ORIGINAL ARTICLE

Effects of *Helicobacter pylori* γ-Glutamyltranspeptidase on Apoptosis and Inflammation in Human Biliary Cells

Wongwarut Boonyanugomol · Chariya Chomvarin · Jea-Young Song · Kyung-Mi Kim · Jung-Min Kim · Myung-Je Cho · Woo-Kon Lee · Hyung-Lyun Kang · Kwang-Ho Rhee · Banchob Sripa · Chariya Hahnvajanawong · Seung-Chul Baik

Abstract

Background Several studies have reported the presence of *H. pylori* in individuals with hepatobiliary diseases, but in vitro and in vivo studies are still needed. Here, we determined the effects of *H. pylori* γ-glutamyltranspeptidase (GGT) on the induction of apoptosis and IL-8 production in a human cholangiocarcinoma cell line (KKU-100 cells).

Methods Cell viability and DNA synthesis were examined by MTT and BrdU assays, respectively. RT-PCR and western blot analysis were performed to assess gene and protein expression, respectively. IL-8 secretion in KKU-100 cells was measured by ELISA.

Results Exposure to the *H. pylori* ggt^+ strain decreased KKU-100 cell survival and DNA synthesis when compared with cells exposed to the *H. pylori* ggt mutant strain. Treatment with recombinant *H. pylori* GGT (rHP-GGT) dramatically decreased cell survival and DNA synthesis, and stimulated apoptosis; these features corresponded to an increased level of iNOS gene expression in KKU-100 cells treated with rHP-GGT. RT-PCR and western blot analyses revealed that rHP-GGT treatment enhanced the expression of pro-apoptotic molecules (Bax, Caspase-9, and Caspase-3) and down-regulated the expression of anti-apoptotic molecules (Bcl-2 and Bcl-xL). The extrinsic-mediated apoptosis molecules, including Fas and activated Caspase-8, were not expressed after treatment with rHP-GGT. Furthermore, rHP-GGT significantly stimulated IL-8 secretion in KKU-100 cells.

Conclusion Our data indicate that *H. pylori* GGT might be involved in the development of cancer in hepatobiliary cells by altering cell kinetics and promoting inflammation.

Keywords *H. pylori* · γ-Glutamyltranspeptidase · Apoptosis · Hepatobiliary diseases · Cholangiocarcinoma

W. Boonyanugomol · C. Chomvarin · C. Hahnvajanawong
Department of Microbiology, Faculty of Medicine,
Khon Kaen University, Khon Kaen 40002, Thailand

W. Boonyanugomol · C. Chomvarin · B. Sripa ·
C. Hahnvajanawong
Liver Fluke and Cholangiocarcinoma Research Center,
Khon Kaen University, Khon Kaen 40002, Thailand

J.-Y. Song · K.-M. Kim · J.-M. Kim · M.-J. Cho · W.-K. Lee ·
H.-L. Kang · K.-H. Rhee · S.-C. Baik (✉)
Department of Microbiology, Gyeongsang National University
School of Medicine, Chiram-dong 90, Jinju,
South Gyeongsang 660-751, Republic of Korea
e-mail: scbaik@gaechuk.gsnu.ac.kr

J.-Y. Song · K.-M. Kim · J.-M. Kim · M.-J. Cho · W.-K. Lee ·
H.-L. Kang · K.-H. Rhee · S.-C. Baik
Research Institute of Life Science, Gyeongsang National
University School of Medicine, Chiram-dong 90, Jinju,
South Gyeongsang 660-751, Republic of Korea

B. Sripa
Department of Pathology, Faculty of Medicine,
Khon Kaen University, Khon Kaen 40002, Thailand

Introduction

Cholangiocarcinoma (CCA) is a fatal cancer of bile duct cells and has a high incidence in northeast Thailand [1]. Liver fluke infection caused by *Opisthorchis viverrini* has been reported to be associated with CCA [2]. However, the mechanism of liver fluke-induced CCA is unclear and requires investigation. Recently, an increasing incidence of CCA has been reported in western countries where there is a low prevalence of liver fluke infection [1]. We hypothesized that other factors may be involved in the

development of CCA. To date, bacteria in the hepatobiliary tract have been proposed as synergistic agents that may be involved in the development of liver diseases [3].

Helicobacter pylori is a well-known pathogen that causes gastrointestinal diseases, especially gastric cancer [4]. Recent reports demonstrate the presence of *Helicobactor pylori*, or *H. pylori*-like DNA, or even other *Helicobacter* species such as *H. hepaticus* or *H. bilis*, in human hepatobiliary specimens [5–8]. These studies suggest a possible involvement of *Helicobacter* species, especially *H. pylori*, in the progression of liver disease [3, 9]. In addition, our previous study found a high incidence of *H. pylori* in hepatobiliary patients, particularly CCA patients, which was found to be significantly higher than the benign and control groups [10]. We suggest that *H. pylori* may play a role or may be a co-factor in the development of hepatobiliary malignancy, especially CCA [10].

Several studies have reported that *H. pylori* virulence factors induce apoptosis in gastric cell lines [11–13], and that apoptosis induced by *H. pylori* plays a crucial role in the development of pathological outcomes in the gastrointestinal tract [14]. In addition, IL-8 production is significantly associated with *H. pylori* infection and correlates with the severity of the disease [15]. A novel protein of *H. pylori*, γ-glutamyltranspeptidase (GGT), has been shown to induce apoptosis in the AGS gastric cell line, indicating a mechanism of GGT involvement in the pathogenesis of *H. pylori* [16].

However, a mechanistic in vitro study of *H. pylori* in hepatobiliary cells has been rarely performed [17, 18], and the specific role of *H. pylori* GGT in hepatobiliary cells has not been investigated. Therefore, the aim of this study was to determine whether *H. pylori* GGT was involved in apoptosis and inflammation in a CCA cell line. We constructed and purified recombinant *H. pylori* GGT (rHP-GGT) to clarify the mechanism of the involvement of this protein in CCA cells.

Materials and Methods

H. pylori Strains and Recombinant *H. pylori* GGT

The *H. pylori* ggt wild-type strain (ggt^+), ggt isogenic mutant strain (ggt^-), and recombinant *H. pylori*-GGT protein (rHP-GGT) were deposited in and supplied from the *H. pylori* Korean Type Culture Collection (Gyeongsang National University School of Medicine). The process of the *H. pylori* ggt mutant construction, GGT protein expression in *E. coli*, and GGT purification were previously reported by Kim et al. [19].

Cell Culture

The human cholangiocarcinoma cell line (KKU-100) was obtained from the Liver Fluke and Cholangiocarcinoma Research Center (Khon Kaen University, Thailand). KKU-100 cells were cultured in Ham F-12 medium supplemented with 10 % FBS, streptomycin (100 µg/ml) and penicillin (1 IU/ml), and incubated at 37°C in a 5 % CO_2 humidified atmosphere.

Cell Viability by MTT Assay

KKU-100 cells were cultured in 96-well plates at a density of 8×10^4 cells/well. After 24 h, cells were co-cultured with *H. pylori* ggt^+ or ggt^- strains, or with/without purified rHP-GGT. Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] assay. Treated and control cells were washed with PBS, and 0.5 µg/ml of MTT was added to each well. Cells were then incubated at 37 °C for 4 h. After this time period, the cell supernatant was removed, formazan crystals were dissolved with DMSO, and the optical density was measured at 520 nm using a spectrophotometer.

DNA Synthesis by BrdU Assay

The KKU-100 cells (8×10^4 cells/well) were cultured in 96-well plate and allowed to adhere overnight. Then, cells were exposed with *H. pylori* ggt^+ and ggt^- strains, and purified rHP-GGT. The treated cells were incubated with BrdU labeling solution at 37 °C for 4 h. After removal of the medium, cells were fixed and denatured as previously described [18]. Cells were incubated with anti-BrdU at room temperature for 1 h and then with a secondary antibody conjugated to HRP for 30 min. Finally, tetramethylbenzidine was added, and the optical density of the chromogenic substrate was measured at 520 nm.

Determination of Apoptotic Cell Death

Nuclear staining by DAPI was used to determine chromatin condensation and fragmentation. After KKU-100 cells were co-cultured with *H. pylori* ggt^+ or ggt^- strains (both at MOI of 100), and with or without rHP-GGT (4.5 µg/ml) for 12 and 24 h, treated cells were washed three times with PBS and fixed in methanol for 15 min. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) for 15 min at 37 °C. Nuclei were visualized under a fluorescence microscope (Olympus, Japan) at ×400 magnification. Apoptotic cells were enumerated by counting a total of 500 cells and represented as a percentage of apoptotic cells.

DNA fragmentation of apoptotic cells was assessed by agarose gel electrophoresis. After cells were treated with rHP-GGT (4.5 μg/ml), cells were washed with PBS, and DNA was extracted using a QIAamp® DNA Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. Treated cells were lysed in lysis buffer containing 10 mg/ml proteinase K and 100 mg/ml RNase A. After DNA precipitation, DNA was transferred to a mini-spin column and washed with washing buffer. Finally, DNA was eluted and electrophoresed in a 1.5 % agarose gel. DNA bands were visualized using a UV illuminator (BioRad, USA).

Gene Expression by RT-PCR

After treatment with rHP-GGT (4.5 μg/ml) for 6, 12, and 24 h, cells were harvested and washed with PBS. RNA was isolated from treated and untreated control cells using TRizol® reagent (Invitrogen, NY, USA) according to the manufacturer's instructions. RNA quantity and purity were measured using a spectrophotometer.

Two micrograms of RNA were reverse transcribed in a 20-μl master mix containing 1× reaction buffer, 0.5 mM dNTPs, 10 mM dithiothreitol (DTT), 40 U RNase inhibitor, 1 pmol random primers, and 200 U of Moloney murine leukemia virus (MMLV) reverse transcriptase at 42 °C for 1 h.

Gene expression of *iNOS*, *p53*, *bax*, *bcl-2*, *caspase-3*, and *GAPDH* was determined as previously described [20–22]. Complementary DNA was amplified in 20 μl of reaction mixture containing reaction buffer (1×), 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 1 pmol each primer, and 1 U *Taq* DNA polymerase. The PCR primers used are shown in Table 1. The cycling conditions were as follows: *iNOS* (94 °C 1 min, 60 °C 1 min, 72 °C 1 min, 35 cycles); *caspase-3* (94 °C 1 min, 68 °C 1 min, 72 °C 1 min, 35 cycles); and *bax*, *bcl-2*, *p53*, and *GAPDH* (94 °C 1 min, 55 °C 1 min, 72 °C 1 min, 35 cycles). The PCR products were run on agarose gels and visualized under UV light after staining with ethidium bromide. The intensity of the PCR product bands was measured using Quantity One software, v.4.6.2 (Bio-Rad, USA). The intensity of the PCR product band was normalized to the intensity of the *GAPDH* band, and data were expressed as the fold change compared with the control (intensity of the PCR product band of rHP-GGT-treated cells divided by intensity of the PCR product band of untreated cells).

Western Blotting

KKU-100 cells were treated with rHP-GGT (4.5 μg/ml) at 6, 12 and 24 h and then harvested and lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, and 1 mM EDTA). Cell lysates were centrifuged at 13,000 rpm at 4 °C for 10 min, and the supernatants were collected. Twenty micrograms of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with nonfat skim milk and incubated overnight at 4 °C with primary antibodies against Bax, Bcl-2, Bcl-xL, Fas, activated caspase-8, β-actin (Santa Cruz Biotechnology, CA, USA), activated caspase-9, and caspase-3 and cleaved PARP (Cell Signaling Technology, MA, USA). HRP-conjugated secondary antibodies were then added, and the immunocomplex was visualized using the enhanced chemiluminescence (ECL) detection system (Thermo Scientific, IL, USA). The intensity of the protein bands was quantified using Quantity One software, v.4.6.2 (Bio-Rad). The intensity of protein bands was normalized to the intensity of β-actin, and data were expressed as the fold change compared with the control (intensity of the protein

Table 1 Primer sequences for RT-PCR reactions

Genes	Primer sequences	Product size (bp)	References
iNOS	5′-CGGTGCTGTATTTCCTTACGAGGCGACGAAGG-3′ 5′-GGTGCTGCT TGT TAGGAGGTCAAGTAAAGGGC-3′	259	[20]
p53	5′-TTCTTGCATTCTGGGACAGCC-3′ 5′-GCCTCATTCAGCTCTCGGAAC-3′	650	[20]
bax	5′-TGGCAGCTGACATGTTTTCTGAC-3′ 5′-CGTCCCAACCACCCTGGTCT-3′	195	[20]
bcl-2	5′-CTGTACGGCCCCAGCATGCG-3′ 5′-GCTTTGTTTCATGGTACATC-3′	231	[21]
caspase-3	5′-TTCAGAGGGGATCGTTGTAGAAGTC-3′ 5′-CAAGCTTGTCGGCATACTGTTTCAG-3′	264	[22]
GAPDH	5′-CGGAGT CAACGGATT TGGTCGTAT-3′ 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′	306	[20]

band of rHP-GGT-treated cells divided by intensity of the protein band of untreated cells).

IL-8 Production by ELISA

KKU-100 cells were treated with *H. pylori ggt*[+] or *ggt*[−] strains (both at MOI of 100) and with or without rHP-GGT (4.5 µg/ml) for 1, 3, 6, 12, and 24 h, and the supernatant was collected and centrifuged at 7,000 rpm for 10 min to remove unattached bacteria. IL-8 protein levels were measured using an IL-8 ELISA (BioSource International, CA, USA) according to the manufacturer's instructions. Cells in Ham F-12 medium without *H. pylori* or rHP-GGT were used as a control. The absorbance value was calculated by comparison with a standard curve of IL-8 production.

Statistical Analysis

The results are expressed as the means ± SE. A two-tailed Student's *t* test was used for statistical analysis, and *p* values <0.05 were considered significant.

Results

Effects of *H. pylori* GGT on KKU-100 Cell Viability and DNA Synthesis

After co-culture with *H. pylori ggt*[+] and *ggt*[−] strains for 24 h, the percentage of viable KKU-100 cells and DNA synthesis significantly decreased in a bacterial dose-dependent manner when compared with untreated control cells. Starting at MOI of 80 and 100, the *H. pylori ggt*[+] strain exhibited a greater inhibitory effect on KKU-100 cell viability and DNA synthesis than the *H. pylori ggt*[−] strain (Fig. 1) ($p < 0.05$). To confirm the effect of *H. pylori* GGT on cell viability and DNA synthesis, KKU-100 cells were treated with rHP-GGT. After 24 h of treatment, rHP-GGT showed significant cytotoxicity and genotoxicity against KKU-100 cells in a dose-dependent manner (Fig. 2a, b).

Determination of Apoptosis

To further investigate the mechanism of rHP-GGT involvement in KKU-100 cell death, the typical characteristics of apoptotic cell death was assessed by DAPI staining and DNA agarose gel electrophoresis (Fig. 3). Nuclear condensation and fragmentation were observed in cells treated with both *H. pylori ggt*[+] and *ggt*[−] strains whereas control-treated cells showed normal nuclei (Fig. 3a). However, when comparing the two strains, significantly higher numbers of apoptotic cells were found in KKU-100 cells treated with the *H. pylori ggt*[+] strain than with the *H. pylori ggt*[−] strain (Fig. 4a).

To clarify the effect of *H. pylori* GGT on apoptosis, KKU-100 cells were co-cultured with rHP-GGT for 12 and 24 h. The treated cells showed chromatin condensation and fragmentation by DAPI staining (Fig. 3b, 4b) and by DNA ladder assay (Fig. 3c) when compared with the untreated control cells.

Fig. 1 Inhibition of cell growth and DNA synthesis in KKU-100 cells after 24 h of treatment with various bacterial concentrations. Cytotoxicity of the *H. pylori ggt*[+] and *ggt*[−] strains against KKU-100 cells as determined by the MTT assay (*upper*). Genotoxicity of the *H. pylori ggt*[+] and *ggt*[−] strains against KKU-100 cells as determined by the BrdU assay (*lower*). Data are presented as the mean ± SE of three independent experiments. *$p < 0.05$, between infected and control cells, #$p < 0.05$, between *H. pylori ggt*[+] and *ggt*[−] strains
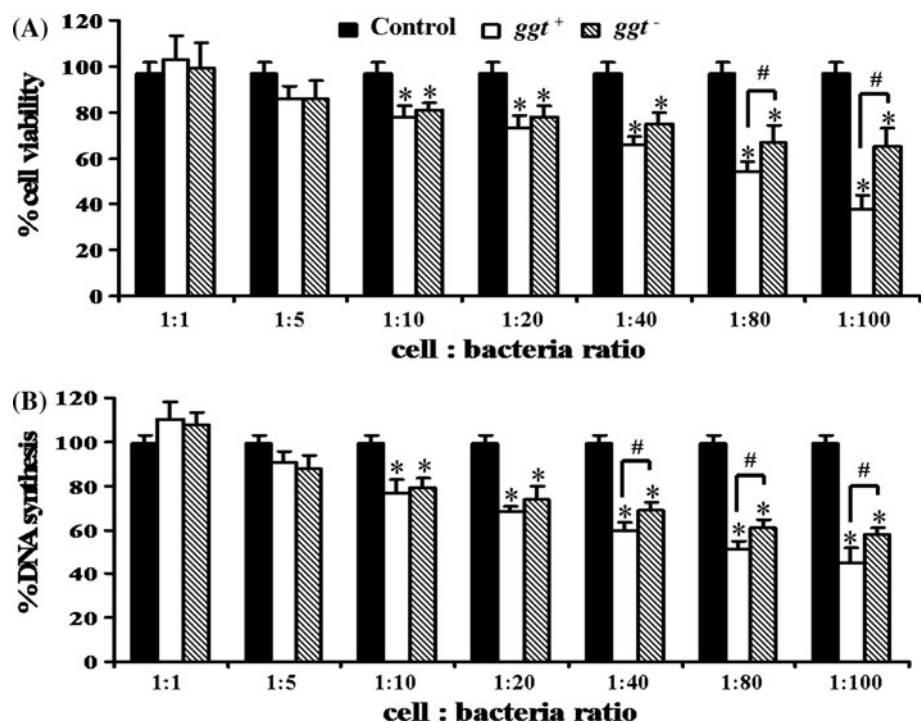
Fig. 2 Suppression of cell growth and DNA synthesis after treatment with rHP-GGT in KKU-100 cells as determined by MTT and BrdU assays, respectively. Data are presented as the mean $\pm$ SE of three independent experiments. $*p < 0.05$, between rHP-GGT-treated and untreated control cells
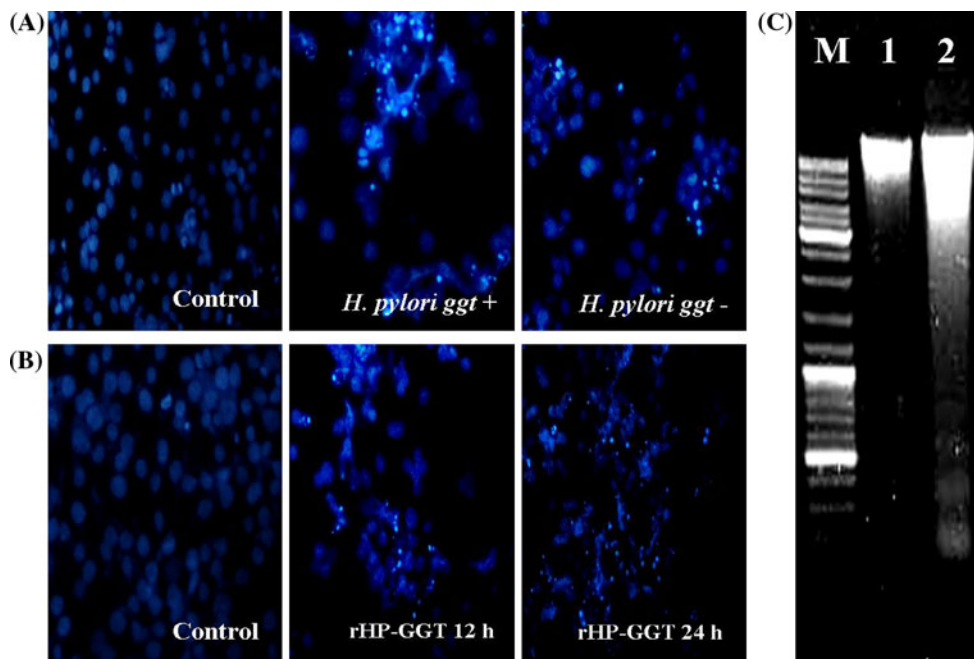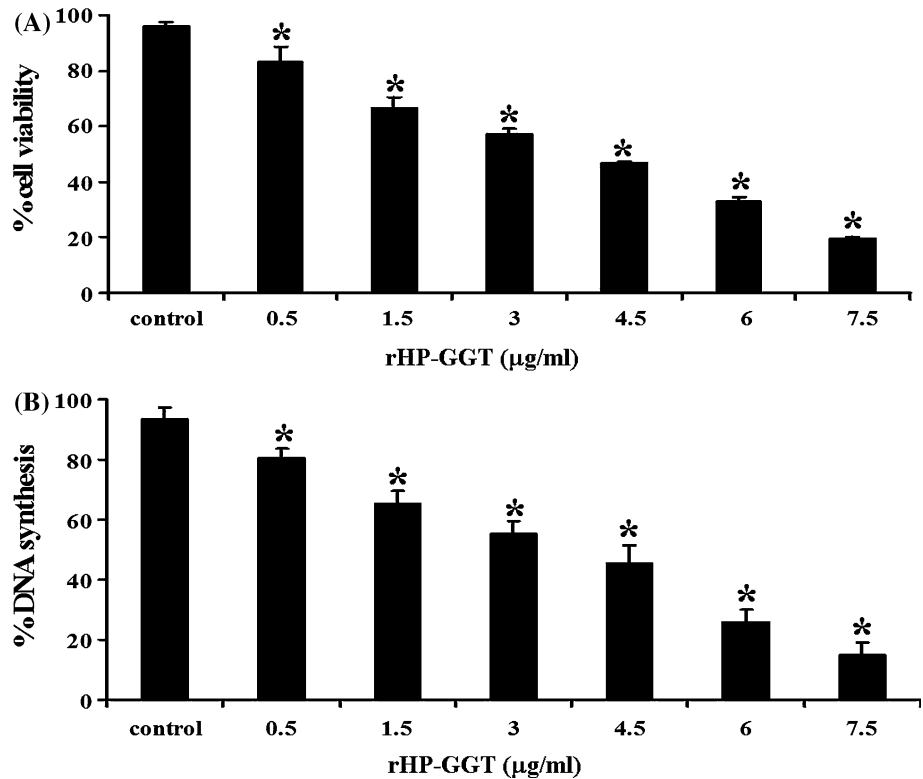

Fig. 3 Determination of apoptosis by DAPI staining in KKU-100 cells infected (24 h) with *H. pylori ggt*[+] and *ggt*[−] strains at MOI of 100 (**a**) and KKU-100 cells treated with 4.5 μg/ml of rHP-GGT (**b**). DNA laddering was determined in KKU-100 cells exposed to rHP-GGT for 24 h (**c**). *Lane M* 100 bp DNA marker; *lane 1* control cells; *lane 2* rHP-GGT-treated cells

Expression of *iNOS* and Apoptosis-Related Genes

To investigate the mechanism of *H. pylori* GGT-induced apoptosis, KKU-100 cells were treated with rHP-GGT, and

iNOS and apoptosis-related gene expression were determined. Figure 5 shows results from the RT-PCR analysis of KKU-100 cells exposed to rHP-GGT for 6, 12, and 24 h. We found that the expression of *iNOS*, *p53*, *bax*, and
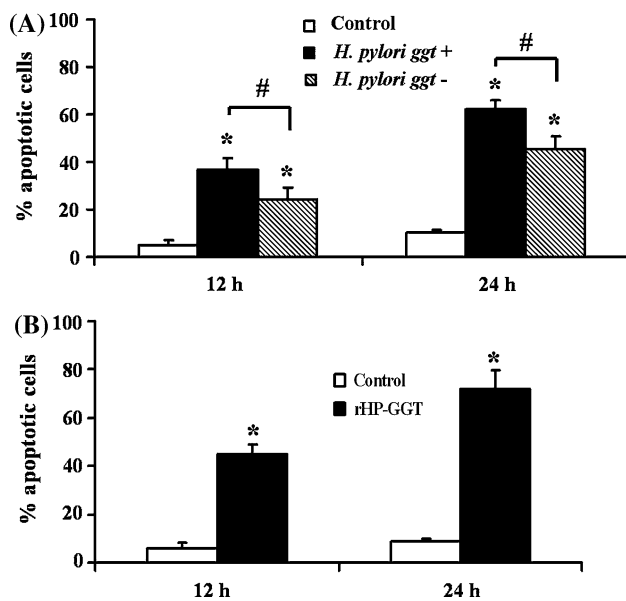
Fig. 4 Evaluation of apoptotic cells in KKU-100 cells exposed to *H. pylori ggt*⁺ and *ggt*⁻ mutant strains at MOI of 100 (**a**) and KKU-100 cells treated with 4.5 μg/ml of rHP-GGT (**b**) for 12 and 24 h. Apoptotic cell death was assessed by DAPI staining, and apoptotic cells were counted from a total of 500 cells. Data are presented as the mean ± SE of three independent experiments. *$p < 0.05$, between treated and control cells, #$p < 0.05$, between *H. pylori ggt*⁺ and *ggt*⁻ strains

Fig. 5 Inducible nitric oxide synthase (*iNOS*), *p53* and apoptosis-related gene (*bax*, *bcl2*, and *caspase-3*) expression in KKU-100 cells treated with rHP-GGT (4.5 μg/ml) for 6, 12, and 24 h as measured by RT-PCR. The density of each PCR band was measured and normalized to *GAPDH* intensity. Data (shown below each *band*) are presented as the fold change of the treated cells compared with the untreated control cells (mean ± SE) and represent three independent experiments. *$p < 0.05$, between treated and untreated control cells

caspase-3 genes was markedly up-regulated in cells treated with rHP-GGT whereas the expression of *bcl-2* was significantly down-regulated.

Expression of Apoptosis-Related Proteins

To explore the molecular mechanisms underlying purified rHP-GGT-induced apoptosis in KKU-100 cells, apoptosis-related protein levels were determined by western blotting. After KKU-100 cells were exposed to rHP-GGT for 6, 12, and 24 h, the levels of Bcl-2 and Bcl-xL were significantly down-regulated whereas the expression of Bax was up-regulated with statistical significance (Fig. 6). The active forms of caspase-9 and caspase-3 were significantly up-regulated in KKU-100-treated cells. To confirm that rHP-GGT induced apoptosis, cleaved PARP was determined. We found increasing levels of PARP in KKU-100 cells after treatment with rHP-GGT (Fig. 6). Proteins involved in the extrinsic apoptotic pathway, including Fas and activated caspase-8, were not expressed in KKU-100 cells after exposure to rHP-GGT when compared with the untreated control cells (Fig. 7).

IL-8 Production

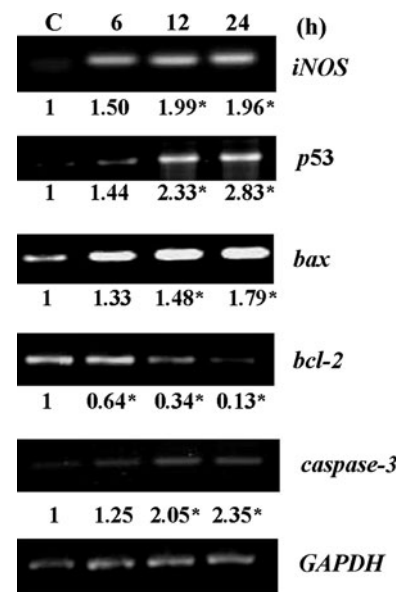After KKU-100 cells were exposed to both *H. pylori ggt*⁺ and *ggt*⁻ strains for various times, the level of IL-8
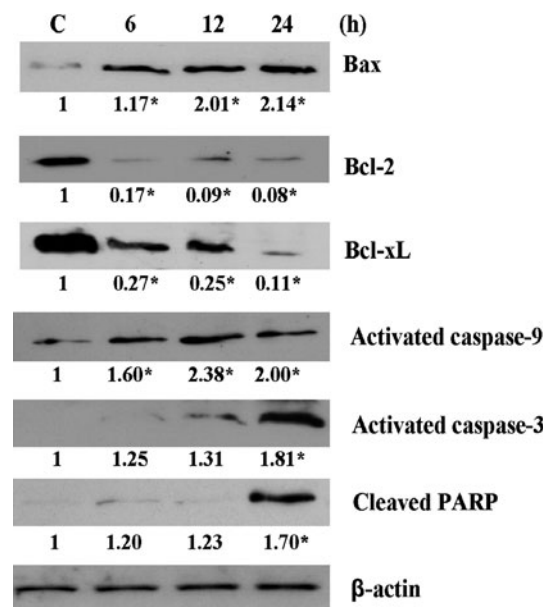

Fig. 6 Apoptosis-related protein expression in KKU-100 cells treated with rHP-GGT (4.5 μg/ml) for 6, 12, and 24 h as determined by Western blot analysis. The density of each protein band was measured and normalized to *β*-actin intensity. Data (shown below each *band*) are presented as the fold change of the treated cells compared with the untreated control cells (mean ± SE) and represent three independent experiments. *$p < 0.05$, between treated and untreated control cells
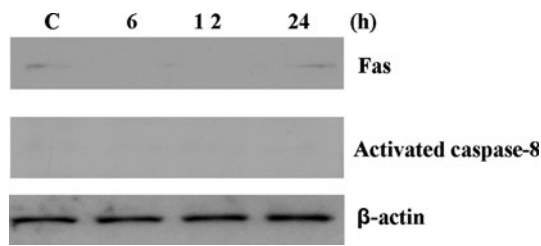
Fig. 7 Protein expression of molecules in the extrinsic apoptotic pathway (Fas and activated caspase-8) in KKU-100 cells treated with rHP-GGT (4.5 μg/ml) for 6, 12, and 24 h as determined by western blot analysis. Three independent experiments were performed
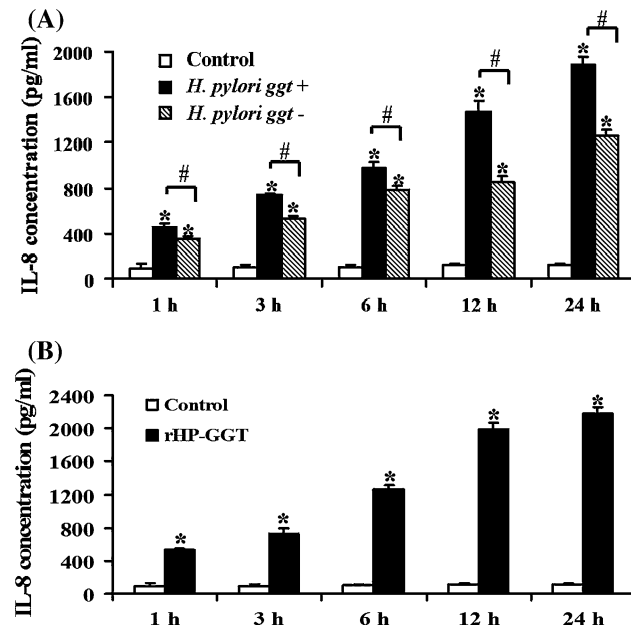

Fig. 8 IL-8 production in KKU-100 cells exposed to *H. pylori ggt*⁺ and *ggt*⁻ strains at MOI of 100 (**a**) or in KKU-100 cells treated with 4.5 μg/ml of rHP-GGT (**b**) for 1, 3, 6, 12, and 24 h. Data are representative of three independent experiments (mean ± SE). *$p < 0.05$, between treated and untreated control cells, #$p < 0.05$, between *H. pylori ggt*⁺ and *ggt*⁻ strains

production was increased ($p < 0.05$). However, cells treated with the *H. pylori ggt*⁺ strain exhibited significantly higher IL-8 production than cells treated with the *H. pylori ggt*⁻ strain (Fig. 8a). To investigate whether rHP-GGT induced IL-8 production, KKU-100 cells were exposed to rHP-GGT for various times. Increasing levels of IL-8 were found in KKU-100 cells treated with rHP-GGT in a time-dependent manner when compared with the untreated control cells (Fig. 8b).

Discussion

Several reports have shown an association between the presence of *H. pylori* and hepatobiliary diseases,

particularly liver carcinoma [5, 6, 23]. However, other reports have demonstrated that the other *Helicobacter* species such as *H. hepaticus* and *H. bilis* were found in patients with hepatobiliary diseases [7, 8], suggesting that *Helicobacter* other than *H. pylori* should not be ignored. Additionally, the effect of *H. pylori* in extra-gastrointestinal systems in animal models has been studied, and this indicates that *H. pylori* may play a role in the hepatobiliary system [24]. These reports are consistent with our previous study in which a significantly higher presence of *H. pylori* was observed in CCA patients (malignant group) than in benign and control groups, while *H. bilis* was also found in a low number [10]. This finding was related to biliary cell inflammation and proliferation, supporting the possibility role of *H. pylori* in hepatobiliary diseases, especially CCA [10]. However, the pathogenesis of *H. pylori* in the hepatobiliary system remains unclear, and in vitro and in vivo studies are needed to explain a pathogenic role for *H. pylori* in the hepatobiliary system. There are many *H. pylori* virulence factors that have been proposed to play an important role in gastrointestinal diseases [25], but in hepatobiliary cells, these have not been fully studied. Recently, we have reported that *H. pylori cagA*-positive strain induces biliary cell proliferation, apoptosis, and inflammation greater than *cagA*-negative strain in a CCA cell line, suggesting that *H. pylori* and the *cagA* gene may be involved in the development of CCA [18]. Therefore, the effects of other *H. pylori* virulence factors should be further examined in hepatobiliary cells.

Gamma-glutamyltranspeptidase (GGT) is an enzyme that functions in glutathione metabolism by catalyzing the transpeptidation and hydrolysis of the γ-glutamyl group of glutathione and related compounds, such as glutamine [26]. This enzyme has been identified in many species of bacteria [27–29]; however, the effect of bacterial GGT on mammalian cells has not been fully elucidated. GGT has been characterized in *H. pylori*, and it is constitutively expressed in all *H. pylori* strains [30]. In an in vitro study, GGT was found to play a significant role in the growth and survival of *H. pylori* [31]. Previous reports have also shown that *H. pylori* GGT promotes apoptosis in gastric epithelial cells [16, 19]. These results indicate that GGT may be involved in *H. pylori* pathogenesis by damaging human gastric cells [32]. Recent reports, including one by Flahou et al., also shed light into our understanding of the mechanism by which *H. pylori* GGT affects gastric cells possibly via apoptosis, oncosis, or other processes [33]. However, the effects of *H. pylori* GGT on human hepatobiliary cells have not been investigated. Thus, the current study examined the role of *H. pylori* GGT in human CCA cells (KKU-100 cells).

Exposure to *H. pylori ggt* isogenic mutant strain decreased cytotoxicity and genotoxicity in the KKU-100

cell line when compared with KKU-100 cells exposed to the wild-type strain. In addition, treatment with rHP-GGT markedly inhibited KKU-100 cell growth and DNA synthesis in a dose-dependent manner, indicating that *H. pylori* GGT exhibited a potential effect on cell survival and DNA synthesis in human biliary cells.

The inhibitory effects of *H. pylori* GGT on cell growth and DNA synthesis were mediated via apoptosis as indicated by chromatin condensation and fragmentation shown by DAPI staining and a DNA ladder assay. Our data are consistent with previous reports demonstrating that *H. pylori* GGT stimulates apoptosis and induces cell cycle arrest at the G1-S phase in gastric cancer cells [19, 34]. To understand the molecular mechanism of *H. pylori* GGT-induced apoptosis in KKU-100 cells, genes and proteins involved in apoptosis were evaluated. There are two main pathways leading to apoptosis, intrinsic and extrinsic, and they are activated by the mitochondria and death receptors, respectively [35]. The Bcl-2 family proteins can be classified into pro- and anti-apoptotic molecules, and these proteins play a crucial role in mediating the delicate balance between cell survival and cell death [36]. Our data demonstrated that rHP-GGT up-regulated *p53*, *bax*, and *caspase-3* gene expression, whereas the anti-apoptotic *bcl-2* gene was significantly down-regulated. We confirmed the mechanism of rHP-GGT-induced apoptosis in KKU-100 cells by western blot analysis. Exposure of KKU-100 cells to rHP-GGT resulted in a reduction in anti-apoptotic Bcl-2 and Bcl-xL protein levels, but Bax levels were markedly increased. Bax is a well-known pro-apoptotic protein that inhibits Bcl-2 activity [37]. Also, we found subsequently up-regulated activated Caspase-9 and Caspase-3 in KKU-100 cells in a time-dependent manner. Furthermore, our data showed that *H. pylori* GGT did not affect the expression of apoptotic molecules in the extrinsic pathway, including Fas receptor and activated caspase-8. These results indicate that *H. pylori* GGT is involved in mitochondria-mediated apoptosis in KKU-100 cells and are similar to results obtained in AGS cells [19]. Recently, one of the mechanisms of *H. pylori*/*H. suis* GGT inducing gastric cell death has been reported [33]. *H. pylori* or *H. suis* GGT supplemented with glutathione mediated an increase of H_2O_2 and lipid peroxidation, resulting in gastric cell damage via apoptosis or necrosis [33], suggesting that glutathione degradation products play a role in the induction of gastric epithelial cell death. It remains to be investigated whether similar mechanisms lead to the results described in the present study. Apoptosis plays an important role in tissue homeostasis. Disturbance of this process may enhance the rate of cell death, resulting in cell hyperproliferation and induction of genetic mutations that promote cancer cell development [38]. In our study, the induction of cell death via apoptosis by *H. pylori* GGT may lead to increased biliary cell proliferation that can promote cancer development.

The pro-inflammatory cytokine, IL-8, is well known to recruit and activate neutrophils, which contribute to mucosal inflammation [39]. IL-8 is mainly secreted from gastric cells upon *H. pylori* infection, and this is an important mechanism of *H. pylori* pathogenesis [40]. In addition, IL-8 exhibits multiple effects in a cancer environment, including stimulating cancer cell proliferation, invasion, and angiogenesis, thereby indicating a crucial role of this cytokine in carcinogenesis [41]. We examined whether *H. pylori* GGT could stimulate IL-8 production in KKU-100 cells. An ELISA revealed that IL-8 production from KKU-100 cells treated with the *H. pylori ggt* mutant strain was significantly lower than that of cells treated with the wild-type strain ($p < 0.05$). We found that treatment with rHP-GGT significantly increased IL-8 production in KKU-100 cells compared with untreated control cells. A previous report has shown that IL-8 production is significantly decreased in AGS cells that are co-cultured with an *H. pylori ggt* mutant strain, and that the *H. pylori* native GGT protein (HP-nGGT) stimulates H_2O_2 and generates IL-8 in primary gastric cells, AGS, and macrophages [42]. Another study has found high production of NO by iNOS, which is involved in the activation of NF-kB, AP-1 and the expression of IL-8 in gastric epithelial cells [43]. In our study, we speculate that *H. pylori* GGT up-regulates iNOS, which may promote the production of IL-8 in KKU-100 cells. Taken together, *H. pylori* GGT may accelerate cancer development by inducing inflammation in KKU-100 cells.

In conclusion, we showed that *H. pylori* GGT inhibited cell growth and DNA synthesis and led to mitochondria-mediated apoptosis in KKU-100 cells. In addition, we found that *H. pylori* GGT exhibited strong immunostimulation of IL-8 production in human biliary cells. In summary, our data are the first to elucidate a possible pathogenic role of *H. pylori* GGT in human biliary cells by disturbing cell kinetics and enhancing biliary cell inflammation. However, in vitro and in vivo studies, particularly animal models, are still required to further clarify the mechanism of *H. pylori* in hepatobiliary diseases.

Conflict of interest　We declare that we have no conflicts of interest.

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