

## Bile Acid Promotes Intestinal Metaplasia and Gastric Carcinogenesis

Masana Tatsugami<sup>1</sup>, Masanori Ito<sup>1</sup>, Shinji Tanaka<sup>2</sup>, Masaharu Yoshihara<sup>3</sup>, Hirofumi Matsui<sup>4</sup>, Ken Haruma<sup>5</sup>, and Kazuaki Chayama<sup>1</sup>

### Abstract

**Background:** Bile acid and *Helicobacter pylori* (*H. pylori*) are important toxic factors for gastric mucosal injury. We examined the role of bile acid in promoting histologic gastritis and gastric carcinoma in Japanese patients.

**Methods:** A total of 767 patients (452 men, mean age 51.1 years) were studied. Gastric juice was collected by gastro-endoscopic examination, and the bile acid concentration was examined by enzymatic method. The grade of histologic gastritis was evaluated by gastric biopsies, and the relationship between the bile acid concentration and the gastritis score was examined. The occurrence of gastric cancer was examined by a retrospective cohort study. *CDX2/CINC1* expression in RGM-1 cells was evaluated by real-time PCR.

**Results:** In *H. pylori*-positive patients, we found significant positive correlation between the bile acid concentration and the grades of atrophy/intestinal metaplasia ( $P < 0.01$ ). However, we found significant negative associations between the bile acid concentrations and the histologic scores of mononuclear cell/neutrophil infiltrations ( $P < 0.01$ ). Patients with a high concentration of bile acid developed gastric cancer more frequently than those with a low concentration ( $P < 0.05$ ). Cholic acid treatment significantly increased *CDX2* expression in RGM-1 cells. *CINC1* expression in RGM-1 cell was significantly induced by coculture with *H. pylori*, and the induction was reduced by glycochenodeoxycholic acid treatment.

**Conclusion:** The bile acid in gastric juice contributes to the progression of histologic atrophy and intestinal metaplasia without inflammatory cell infiltration, followed by carcinogenesis in *H. pylori*-positive patients.

**Impact:** Bile acid promotes intestinal metaplasia and gastric carcinogenesis without inflammatory cell infiltration. *Cancer Epidemiol Biomarkers Prev*; 21(11); 2101–7. ©2012 AACR.

### Introduction

*Helicobacter pylori* (*H. pylori*) plays an important role in the induction of chronic gastritis, and there is a strong association between *H. pylori*-associated gastritis and gastric diseases including peptic ulcer, functional dyspepsia, and gastric cancer (1). The basic components of the process are chronic active nonatrophic gastritis → multifocal atrophy → intestinal metaplasia → dysplasia → invasive carcinoma (2). However, in *H. pylori*-positive patients, the progression of atrophic gastritis is quite different among patients with infection. It is still unclear whether some patients showed advanced progression of gastritis fol-

lowed by gastric carcinogenesis. Many studies have revealed that multiple factors including bacterial factors, environmental factors, and the host immune response, contribute to the progression of mucosal atrophy, metaplasia, and dysplasia toward gastric cancer (3, 4).

Bile acid is another important toxic factor involved in the injury of gastric mucosa (5). In the remnant stomach of rats after gastrectomy, bile acid, the main component of the duodenal juice, has been implicated in gastric cancer due to duodenogastric reflux (6). In humans, duodenogastric reflux also seems to be implicated in gastric stump carcinoma (7). Previous studies have shown that bile acid was associated with intestinal metaplasia in the cardia (8) and with the degree of esophageal mucosal injury (9). Several clinical studies have emphasized the role of chronic duodenogastro-esophageal reflux in the development of Barrett esophagus with intestinal metaplasia (10, 11). We previously reported that the bile acid caused DNA damage, which may contribute to the carcinogenesis (12).

The *CDX1* and *CDX2* homeobox transcription factors show intestine-specific expression in adult tissues and seem to have critical functions in intestinal development as well as in specification and maintenance of the intestinal phenotype in adults (13). The mouse *CDX1* and *CDX2* genes are expressed rather broadly in caudal

**Authors' Affiliations:** <sup>1</sup>Department of Gastroenterology and Metabolism, Graduate School of Biomedical Sciences, Hiroshima University; <sup>2</sup>Department of Endoscopy, Hiroshima University Hospital, Hiroshima; <sup>3</sup>Health Service Center, Hiroshima University, Higashi-Hiroshima; <sup>4</sup>Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba; and <sup>5</sup>Division of Gastroenterology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki, Japan

**Corresponding Author:** Masanori Ito, Department of Gastroenterology and Metabolism, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Phone: 81-82-257-5191; Fax: 81-82-257-5194; E-mail: maito@hiroshima-u.ac.jp

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structures during the early stages of embryonic development, but in the later stages of development and in normal adult tissues, expression of these genes are restricted to the epithelium of the small intestine and colon (14, 15). Mutoh and colleagues directly showed that *CDX2* contributes to the generation of intestinal metaplasia of gastric epithelial cells in transgenic mice (16). Cytokine-induced neutrophil chemoattractant-1 (*CINC1*), which corresponds to human interleukin-8 (IL-8), is a key cytokine in the rat. Previous studies showed that *CINC1* protein plays an important role in rat gastric inflammation induced by *H. pylori* infection (17).

In the present study, we tried to clarify the role of the bile acid in gastric juice in gastric inflammation/carcinogenesis. We examined (1) the relationship between the bile acid concentration in gastric juice and histologic gastritis in a large-scale cross-sectional study (2), the relationship between the bile acid concentration in gastric juice and gastric cancer occurrence in Japanese patients with *H. pylori* in a retrospective cohort study. Furthermore, we investigated whether bile acid influences intestine-specific gene expression (*CDX2*) and inflammation-related cytokine (*CINC1*) in rat gastric epithelial cells cultured *in vitro*.

## Materials and Methods

### Patients

Seven hundred and sixty-seven patients with dyspepsia (452 men, mean age 51.1 years) were included in this study. We excluded patients with previous gastrectomy or with previous *H. pylori* eradication therapy. No patients who were continuously taking proton pump inhibitors were included in the study. All patients received routine gastroduodenal endoscopic examination between October 1996 and July 2006. We confirmed that no patient had gastric neoplasm, and the endoscopic features were recorded in a database. Gastric juice was collected by aspiration before routine observation. It was stored at  $-20^{\circ}\text{C}$  until use, and then pH and the concentration of bile acid were evaluated with the use of an enzymatic method (18). Type of bile acid was evaluated by gas chromatography. *H. pylori* infection was examined by histologic examination and antibody titer (E-plate). *H. pylori* was regarded as negative when both tests showed negative results. A total of 357 patients (men 223, mean age 60.0 years) out of 767 patients received endoscopic examination for cancer screening for less than 3 years (range 3–30 years, mean 10 years), and these data were recorded in the database in Hiroshima University Hospital (Hiroshima, Japan). Ethics Committee of Hiroshima University Hospital approved our protocol (Hiroshima University Hospital #222).

### Histologic gastritis score

In upper gastrointestinal endoscopy, gastric biopsies (2 from the antrum and 2 from the corpus) were obtained. Biopsy specimens were taken from a site without localized lesions. Specimens were fixed with formalin and stained with hematoxylin and eosin. Gastritis scores were evalu-

ated by 2 specialists (M. Ito and M. Tatsugami) independently, using the updated Sydney system (19).

### Cell culture and RNA extraction

Rat gastric epithelial cell line (RGM-1), which was authenticated by RIKEN BioResource Center, was purchased from RIKEN Cell Bank. These cells were routinely cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham F12 medium supplemented with 10% heat-inactivated FBS. In the examination of *CDX2*/*CINC1* expression, 4 kinds of bile acid [200  $\mu\text{mol/L}$  cholic acid, chenodeoxycholic acid (CDC), glycocholic acid (GCA), or glycochenodeoxycholic acid (GCDC)] with *H. pylori* were added to the culture medium for 24 hours before harvesting. *H. pylori* strain was derived from a patient with gastric ulcer, and we confirmed that it was CagA-positive (East Asian type; ref. 20). After incubation in the liquid culture medium for 24 hours, *H. pylori* was added to the culture supernatant of RGM-1 cells.

### RNA extraction and PCR reaction

RNA was extracted from RGM-1 cells with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was conducted using a first-strand cDNA kit (GE Healthcare UK Ltd.). One-microliter aliquots of the synthesized cDNA were mixed with SYBR Green PCR master mix (Applied Biosystems) with appropriate primers and amplified using a real-time PCR system 7300 (Applied Biosystems). Sense and antisense primers used were 5'-aga aca tcc aga gtt tga agg tga t-3' and 5'-gtg gct atg act tgg gtt tgg-3' (21) for *CINC1* (67 bp), 5'-tgt gtc gta aat gcc aga gc-3' and 5'-acc ctc ata gat ggg cac ag-3' (NCBI database NM-023963) for *CDX2* (77 bp), and 5'-cag caa ttc cgg tct tct tc-3' and 5'-acc ctc ata gat ggg cac ag-3' (22) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 288 bp), respectively. The specificity of each amplified product was confirmed by dissociation analyses giving a single sharp dissociation peak, the absence of the amplified product without reverse transcription, and the appearance of a band of the expected size on electrophoresis of the amplified product.

The expression of *GAPDH* was used as an internal control. All quantified values were normalized to those of *GAPDH* (quantified value for a certain target/quantified value for *GAPDH*).

### Statistical analysis

Statistical analysis was conducted using the Mann-Whitney *U* test, Kaplan-Meier method, Kruskal-Wallis test, and Steel test with Stat View (SAS Institute Inc.). A value of  $P < 0.05$  was considered significant.

## Results

### Relationship between bile acid concentration and histologic gastritis

Out of 767 patients examined, 612 were regarded as *H. pylori*-positive. First, we examined the relationship

between histologic gastritis and bile acid concentration in the gastric juice in *H. pylori*-positive patients. As shown in Fig. 1, bile acid concentration was significantly higher in the group with high atrophy/intestinal metaplasia and with low activity/inflammation in the gastric antrum. The same result was obtained for the gastric corpus (data not shown). However, in *H. pylori*-negative patients, the bile acid concentration was significantly low, and such a tendency was not detected either in the gastric antrum or in the corpus (Fig. 2). Next, we excluded the patients with marked atrophy, and carried out the same examination to refute the influence of natural clearance of *H. pylori* followed by decreased cell infiltration. Surprisingly, the bile acid concentration was significantly lower in the group with a high score for gastric inflammation (Fig. 3).

### Bile acid concentration and gastric carcinogenesis

Next, we conducted a retrospective cohort study to investigate the role of bile acid in gastric carcinogenesis. Retrospectively, we enrolled 357 patients who were followed by endoscopic examination for cancer screening for less than 3 years, and conducted a cohort study using these patients. We set the cut-off at 1,000  $\mu\text{mol/L}$  and grouped the patients by high and low concentrations of bile acid. As illustrated in Fig. 4, on the basis of the Kaplan-Meier method, we found that the prevalence of gastric cancer development was statistically higher in patients with high concentrations of bile acid. Gastric

cancers that developed in these patients were 28 intestinal-type and 7 diffuse-type cancers.

### Effect of bile acid on the expression of CDX2 and CINC1 in RGM-1 cells

We next examined the effect of bile acid on gastric mucosa cells, which were treated with 200  $\mu\text{mol/L}$  cholic acid, CDC, GCA, and GCDC, and then we examined the *CDX2/CINC1* expression in gastric epithelial cells *in vitro*. *CDX2* expression level was not altered by coculture with *H. pylori*. However, *CDX2* level was statistically higher in the group that underwent cholic acid treatment than in the control ( $P < 0.05$ , Fig. 5A). This effect was not clear for other kinds of bile acids on RGM-1. On the other hand, *CINC1* expression level was extremely low in RGM-1 cells at the basal status; however, its expression was significantly induced by coculture with *H. pylori* (Fig. 5B). When GCDC was added, *CINC1* expression was significantly suppressed ( $P < 0.05$ ). In contrast, unconjugated bile acids (cholic acid and CDC) increased *CINC1* expression. The effect on *CINC1* expression was significantly different between glycine-conjugated and unconjugated bile acids.

### Discussion

In the present study, we showed that in *H. pylori*-positive patients (1) a significant correlation was found between the bile acid concentration and atrophy/intestinal metaplasia (2), a significant negative association was disclosed

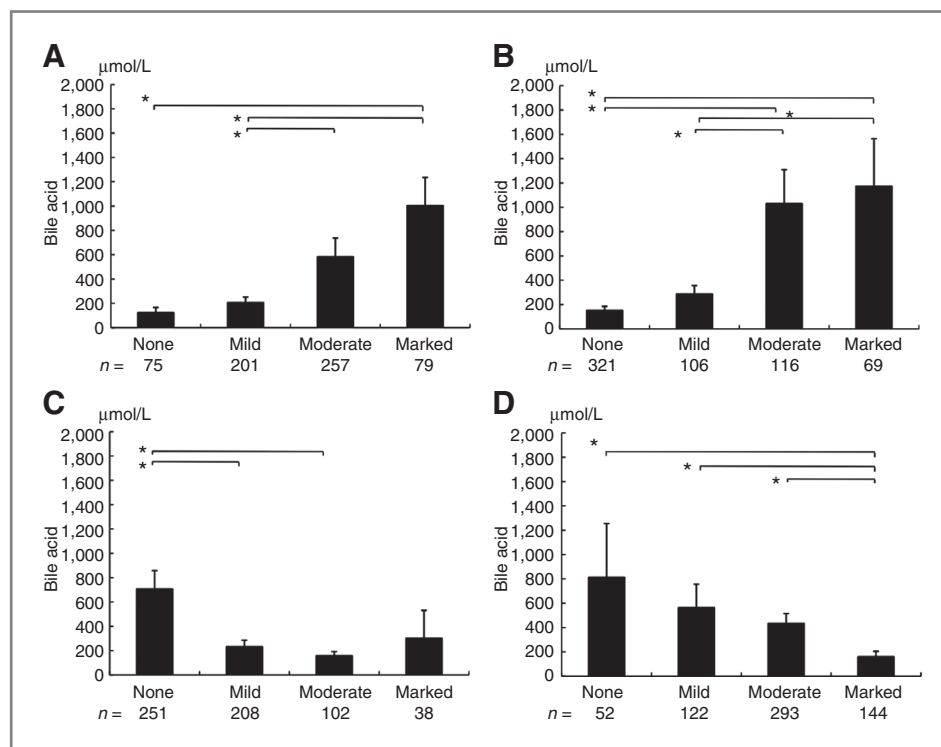
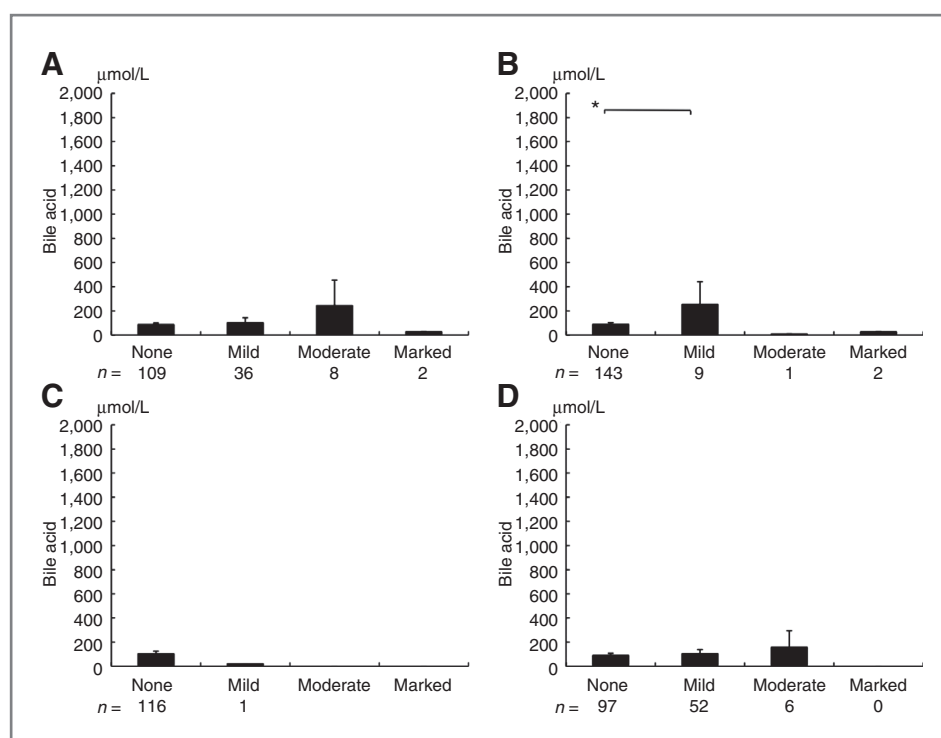


Figure 1. Relationship between bile acid concentration and gastritis score [atrophy (A), intestinal metaplasia (B), inflammation (C), activity (D)] in the gastric antrum of *H. pylori*-positive patients. The titer of bile acid concentration is illustrated by a linear scale. \*,  $P < 0.05$ .



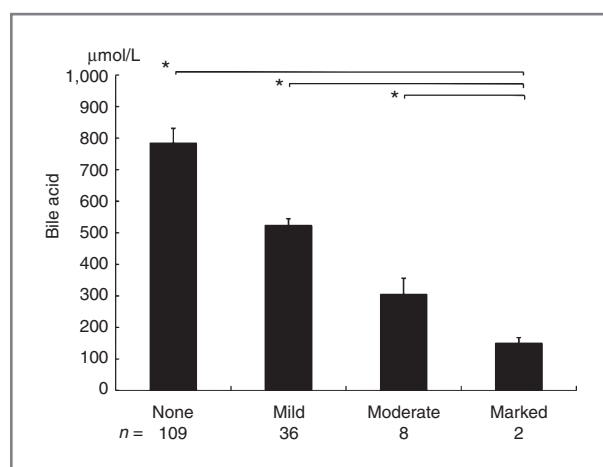
**Figure 2.** Relationship between bile acid concentration and gastritis score [atrophy (A), intestinal metaplasia (B), inflammation (C), activity (D)] in the gastric antrum of *H. pylori*-negative patients. The titer of bile acid concentration is illustrated by a linear scale. \*,  $P < 0.05$ .

between bile acid concentration and inflammatory score as histologically assessed in gastric mucosa biopsy samples. In addition, we found that (3) patients with a high concentration of bile acid developed gastric cancer more frequently than those with a low bile acid concentration. These suggest that bile acid in gastric juice contributes to the progression of histologic atrophy and intestinal metaplasia without inflammatory cell infiltration, followed by carcinogenesis, in *H. pylori*-positive patients.

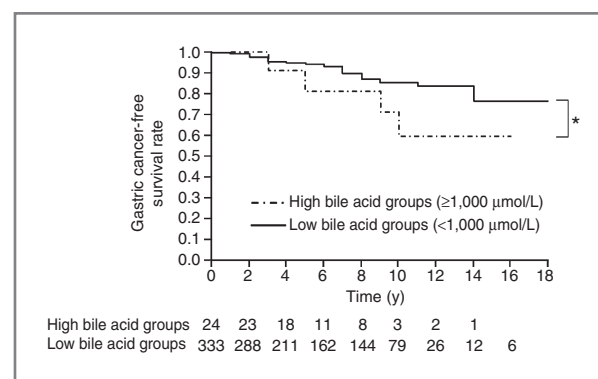
In addition, we tried to clarify the mechanism of these alterations using RGM-1 cells *in vitro*. We confirmed

*CDX2* induction in RGM-1 cells after cholic acid treatment. Xu and colleagues recently reported that CDC induced *CDX2* expression in RGM-1 cells (23). Recent studies focused on the development of intestinal metaplasia on esophago-gastric junction leading to Barrett esophagus (24). Our previous work showed that bile acid has an effect in promoting gastric atrophy and intestinal metaplasia (25).

However, the inhibition of inflammatory cell infiltration was quite unexpected. As far we know, this is the first report describing the inhibitory effect of bile acid on gastric inflammation induced by *H. pylori* infection. It is hypothesized that decreased inflammation may arise from severe atrophy and natural clearance of *H. pylori*.

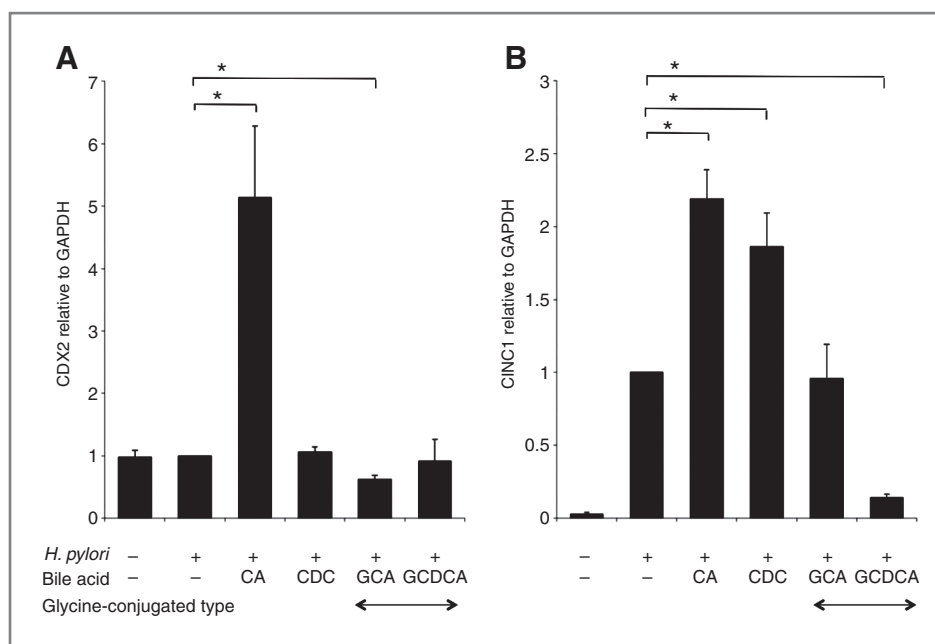


**Figure 3.** Relationship between bile acid concentration and inflammation score in the corpus in patients with no to moderate atrophy. The titer of bile acid concentration is illustrated by a linear scale. \*,  $P < 0.05$ .



**Figure 4.** Kaplan-Meier analysis of gastric cancer development. Patients were subclassified into 2 groups: high bile acid ( $\geq 1,000$   $\mu\text{mol/L}$ ) and low bile acid ( $< 1,000$   $\mu\text{mol/L}$ ) groups.

**Figure 5.** The expression of CDX2 and CINC1 in RGM-1 cells. Cells were incubated with or without 200  $\mu\text{mol/L}$  bile acid/*H. pylori* for 24 hours, and then real-time PCR was conducted. A, expression level of CDX2 evaluated by real-time PCR ( $n = 4$ ). B, expression level of CINC1 evaluated by real-time PCR ( $n = 5$ ). The relative expression level (CDX2/GAPDH or CINC1/GAPDH) was expressed by setting the standard (*H. pylori*-positive and no bile acid) as 1.0. Bars, SEM. \*,  $P < 0.05$ .



In addition, it is well known that bile acid has an anti-*H. pylori* effect (26). However, surprisingly, our additional result (Fig. 3) contradicts this hypothesis because we could also show a negative association between bile acid inflammation and grades of mononuclear cell infiltration, even in patients with no or mild atrophy, in whom *H. pylori* was not cleared. These results support the anti-inflammatory effect of bile acid in the gastric juice.

In the present study, we showed that *CINC1*, which encodes the rat cytokine similar to human IL-8 (27), was dramatically induced by *H. pylori* infection and was inhibited by GCDCA treatment in RGM-1 cells. As is well known, IL-8 is a key cytokine that enhances *H. pylori*-induced gastric inflammation showing mononuclear cell/neutrophil infiltration in the mucosal layer (28). We showed the possibility that bile acid first has an inhibitory effect on *CINC1* expression induced by *H. pylori* infection, and second, inhibits the infiltration of inflammatory cells in the gastric mucosa. Of course, the antibacterial effect of bile acid may play a partial role in inhibiting the infiltration of inflammatory cells.

It is questionable whether intestinal metaplasia without inflammatory cell infiltration can be linked to gastric carcinogenesis. The intestinal metaplasia induced by bile acid was not a result of a secondary event following active inflammation but rather a direct effect of bile acid. Therefore, we conducted a retrospective cohort study in our subjects to analyze gastric cancer development. Interestingly, we could show a positive relationship between bile acid concentration and gastric cancer development, suggesting the carcinogenic role of bile acid-induced gastritis or intestinal metaplasia. Because this mucosa has few inflammatory cells, the indirect effect from inflammatory cells seems to be unlikely by, for example, nitric oxide release by inflammatory cells (29). A recent study showed

that bile acid directly affects intracellular signaling of gastric epithelial cells (30). Besides *CDX2*, the alteration of carcinogenic pathways should be clarified in future studies.

As shown in our results, the proportion of different fractions in bile acid is quite important. From our previous results, the major components of bile acids were cholic acid and CDC (31); however, it was shown that the greater part in gastric juice was glycine-conjugated type (GCA and GCDCA; ref. 32). Therefore, we used these 4 bile acids in the present study. It is well known that bile acids do not equally regulate gastric inflammation or carcinogenesis (33). For example, we confirmed that cholic acid selectively increased *CDX2* expression *in vitro*. On the other hand, the decrease of *CINC1* expression was clear in glycine-conjugated bile acids, especially in GCDCA. About the *CINC1*-related inhibition of inflammatory cell infiltration in particular, the process of glycine conjugation may be important. In addition, we previously showed the specific carcinogenic role of GCDCA, suggesting the exceptional role of GCDCA in gastric inflammation and carcinogenesis (12). It will be important to evaluate bile acid proportions and clinical outcome in future studies. Because our study design was a cross-sectional retrospective cohort, a prospective cohort study is needed to clarify the anti-inflammatory but carcinogenic effect of bile acid.

In the present study, the bile acid titer was significantly lower in *H. pylori*-negative patients. The reason for this difference is unknown. We previously showed that the degree of duodenal-gastric reflux was statistical higher in *H. pylori*-positive patients than in -negative patients by ultrasonographic examination (34). However, this difference was not so pronounced. While this may be a partial explanation, the main reason is still unclear. The difference in gastric juice pH may be important (35).

In Japan, the prevalence of *H. pylori* infection is decreasing gradually and the spread of eradication therapy has accelerated the disappearance of this bacterium. We will be able to control the carcinogenic effect of *H. pylori* in the near future (36, 37). Although *H. pylori*-negative gastric cancer is rare (38), the role of bile acids should become important in gastric carcinogenesis in the next generation. Therefore, we should clarify the detailed mechanism by which bile acid induces gastric inflammation and carcinogenesis.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Authors' Contributions

**Conception and design:** M. Tatsugami, M. Ito, K. Haruma  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Haruma  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Haruma  
**Writing, review, and/or revision of the manuscript:** K. Chayama  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H. Matsui, K. Chayama  
**Study supervision:** S. Tanaka, M. Yoshihara, K. Chayama

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