**Helicobacter pylori Eradication Downregulates Cellular Inhibitor of Apoptosis Protein 2 in Gastric Carcinogenesis**

Hyuk Yoon, Sang Gyun Kim, Bo Kyoung Kim, Eun Shin, Nayoung Kim, Hyuk-Joon Lee, Gyeong Hoon Kang, and Hyuk Chae Jung

1Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, 2Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, 3Department of Pathology, Seoul National University Bundang Hospital, Seongnam, Departments of 4Surgery and 5Pathology, Seoul National University College of Medicine, Seoul, Korea

**Background/Aims:** To evaluate the expression of cellular inhibitor of apoptosis protein 2 (cIAP2) during gastric carcinogenesis after Helicobacter pylori (HP) infection and after HP eradication. **Methods:** We divided non-cancer patients into four groups according to the status of HP infection and atrophic gastritis (AG)/intestinal metaplasia (IM). We compared cIAP2 mRNA expression among these four groups and patients with HP-positive early gastric cancer (EGC) by using real-time polymerase chain reaction (PCR). We evaluated the expression of cIAP2 messenger RNA (mRNA)/protein by using real-time PCR/immunohistochemistry and the degree of apoptosis with a terminal deoxynucleotidyl transferase-mediated nick end labeling assay before and 12 months after endoscopic submucosal dissection (ESD) in HP-positive EGC patients, regardless of whether they had undergone eradication therapy. **Results:** The expression of cIAP2 mRNA was significantly higher in the groups with HP(+), AG/IM(+), and HP-positive EGC than in the control, HP(+), and AG/IM(−) groups (p<0.005). In the HP eradication group, the expression of cIAP2 mRNA/protein significantly decreased (p=0.006) and apoptosis increased at the 12-month follow-up after ESD. In the HP noneradication group, the aforementioned changes were not found during the same follow-up period. **Conclusions:** The expression of cIAP2 increased during gastric carcinogenesis after HP infection; HP eradication in the patients who had undergone ESD for EGC reversed overexpression of cIAP2 and suppressed cell apoptosis. (Gut Liver 2017;11:79-86)

**Key Words:** Helicobacter pylori; Cellular inhibitor of apoptosis protein 2; Gastric carcinogenesis

**INTRODUCTION**

Inhibitor of apoptosis protein (IAP) is defined as a protein containing one or more repeats of the baculovirus IAP domain. IAP is one of the major proteins controlling apoptosis, which plays an important role in both normal development and diseases. Until now, eight types of IAP family proteins have been identified. Among these, cellular inhibitor of apoptosis protein 2 (cIAP2) is known to play an important role in carcinogenesis. cIAP2 is induced by nuclear factor-κB (NF-κB), and is reported to inhibit apoptosis by downregulating certain caspases. Overexpression of cIAP2 has been reported in colon and pancreatic cancer. However, studies investigating the role of cIAP2 in gastric carcinogenesis are scarce.

Causing an imbalance between cell proliferation and apoptosis is one of several mechanisms through which Helicobacter pylori induces gastric cancer. H. pylori is known to either activate or inhibit apoptosis depending on the degree, duration, and nature of infection. For example, H. pylori expressing CagA and VacA stimulates apoptosis in gastric epithelial cells. Proapoptotic genes were overexpressed in H. pylori-infected patients, and apoptosis increased in the early stage of gastric carcinogenesis due to H. pylori infection. Conversely, it was also reported that H. pylori can inhibit apoptosis. It is known that H. pylori activates NF-κB in a Cag pathogenicity island-dependent manner and subsequently induces the up-regulation of cIAP2 expression. Expression of both NF-κB and cIAP2 increased when gastric cancer cell lines were cocultured with CagA-positive H. pylori. Conversely, knockdown of cIAP2 in gastric cancer cell lines resulted in increased apoptosis, decreased cell proliferation, and delayed cell migration. In mouse models, H. pylori activates NF-κB, and cIAP2 increases, leading to increased apoptosis and decreased cell proliferation.
pylori infection induced increased cIAP2 in the gastric mucosa. Finally, cIAP2 was reportedly overexpressed in more than 70% of human gastric cancer tissues; overexpression was more prominent in cancer tissues than in adjacent non-cancer tissues, and in H. pylori-positive patients than in H. pylori-negative patients. Therefore, it was suggested that cIAP2 overexpression plays an important role in H. pylori-induced gastric carcinogenesis. However, the exact stage at which overexpression of cIAP2 commences during progression from H. pylori infection to gastric cancer is unclear.

The first aim of this study was to evaluate cIAP2 overexpression during human gastric carcinogenesis after H. pylori infection in the context of atrophic gastritis (AG) and intestinal metaplasia (IM). The second aim was to determine whether eradication of H. pylori in patients who underwent endoscopic submucosal dissection (ESD) for early gastric cancer (EGC) reverses cIAP2 overexpression.

MATERIALS AND METHODS

1. Subjects and study design

This study was carried out in three stages. First, we determined the expression of several IAP family genes, including cIAP2, in the gastric mucosal samples of control subjects who were H. pylori-negative and showed no evidence of AG and IM in either the antrum or the body, and in patients with H. pylori-positive EGC. In the second step, we divided non-cancer patients into four groups according to the presence or absence of H. pylori infection and AG/IM, and we compared the expression of cIAP2 mRNA among them as well as among H. pylori-positive EGC patients by real-time polymerase chain reaction (PCR). Lastly, we evaluated the expression of cIAP2 mRNA/protein and the degree of apoptosis before and 12 months after ESD in H. pylori-positive EGC patients irrespective of whether they received eradication therapy. In this stage, the expression levels of cIAP2 protein and the degree of apoptosis were analyzed by immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, respectively.

In the present study, gastric tissues of patients without EGC were acquired from subjects who underwent esophagogastroduodenoscopy (EGD) and were healthy or diagnosed with gastritis between October 2005 and October 2006. Gastric tissues of EGC patients were acquired from samples that were previously collected for another study; the summary of that study is as follows. Negative AG/IM was defined as no evidence of AG and IM in both the antrum and body. Positive AG/IM was defined as moderate or high AG or IM in either the antrum or body.

2. Gastric tissue collection

We collected two sections of gastric mucosal tissue from the lesser curvature of the antrum and body by endoscopic biopsy from all subjects; in patients with EGC, noncancerous tissues were collected. H. pylori infection status was determined by histologic evaluation and the rapid urease test, which was performed in another tissue sample from the lesser curvature of the antrum. H. pylori infection was deemed positive if at least one of two tests was positive. The degree of AG and IM in the gastric mucosa was classified according to the updated Sydney system. Negative AG/IM was defined as no evidence of AG and IM in both the antrum and body. Positive AG/IM was defined as moderate or high AG or IM in either the antrum or body.

3. Real-time PCR microarray for IAP family genes

We collected four samples each from control subjects who were H. pylori-negative and showed no AG and IM, and from subjects with H. pylori-positive EGC. We then extracted the total RNA and performed quality control tests; two samples from each group were selected. Briefly, total RNA was extracted using the RNasy midi kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and treated with RNase-free DNase I (Promega, Madison, WI, USA) to reduce DNA contamination. Total RNA concentration and purity were determined by measuring the 260:280 nm absorbance ratio using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA samples was confirmed by the appearance of distinct 28S and 18S bands of ribosomal RNA using a Bioanalyzer 2100 system (Agilent Technology, Santa Clara, CA, USA).

We then analyzed mRNA expression of several IAP family genes including cIAP2 by real-time PCR microarray. Briefly, complementary DNA (cDNA) was synthesized from 1 μg total RNA according to the manufacturer’s instructions (RT2 First Strand kit; Qiagen). For quantitative comparison of mRNA levels, real-time PCR was performed using Human Apoptosis RT Profiler™ PCR Array (Qiagen Korea Ltd., Seoul, Korea). This real-time PCR microarray consisted of a 96-well plate and included 84 genes closely related to apoptosis and cell survival, in addition to positive/negative controls. Two independent assays were performed for each condition. Gene expression was measured relative to the mean expression of β-actin as the endogenous control. Reactions were carried out on a StepOnePlus™ Real-
Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR detection was performed under the following thermal cycling conditions: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For analysis using the \( \Delta \Delta \text{CT} \) method, we used the PCR Array Data Analysis Software (SABiosciences; http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

4. Real-time PCR for cIAP2 mRNA

Gastric mucosal specimens for real-time PCR were collected from the lesser curvature of the antrum. Total RNA was extracted from the specimens using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions and purified using RNasy mini kits (Qiagen, Valencia, CA, USA).\(^{15}\) RNA samples were then treated with DNase I (Invitrogen) and quantified by spectrophotometry. Total RNA was amplified and then labeled in the presence of fluorescent dUTP (Cy3 dUTP or Cy5 dUTP; GE Healthcare, Piscataway, NJ, USA). RNA samples were diluted to a final concentration of 0.5 mg/mL in RNase-free water and stored at -80°C until use. cDNA synthesis was performed with 1 ng of total RNA with M-MLV reverse transcription reagents (Invitrogen), and real-time PCR was conducted using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Real-time PCR detection was performed under the following thermal cycling conditions: 95°C for 15 seconds and 60°C for 1 minute. The human GAPDH gene was used as an endogenous reference when determining from the Ct values using the 2\(^{-\Delta \Delta \text{CT}}\) method.\(^{16}\)

5. IHC for cIAP2 protein

Tissues from the antrum were fixed in 10% neutral buffered formalin, paraffin-embedded, and then cut into 4-μm sections. Slides were stained using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems Inc., Tucson, AZ, USA) and detection was performed using the Ventana Chromo Map Kit (Ventana Medical Systems). Sections were deparaffinized using the EZ Prep solution. CC11 (pH 8.4 buffer) for antigen retrieval. Endogenous peroxidases were blocked with Inhibitor D (3% H\(_2\)O\(_2\)) for 4 minutes at 37°C temperature. Slides were incubated with primary antibodies for 32 minutes at 37°C and a secondary antibody for 20 minutes at 37°C. Slides were incubated in diaminobenzidine (DAB) plus H\(_2\)O\(_2\) substrate for 8 minutes at 37°C followed by hematoxylin and bluing reagent counterstain at 37°C. Reaction buffer (pH 7.6 Tris buffer) was used as a washing solution. A monoclonal antibody for cIAP2 (Santa Cruz Biotechnology; Santa Cruz, CA, USA; dilution 1:30) was used as a primary antibody, and biotin-labeled anti-mouse immunoglobulin G (UltraMap anti-Ms HRP Roche; Ventana Medical Systems, Inc.) was used as a secondary antibody. Negative controls were treated similarly while excluding the primary antibodies. The whole slide area was assessed and positive staining was qualitatively evaluated by an experienced pathologist (E.S.).

6. TUNEL assay

To evaluate apoptosis, TUNEL staining was performed using the Apoptag® Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore Corp., Billerica, MA, USA).\(^{19}\) After deparaffinization with xylene and graded concentrations of alcohol, gastric mucosal tissue sections were exposed to Proteinase K for 15 minutes at room temperature. Endogenous peroxidase activity was quenched with Inhibitor D for 5 minutes at room temperature. Sections were incubated with terminal deoxynucleotidyl transferase (TdT) in a humidified chamber at 37°C for 1 hour. After incubation with anti-digoxigenin-conjugate for 30 minutes at room temperature, peroxidase substrate and 0.05% DAB was applied to develop color. The specimens were then washed with distilled water and counterstained with 0.5% methyl green for 10 minutes at room temperature. Sections were counterstained with Mayer’s hematoxylin. Identically-treated slides not exposed to TdT served as negative controls. In all cases, positive staining was qualitatively evaluated on the entire slide by an experienced pathologist (E.S.).

7. Statistical analysis

SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Because the data were not normally distributed (as demonstrated by the Shapiro-Wilk test), the relative expression of cIAP2 mRNA among several groups was compared using a Kruskal-Wallis test. Post hoc comparisons of pairwise differences between two groups were evaluated by the Mann-Whitney U test using the modified Bonferroni procedure for multiple comparison adjustment. To compare the expression levels of cIAP2 mRNA in H. pylori-positive EGC before and at 12 months after ESD, a Wilcoxon signed-rank test was performed. Results with p-values less than 0.05 were considered significant.

RESULTS

1. Real-time PCR microarray for IAP family genes

Fig. 1 shows the heat map of PCR microarray. Among the five IAP family genes analyzed (Survive, cIAP1, cIAP2, XIAP, and NAIP), only the cIAP2 (BIRC3) mRNA was 1.85 times higher in subjects with H. pylori-positive EGC than in H. pylori-negative controls without AG/IM.
2. Real-time PCR for cIAP2 mRNA

We performed cIAP2 mRNA real-time PCR for 22 samples in each group. However, the results of several samples were not interpretable, and were thus excluded. Therefore, the number of samples in each group varied, ranging between 18 and 22 (normal control, both H. pylori and AG/IM-positive group; H. pylori-positive early gastric cancer compared with the expression in H. pylori-positive gastric mucosal tissues by real-time PCR. There was a significant difference in the relative expression of cIAP mRNA among the five groups (p<0.001). Post hoc analysis showed that the expression of cIAP2 mRNA was significantly higher in patients positive for both H. pylori and AG/IM as well as in H. pylori-positive EGC patients than in control patients and those positive for H. pylori but negative for AG/IM (p<0.005).

3. Effect of H. pylori eradication on cIAP2 expression and apoptosis

The effect H. pylori eradication on cIAP2 expression and apoptosis in the EGC patients after ESD was evaluated in 27 subjects (eradicaton group, n=12; noneradication group, n=15). Fig. 3 shows the expression of cIAP2 mRNA by real-time PCR in patients with H. pylori-positive EGC before ESD and at 12 months following the procedure. The cIAP2 mRNA levels before ESD were not significantly different between the eradication and noneradication groups (p=0.354). In the eradication group, however, there was no difference in the expression of cIAP2 mRNA before and 12 months after ESD (p=0.14) (Fig. 3B). Fig. 4 shows representative IHC staining for cIAP2 protein in patients with H. pylori-positive EGC before ESD and at 12 months following the procedure. In the eradication group, the expression of cIAP2 protein decreased at the 12-month follow-up.
up time (Fig. 4B). In the noneradication group, there was no difference in the expression of cIAP2 protein before ESD and 12 months after (Fig. 4D). Fig. 5 shows representative TUNEL staining in patients with H. pylori-positive EGC before and at 12 months after ESD. In the eradication group, cell apoptosis had increased 12 months following the ESD procedure (Fig. 5B). In the noneradication group, there was no difference in cell apoptosis before and 12 months after ESD (Fig. 5D).

**DISCUSSION**

Previous investigations of the relationship between H. pylori infection and cIAP2 expression during gastric carcinogenesis were performed in gastric cancer cell lines cocultured with H. pylori or in a mouse model of H. pylori infection. In this study, we explored the role of cIAP2 in H. pylori-induced gastric carcinogenesis in humans. We found that overexpression of cIAP2 during gastric carcinogenesis requires not only H. pylori infection, but also progression to AG/IM. These results are consistent with previous reports showing that H. pylori has both apoptotic and antiapoptotic effects. When H. pylori infection becomes chronic, antiapoptotic signaling pathways becomes predominant. Moreover, the expression of cIAP2 mRNA mildly decreased in patients with negative H. pylori infection and positive AG/IM compared to control patients. This indicates that progression of AG/IM is not sufficient to induce overexpression of cIAP2 mRNA in gastric carcinogenesis; H. pylori infection is also required. Although we could not perform additional experiments to clarify the ring connecting H. pylori infection, AG/IM, and expression of cIAP2, we postulate that activation of NF-κB by CagA may be the primary mechanism for the development of H. pylori-specific gastric carcinogenesis.

Several studies reported that eradication of H. pylori after ESD in patients with EGC decreases the incidence of metachronous gastric cancer. However, the underlying molecular mechanism has not been widely explored. Tsai et al. reported that several genes related to cell proliferation were down-regulated in the gastric mucosa 1 year after H. pylori eradication. In the present study, we found that cIAP2 overexpression and apoptosis suppression could be reversed within 1 year after H. pylori eradication in patients who underwent ESD for EGC. Taken together, our results suggest that risk reduction of metachronous
gastric cancer by *Helicobacter pylori* eradication after ESD in EGC patients may be related to changes in the expression of genes controlling cell proliferation and apoptosis.

There are two distinct apoptotic pathways: a death receptor (extrinsic) pathway and a mitochondrial (intrinsic) pathway. Some reports claim that overexpression of cIAP2 inhibits apoptosis through the suppression of caspases 3, 7, and 9 activity. Others suggest that the underlying mechanism involves the suppression of caspase 8 activity. In this study, several caspase genes were probed with a real-time PCR microarray. We found that caspases 3, 8, and 9 were suppressed but caspase 7 was slightly overexpressed in subjects with *H. pylori*-positive EGC compared to that in *H. pylori*-negative individuals without AG/IM. These findings were somewhat counterintuitive. Moreover, Bcl2 and Bax, which play opposing roles in the intrinsic pathway, were equally overexpressed in *H. pylori*-positive EGC patients. Because we examined the expression of general apoptosis-related genes in a very limited number of samples, and the interactions of the intrinsic and extrinsic apoptosis pathways are complex, we could not determine whether Bax was overexpressed to counteract the overexpression of Bcl2. Further experiments are required to expose the pathways key to *H. pylori*-infected and cIAP2-induced gastric carcinogenesis.

This study had several limitations. First, we did not determine the status of CagA in the *H. pylori* strains in our study; hence, it was not possible to ascertain whether only CagA-positive *H. pylori* induces cIAP2 overexpression during gastric carcinogenesis. Moreover, lack of assessment of other virulent factors such as VacA and OipA, which are frequently found in Asian strains, is also a limitation. Second, because there was a limit to the number of available tissues from noncancerous subjects, we could not evaluate the effects of AG and IM on the expression of cIAP2 separately. Although evidence suggested that cIAP2 was overexpressed at a relatively late stage during *H. pylori*...
induced gastric carcinogenesis, the specific stage at which cIAP2 is overexpressed remains unknown. Finally, we did not examine more potent anti-apoptotic factors such as Bcl2-family member Mcl1.\textsuperscript{28} We also did not evaluate the status of tumor necrosis factor-related apoptosis-inducing ligand signaling and cIAP2-counteracting molecules such as Smac/DIABLO and Omi/Htr2A.\textsuperscript{29} Therefore, it remains uncertain whether H. pylori eradication-mediated cIAP2 downregulation alone induces apoptosis in the eradication group, as other signaling events may be involved.

In conclusion, the expression of cIAP2 increased during gastric carcinogenesis after H. pylori infection and progression to AG/IM, and eradication of H. pylori in the patients who underwent ESD for EGC reversed the overexpression of cIAP2 and suppressed cell apoptosis.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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