C, and 72°C for Gli1 and 18 cycles of 30 sec each at 95°C, 60°C, and 72°C for β-actin. The following primer pairs were used: β-actin, 5′-TTG CCG ACA GGA TGC AGA AGA-3′ and 5′-AGG TGG 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′; Ptc1, 5′-ATG CTG GCG GGA TCT GAG TTC GAC T-3′ and 5′-GGG TGT GGG CAG GCG GTT CAA G-3′; Gli1 5′-TAT GGA CTT CCC ACC TAC TG-3′ and 5′-AAT GTT CAA GTC GAG GAC AC-3′.

The real time RT-PCR analysis 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 the ABI PRISM 7000 Quantitative PCR system (Applied Biosystems[16]). Each sample was examined in triplicate and the amounts of the PCR products produced were normalized with respect to β-actin as an internal control.

6. Statistical analysis

Statistical analyses were conducted using Student *t*-test for real time PCR and Pearson's chi-square test for immunohistochemistry. *p*-values < 0.05 were regarded as statistically significant. All statistical analyses were performed using SPSS software (SPSS 12.0, Chicago).

Ⅲ. *RESULTS*

1. Shh expression correlated with H. pylori infection in gastric cancer cells.

To examine whether *H.* pylori infection was associated with expression of Shh mRNA and protein, we performed *in vitro* experiments with three human gastric cancer cell lines, AGS and MKN74. We firstly examined whether *H.* pylori infection altered Shh expression in these cell lines. We infected these cell lines with 8822 (PAI-negative strain), 60190 (PAI-positive strain) and isogenic mutants △cagA for 6 or 24 hr to induce Shh expression. The expression of Shh mRNA was examined by real-time reverse transcription PCR or RT-PCR in gastric cancer cells. On the whole, *H. pylori* infection increased the expression of Shh mRNA, and 60190 infection enhanced the expression Shh mRNA more significantly than 8822 and △cagA (Fig. 1, 2).

To confirm that the Shh production related to *H. pylori* infection in gastric cancer cells, we performed immunoblotting analysis. *H. pylori* infection increased Shh levels in AGS and MKN45 cells (Fig. 3). Furthermore, infection by *cagA* positive strains showed higher level of Shh expression when infected (Fig. 3). These findings indicated that *H. pylori* infection affected Shh mRNA expression and enhanced Shh protein production in these cell lines.

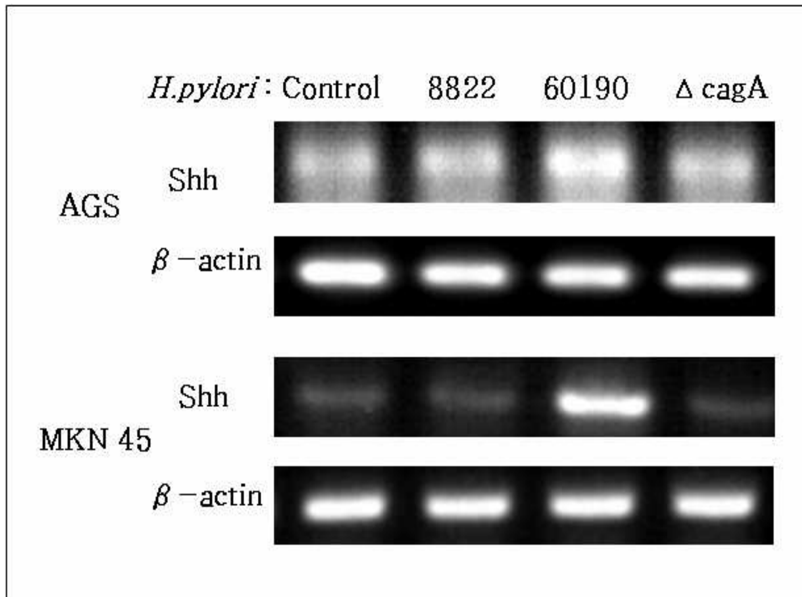

Fig. 1. Shh mRNA correlated positively with *H. pylori* infection in gastric cancer cells. AGS and MKN45 cells were infected by *H. pylori* CagA positive (60190) and negative (△cagA, 8822) strains for 6 hr and RT-PCR were performed. β-actin served as an internal control. 60190 significantly enhanced expression Shh mRNA.

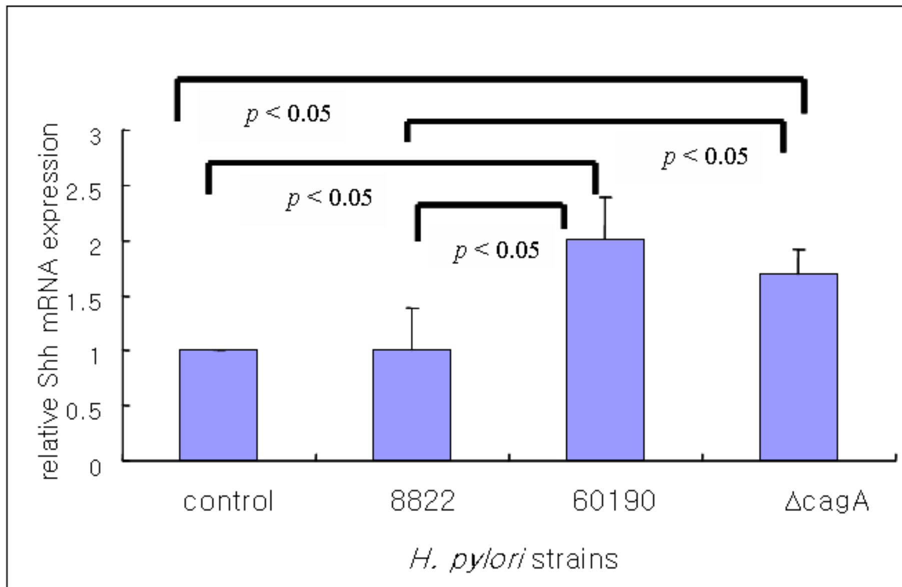

Fig. 2. Shh mRNA level positively correlated with *H. pylori* infection in gastric cancer cells. Shh mRNA expressions by AGS cells were examined by real-time RT-PCR. Relative Shh mRNA level after normalization to the corresponding β-actin mRNA expression were shown. *H. pylori* increased Shh mRNA levels in AGS cell. Furthermore, *cagA* positive strains showed greater level of Shh mRNA expression when infected. Columns, mean of the three independent experiments; *bars*, SD.

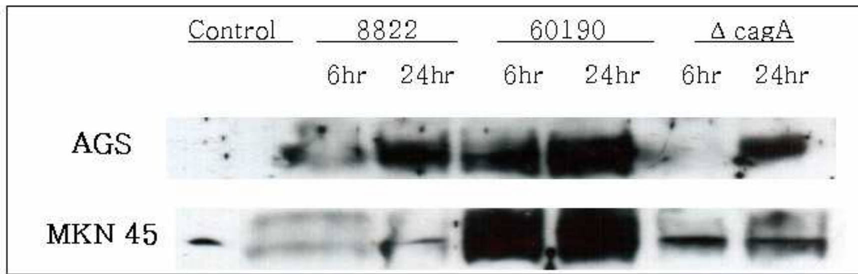

Fig. 3. Immunoblotting of Shh expression in two gastric cancer cell lines. AGS and MKN45 cells were infected with 8822, 60190 and △cagA for 6 or 24 hr to induce Shh expression. *H. pylori* infection increased expression of Shh protein, and 60190 other than 8822 and △cagA significantly increased the expression of Shh protein in gastric cancer cells when infected.

2. Overexpression of Shh induced by H. pylori led to enhanced Hh pathway activation in gastric cancer cell.

We examined the ligand-dependent Hh pathway activation in gastric cancer cells. The gastric cancer cells were infected with *H. pylori* to overexpress Shh, and the Gli1 expression, a transcriptional target of the Hh pathway, was then monitored by RT-PCR and immunoblotting.[16] RT-PCR revealed that cells infected with *H. pylori* expressed higher level of Gli1 mRNA than uninfected control (Fig. 5) and Immunoblotting showed that 60190 strain significantly induced greater magnitude of Gli1 protein in AGS cells (Fig. 6).

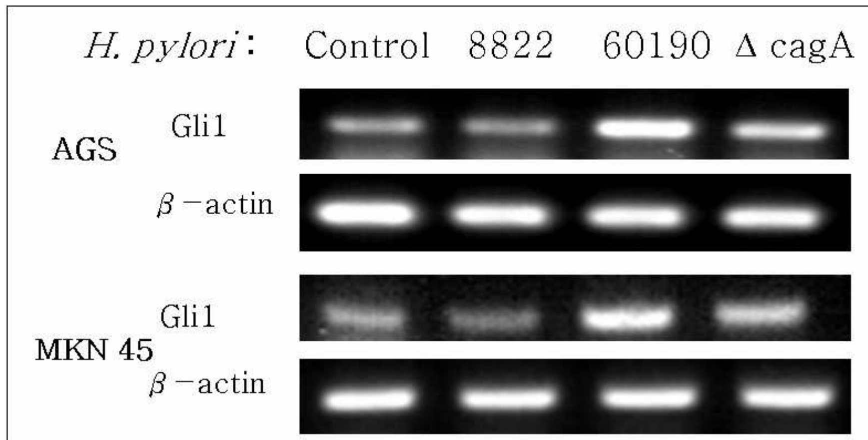

Fig. 5. Effects of *H. pylori* infection on Gli1 mRNA expression. AGS and MKN45 cells were infected with *H. pylori* for 6 hr. RT-PCR analysis of the expression of Gli1 mRNA were performed. *H. pylori* increased Gli1 mRNA levels in these cells. Furthermore, *cagA* positive strains showed higher level of Gli1 mRNA expression when infected. *H. pylori* infection induced Gli1 mRNA expression implying enhanced Hh pathway activation in gastric cancer cells.

These data suggested that overexpression of Shh enhanced Hh pathway activation within the gastric cancer cells.

To confirm these results, we treated AGS cell with cyclopamine to suppress Smo (Fig. 7). Immunoblotting revealed that cyclopamine significantly suppressed the expression of Gli1 protein (Fig. 7). These data again confirmed that *H. pylori*-induced Shh expression activated the Hh pathway in gastric cancer cells.

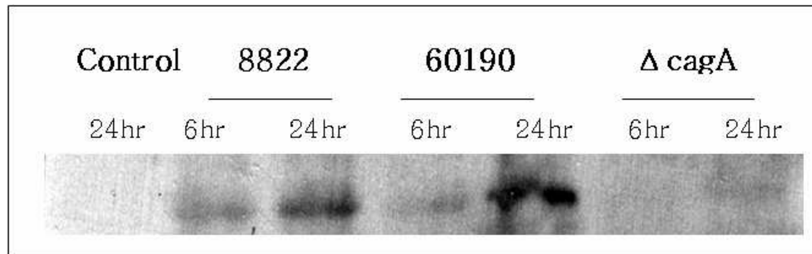

Fig. 6. Effects of *H. pylori* infection on Gli1 protein expression. AGS cells were infected with 8822, 60190 and △cagA for 6 or 24 hr to induce Gli1 expression. Gli1 protein by immunoblotting in AGS cells. 60190 significantly induced greate amount of Gli1 protein expression compare to 8822 and △cagA. *H. pylori* infection induced Gli1 protein implying enhanced Hh pathway activation in gastric cancer cells.

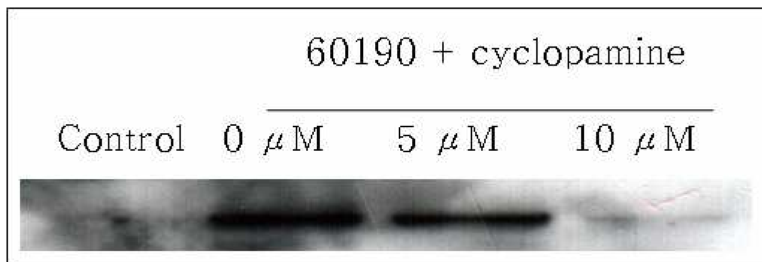

Fig. 7. Inhibition of *H. pylori*-induced Gli1 protein in AGS cells. AGS cells were infected with *H. pylori* for 12 hr after they were pretreated with cyclopamine for 6 hr. Immunoblotting was done with anti-Gli1. Cyclopamine dose dependently inhibit the expression of Gli1 protein gastric cancer cells when infected by *H. pylori*.

3. H. pylori-induced Shh correlated with a reduction in the level of Ptc1 protein.

We examined whether Hh signaling has the paradoxical effect of both blocking Ptc protein function and activating *ptc* gene expression. We, unexpectedly observed that *H. pylori* infection had opposing effects on Ptc1. Ptc1 expression was reduced in AGS cell infected with *H. pylori* and 60190 strain was sufficient to reduce Ptc1 expression (Fig. 8-10). These observations suggest that *H. pylori*-induced Shh caused down regulation of Ptc1 protein in gastric cancer cells.

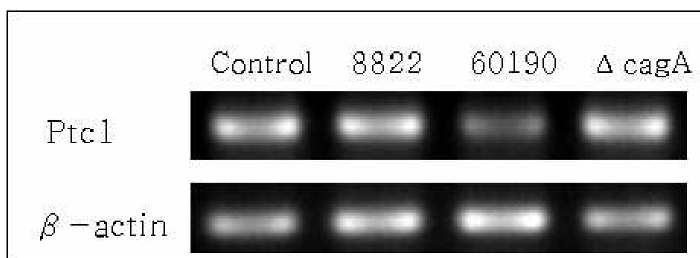

Fig. 8. *H. pylori*-induced Shh correlated with a reduction in the level of Ptc1 mRNA. AGS cells were infected by *H. pylori* CagA positive (60190) and negative (△cag, 8822) strains for 6 hr and RT-PCR were performed. β -actin served as an internal control. *H. pylori* starin of 60190 significantly reduced expression Ptc1 mRNA. Ptc1 mRNA reduction was dependent on CagA protein in gastric cancer cells.

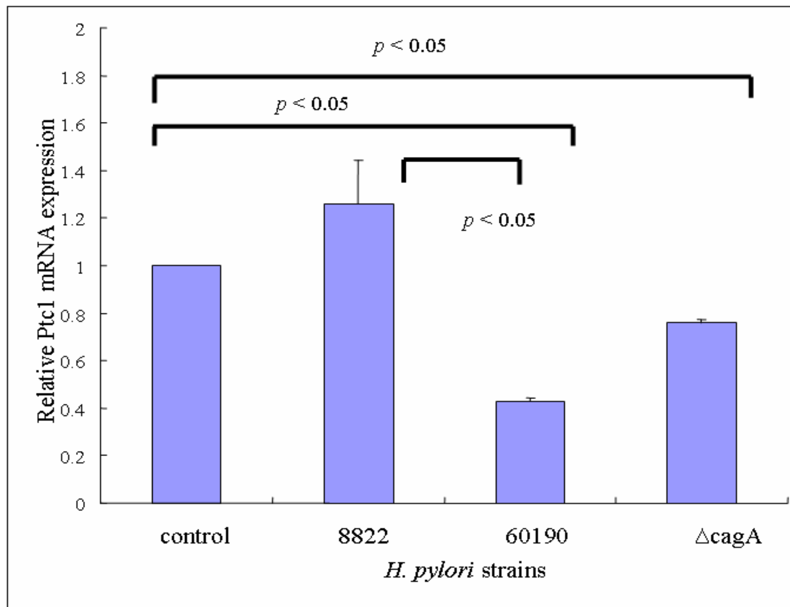

Fig. 9. Real time RT-PCR mRNA expression of Ptc1. AGS cells were infected by *H. pylori* CagA positive (60190) and negative (△cag, 8822) strains for 6 hr and Real time RT-PCR were performed. Relative Ptc1 mRNA level after normalization to the corresponding β-actin mRNA expression were shown. *H. pylori* (*cagA* positive strain) infection reduced Ptc1 mRNA levels in AGS cell. Columns, mean of the three independent experiments; *bars*, SD.

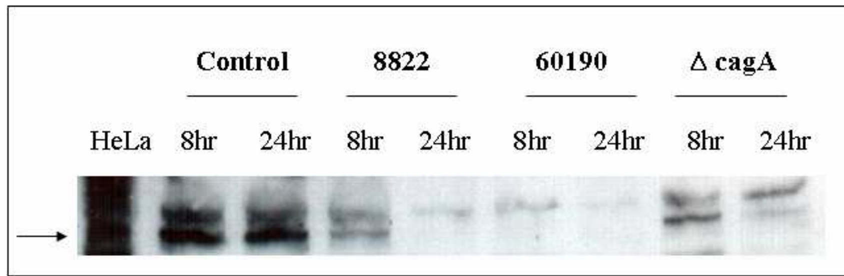

Fig. 10. Immunoblotting of Ptc1 expression in AGS cells. AGS cells were infected by *H. pylori* strains for 8 hr and 24 hr and analyzed by immunoblotting with anti-Ptc1. *H. pylori* reduced expression of Ptc1 protein and moreover 60190 significantly reduced expression of Ptc1 protein in gastric cancer cells. HeLa cell is a positive control.

4. H. pylori induce Shh protein is related to NF-κB pathway.

H. pylori has been demonstrated to induce chemokine and NF-κB activation through TLR2 and TLR5.[12] We questioned whether *H. pylori*-induced overexpression in Shh protein would be blocked by NF-κB inhibition. AGS cells were pretreated with the NF-κB inhibitor, MG-132 (Sigma-Aldrich, St. Louis, MO), before infection with *H. pylori* for 6 hours. The expression of Shh protein was analyzed by immunoblotting (Fig. 11). MG-132 treatment resulted in a near complete inhibition of Shh protein expression. These results indicate that *H. pylori*-induced Shh expression in AGS cells is mediated through NF-κB activation. To confirm these results, AGS cells were treated with *H. pylori* for 6 hr after they were pretreated with another specific NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) for 6 hr (Fig. 12).[17] When treated with *H. pylori* in the presence of PDTC, levels of Shh were reduced. These data suggested that the *H. pylori*-induced Shh protein expression was mediated through NF-κB pathway in gastric cancer cells.

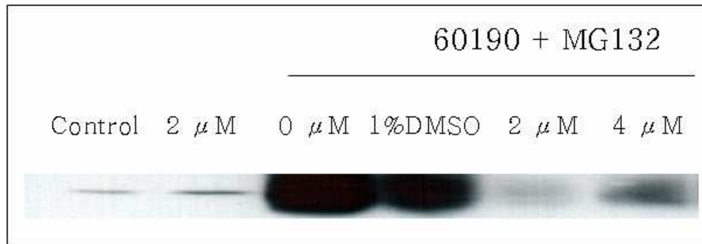

Fig. 11. Level of *H. pylori*-induced Shh protein dose dependently decreased with NF-κB inhibition in AGS cells. AGS cells were infected with *H. pylori* for 12 hr after they were pretreated with MG-132 for 1 hr. Immunoblotting was done with anti-Shh. When the concentration of MG-132 was higher than 2 μM MG-132 could inhibit expression of Shh protein induced by *H. pylori.*

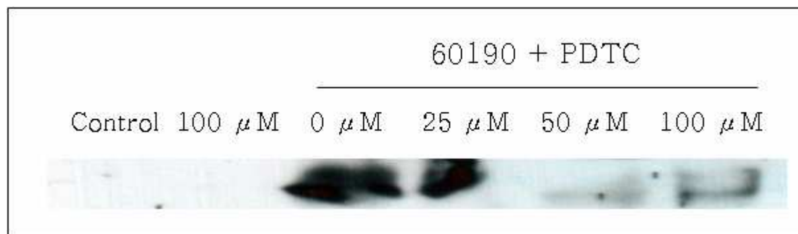

Fig. 12. Level of *H. pylori*-induced Shh protein dose dependently decreased with NF-κB inhibition in AGS cells. AGS cells were infected with *H. pylori* for 6 hr after they were pretreated with PDTC for 6 hr. Immunoblotting was done with anti-Shh. When the concentration of PDTC was higher than 50 μM PDTC could inhibit expression of Shh protein induced by *H. pylori.*

5. Shh expression correlated positively with H. pylori infection in specimens of human gastric cancers.

To examine whether *H. pylori* infection was associated with expression of Hh pathway *in vivo*, we stained a series of 20 paraffin-embedded surgical specimens, including 10 specimens of well differentiated carcinomas (*H. pylori*-positive group and negative group) and 10 specimens of poorly differentiated carcinomas (*H. pylori*-positive group and negative group). Expressions of Shh and Gli1 were examined immunohistochemically. Shh ligand was expressed mainly in the cytoplasm (Fig. 13) and Gli1 was strongly expressed in both the cytoplasm and nuclei of gastric cancer cells (Fig. 14). *H. pylori*-positive group showed higher Shh positive staining ratios than *H. pylori*-negative (Table 1.). The percentage of cells with nuclear staining of Gli1 showed similar results (Table 2.). In addition, the staining ratios of well differentiated carcinomas were higher than those of poorly differentiated cancer and this association was statistically significant[18]. A strong positive correlation was detected between Shh staining ratio and Gli1. These data suggested that 1) there was a correlation between *H. pylori* infection and Shh expression, and 2) overexpression of Shh induced by *H. pylori* infection may induce enhanced Hh pathway activation in gastric cancer tissues.

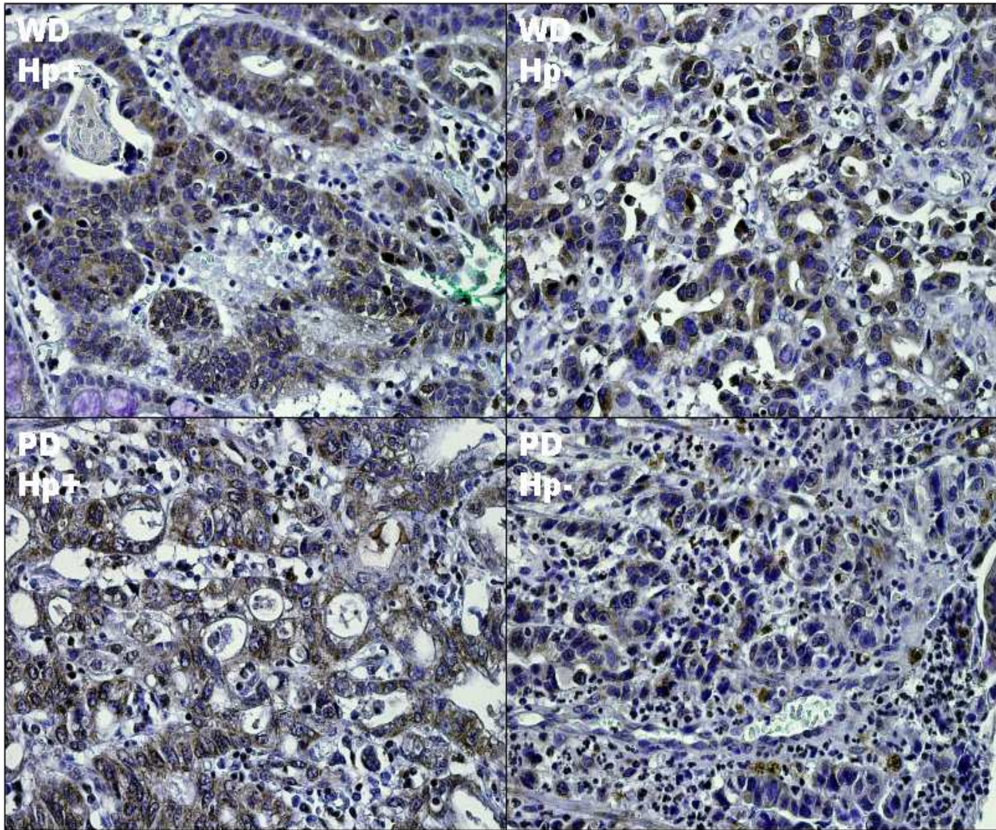

Fig. 13. Expressions of Shh in gastric cancer tissues. Shh protein was detected by immunohistochemistry with anti-Shh in gastric cancer tissues. Shh ligand was expressed mainly in the cytoplasm of gastric cancer cells. *H. pylori*-negative tissue was less intense than *H. pylori*-positive. Shh expression in well differentiated cancer was stronger than that in poorly differentiated cancer. Magnification: × 400

WD Hp+: Well differentiated and *H. pylori* positive.
WD Hp-: Well differentiated and *H. pylori* negative.
PD Hp+: Poorly differentiated and *H. pylori* positive.
PD Hp-: Poorly differentiated and *H. pylori* negative.

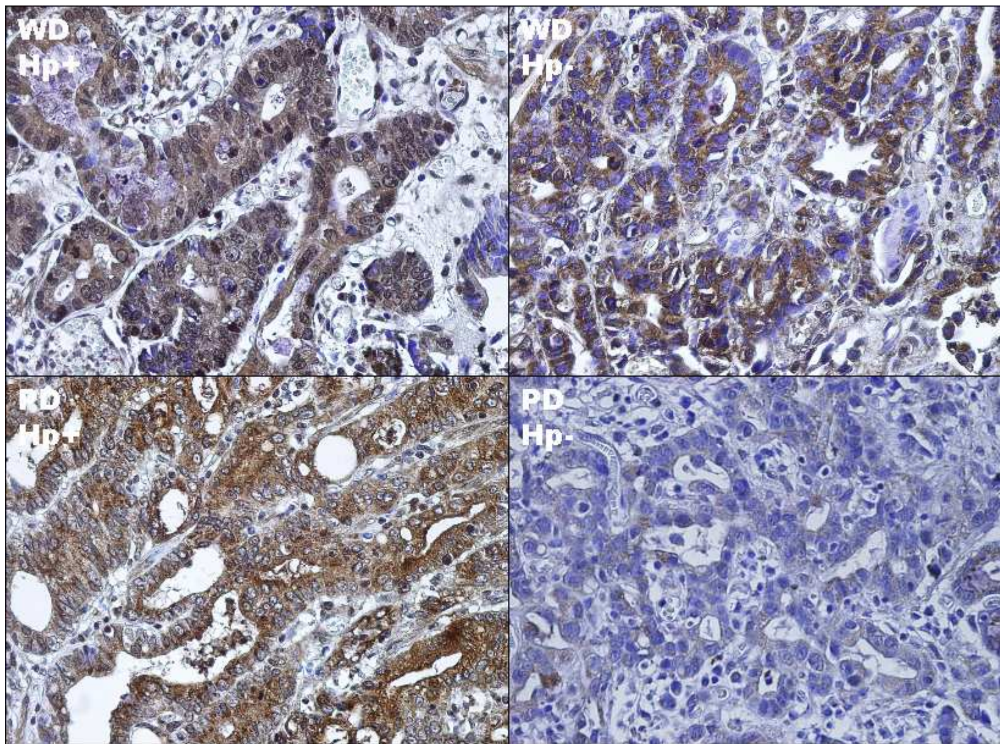

Fig. 14. Expressions of Gli1 in gastric cancer tissues. Gli1 protein was detected by immunohistochemistry with anti-Gli1 in gastric cancer tissues. Gli1 was strongly expressed in both the cytoplasm and nuclei of gastric cancer. *H. pylori*-negative tissue was less intense than *H. pylori*-positive. Gli1 expression in well differentiated cancer was stronger than that in poorly differentiated carcinomas. Magnification: × 400

WD Hp+: Well differentiated and *H. pylori* positive.

WD Hp-: Well differentiated and *H. pylori* negative.

PD Hp+: Poorly differentiated and *H. pylori* positive.

PD Hp-: poorly differentiated and *H. pylori* negative.

Table 1. Relationship between Shh expression levels and *H. pylori* and differentiation.

	Expression intensity of Shh (No. %)		
	Negative	Positive	Total cell
WD+	87 (17.4%)	413 (82.6%)	500
WD-	137 (27.4%)	363 (72.6%)	500
PD+	158 (31.6%)	342 (68.4%)	500
PD-	210 (42%)	290 (58%)	500

For immunostaining of Shh, cancer cells showing no detectable cytoplasm staining were given 'negative' and strong cytoplasm staining were given 'positive'.

$p < 0.05$, p-values calculated using: chi-square analysis.

WD: well differentiated　　+: *H. pylori* positive

PD: poorly differentiated.　　-: *H. pylori* negative

Table 2. Relationship between Gli1 expression levels and *H. pylori* and differentiation.

	Expression intensity of Gli1 (No. %)		
	Negative	Positive	Total cell
WD+	231 (30.8%)	519 (69.2%)	750
WD-	386 (51.5%)	364 (48.5%)	750
PD+	399 (53.2%)	351 (46.8%)	750
PD-	429 (57.2%)	321 (42.8%)	750

For immunostaining of Gli1, cancer cells showing no detectable nuclear staining were given 'negative' and strong nuclear staining were given 'positive'.

$p < 0.05$, p-values calculated using: chi-square analysis.

WD: well differentiated　　+: *H. pylori* positive

PD: poorly differentiated.　　-: *H. pylori* negative

Ⅳ. *DISCUSSION*

It was recently reported that aberrant activation of Hedgehog signaling pathway lead to pathological consequences in human gastric cancer, pancreatic cancer, esophageal cancer, prostate cancer, small cell lung cancer, basal cell carcinoma, medulloblastoma and glioma.[6, 19, 20-22] The Shh-Smo-Gli1 pathway has a pivotal role in the growth of gastric cancer cells and the stimulation of Hh signaling by Shh ligand occurs in an autocrine/paracrine manner in gastric cancer cells.[16] However, the molecular mechanisms underlying this abnormal activation remain unclear. Cyclopamine, a steroidal alkaloid that interacts directly with Smo to inhibits Hh signaling, effectively retards the growth of various tumors, including gastric carcinoma, indicating that Hh signaling is involved in tumor growth.[19, 23-25] Nevertheless, it remains unclear how the blockade of Hh signaling leads to tumor growth suppression in gastric cancer. If so, it is important to elucidate the identities of the molecules that regulate expression of Shh. Our data that *H. pylori* infection is one of the mechanisms responsible for Shh overexpression seem to have broad meaning.

H. pylori primarily infects individuals during childhood, and can cause gastric diseases such as chronic atrophic gastritis and peptic ulcers later in life.[8] Recent epidemiological studies have indicated that *H. pylori* is involved in the development of gastric adenocarcinoma. A large-scale prospective study revealed that the risk for development of gastric carcinoma was much greater in the *H. pylori*-infected population than in the *H. pylori*-uninfected population.[26] However, molecular mechanisms by which *H. pylori* triggers the process leading to gastric carcinoma remain largely unknown.

We focused on the role of *H. pylori* infection in Shh expression in gastric cancer for the following reason. Chronic persistent infection with *H. pylori* over a few decades leads to chronic atrophic gastritis, and then to gastric cancer.[7] *H. pylori* with CagA virulence factors causes mucosal damage, and following repair in the stomach. Shh, ensuring stem/progenitor cell restitution of damaged gastric mucosa, is implicated in the repair process during chronic persistent *H. pylori* infection. Our data showed that CagA positive strains showed higher level of Shh expression when infected. The vast majority of *H. pylori* strains contain the cag pathogenicity island (cag-PAI), a 40 kb genomic fragment containing 31 genes. This stretch of DNA codes for type IV secretion (TFSS) apparatus used to inject bacterial proteins such as the 120 kD protein CagA into host epithelial cells. A series of in vitro reports have now established that injection of CagA into host cells leads to phosphorylation of CagA by host cell kinases (such as c-Src), resulting in activation of SHP-2 tyrosine phosphatase and MAP kinase signaling pathways.[27] CagA is an important mediator for *cag*PAI-dependent induction of potent proinflammatory responses during *H. pylori* infection. Transfected and translocated CagA from a subset of *H. pylori* strains are able to induce IL-8 release through NF-κB activation.[28]

NF-κB is a transcription factor that is involved in inflammation,[29] cell proliferation,[30] angiogenesis,[31] and apoptosis.[32] NF-κB is constitutively activated in gastric carcinoma and NF-κB activation is related to the invasive ability of carcinoma cells.[33] In most unstimulated, normal cells, NF-κB is present in the cytoplasm as an inactive heterodimer composed of the p50, p65, and IκBα subunits. After activation, IκBα undergoes phosphorylation and ubiquitination dependent degradation by the

proteasome. Consequently, nuclear localization signals on the p50-p65 heterodimer are exposed, leading to nuclear translocation and binding to a specific consensus sequence that activates gene transcription, including genes encoding inflammatory cytokines, chemokines, growth factors, cell adhesion molecules, and cytokine receptors.[34] NF-κB activation is one of the mechanisms underlying Shh overexpression in pancreatic cancer and that proliferation of pancreatic cancer cells is accelerated by NF-κB activation in part through Shh overexpression.[13] To examine whether *H. pylori* induce Shh protein via activation of NF-κB, we used proteasome inhibitor MG-132 and PDTC. PDTC is a specific inhibitor of NF-κB and belongs to an antioxidant. It functions through two pathways, one is inhibiting NF-κB p65 protein activation, another is reducing NF-κB nuclei translocation by inhibiting degradation of κB inhibitory molecule. When the concentration of MG-132 was higher than 2 μM, MG-132 could inhibit expression of Shh protein induced by *H. pylori.* and 50 μM PDTC could inhibit expression of Shh protein via activation of NF-κB. Together with these results, our data raised a possibility that CagA induced Shh protein through NF-κB activation.

The Hh signaling pathway is a tale of two transmembrane proteins Patched (Ptc1), a twelve-pass membrane protein binds Hedgehog ligand. Smoothened (Smo), a seven-pass membrane protein is a signal transducer. It had been shown that Shh correlated with a reduction in the level of Ptc1 protein. In the absence of ligand, Ptc interacts with and inhibits Smo, either directly or indirectly. This repression culminates in a transcription factor acting as a transcriptional repressor. The transcription factor is called Cubitus interruptus (Ci) in Drosophila and Gli in vertebrates. Cells that receive the Hh signal, in contrast, express these genes because Ptc cannot function. The relative

levels of opposing Hh and Ptc activities thus control where and when target genes are expressed. Interestingly, *ptc* itself is nearly always induced by Hh.[35] Therefore, Hh signaling has the paradoxical effect of both blocking Ptc protein function and activating *ptc* gene expression. Shh elevates *ptc1* expression in most tissues, confirming that *ptc1* is an excellent indicator of Hh signaling.[36] Conversely, persistent and high-level Ptc expression from a transgene expressed throughout the neural tube inhibits transcription of endogenous *ptc1*.[37] Our data showed that Ptc1 protein and mRNA were reduced in AGS cell infected with 60190. The detailed mechanisms that down-regulation of Ptc expression by *H. pylori* infection remains to be investigated.

In the present study, we showed that the expression of Shh in gastric epithelial cells infected by *H. pylori* was increased which were confirmed by immunoblot and real time RT-PCR. Also, CagA positive strains showed higher level of Shh expression by *H. pylori* infected gastric cancer cells. Gli1 mRNA and protein were increased in expression in cells infected with *H. pylori* implying the activation of Shh signal pathway in gastric cancer cells. *H. pylori* infection contributes to Hh pathway activation through up-regulation of Shh expression in gastric cancer cells. We demonstrated a positive correlation between *H. pylori* infection and Shh expression in clinical tissue samples. Cells infected with *H. pylori* expressed higher levels of Shh and Gli1 than the uninfected control cells. These finding indicate that *H. pylori*-induced overexpression of Shh activated the Hh pathway in gastric cancer cells in a ligand-dependent manner. The reduction in Shh protein expression by inhibitors of NF-κB signal pathway suggest the possible role of NF-κB pathway in Shh expression by *H. pylori* infected gastric cancer.

In conclusion, Shh expression correlates with the status of *H. pylori* infection and *H. pylori* may activate the Hh signal pathways through up-regulation of Shh in gastric epithelial cells. CagA may influence the expression of Shh in gastric epithelial cells. This novel finding that *H. pylori* infection influences Shh expression improves our understanding of the mechanism of Hh signaling activation in gastric cancer.

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Abstract (In Korean)

위암상피세포에서 Helicobacter pylori 감염에 따른 Sonic hedgehog 신호 조절

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최 연 정

최근 암의 발생과정은 분화가 끝난 세포에서 생기는 것이 아니라 각 장기에 위치한 성체 줄기세포에서 생긴다는 보고들이 나오면서 정상적인 분화에 관여되는 신호전달체계에 관심을 가지게 되었으며, 여러 연구들을 통하여 정상적인 분화에 관여되는 신호전달과정이 발암과정에 관여됨이 보고 되었다. 이중 대표적인 것이 Hedgehog (Hh) 신호전달과정이다. 만성적인 자극이 발생, 분화에 관여하는 신호체계를 비정상적으로 자극한다는 개념에서 발암과정에 관여된다는 관점이나, 실제로 이의 기전자체를 조사한 연구들은 거의 없다. 따라서 이번 연구과제에서는 위의 대표적 만

성자극중의 하나인 *Helicobacter pylori* (*H. pylori*) 감염이 위암발생에 있어 Hh 신호전달과정에 미치는 영향을 밝힘으로써 성체 줄기세포에서의 암기원에 대한 기전 연구에 일익을 담당하고자 하였다. 위암상피세포주 AGS, MKN45 각각에 여러 종류의 *H. pylori* 감염시켰다. *H. pylori* 균주로는 60190 (*cag*PAI positive), 8822 (*cag*PAI negative) 그리고 60190 균주에 *cagA, cagE* 유전자를 각각 knock-out 시킨 변이된 균주도 사용하였다. *H. pylori* 감염 후 위암상피세포주에서 Shh, Ptc1 과 Gli1의 전사수준에서의 조절과 단백질의 발현정도를 관찰하기 위해 Real time RT-PCR과 western blotting을 수행하였다. *in vivo* 연구로는 *H. pylori* 감염이 확인된 위암환자에서 추출한 위점막조직을 이용하여 면역화학염색 실시하였고, *H. pylori* 감염여부에 따른 발현의 변화를 분석하였다. Western blotting과 Real time RT-PCR의 결과를 통해 *H. pylori* 감염이 Shh의 발현을 증가시키는 것을 알 수 있었다. 게다가 *cagA* positive 균주가 Shh의 발현을 더욱 증가시키는 것이 관찰되었다. Hh 신호전달과정에서 transcriptional target인 Gli1의 단백질과 mRNA가 *H. pylori* 감염시킨 후 증가하였다. 이는 위암상피세포에서 *H. pylori* 감염이 Hh 신호전달과정을 활성화 시킨다는 것을 의미한다. *in vivo* 연구에서 위암환자로부터 얻은 위점막조직의 면역화학염색 결과, *H. pylori* 감염 된 환자의 조직에서 *H. pylori* 감염이 되지 않은 환자의 조직보다 Shh와 Gli1의 발현이 높았다. 이번 연구의 결과로 *H. pylori* 감염과 Shh 발현은 상호연관성이 있는 것으로 밝혀졌다. *H. pylori* 감염은 위암상피세포주에서 Shh 발현을 증가시킴으로서 Hh 신호전달과정을 활성화 시킨다. 그리고 *H. pylori*의 중요단백인 CagA가 위암상피세포주에서 Shh 발현에 영향을 미친다는 것을 알 수 있었다.

핵심되는 말 : 위암, Hedgehog 신호조절, *Helicobacter. pylori*