CsA treatment; Fig. 5D; Supplementary Fig. S7). These results showed that the CsA treatment suppressed inflammatory responses but not *HP* colonization, and that the suppression of inflammatory responses markedly repressed methylation induction.

***Expression analysis of genes with promoter methylation in* HP*-infected GECs.*** HG2, SC3, and SD2 were located in the promoter regions of *Gpr37*, *Rnf152*, and *Nptx2*, respectively. Promoter CGIs are generally resistant to DNA methylation (29), and only when genes are transcribed at low levels are they susceptible to DNA methylation (30–32). To confirm the low expression and the effect of methylation on gene expression, we analyzed their expression levels in GECs isolated from gerbils with and without *HP* infection (10 and 50 weeks

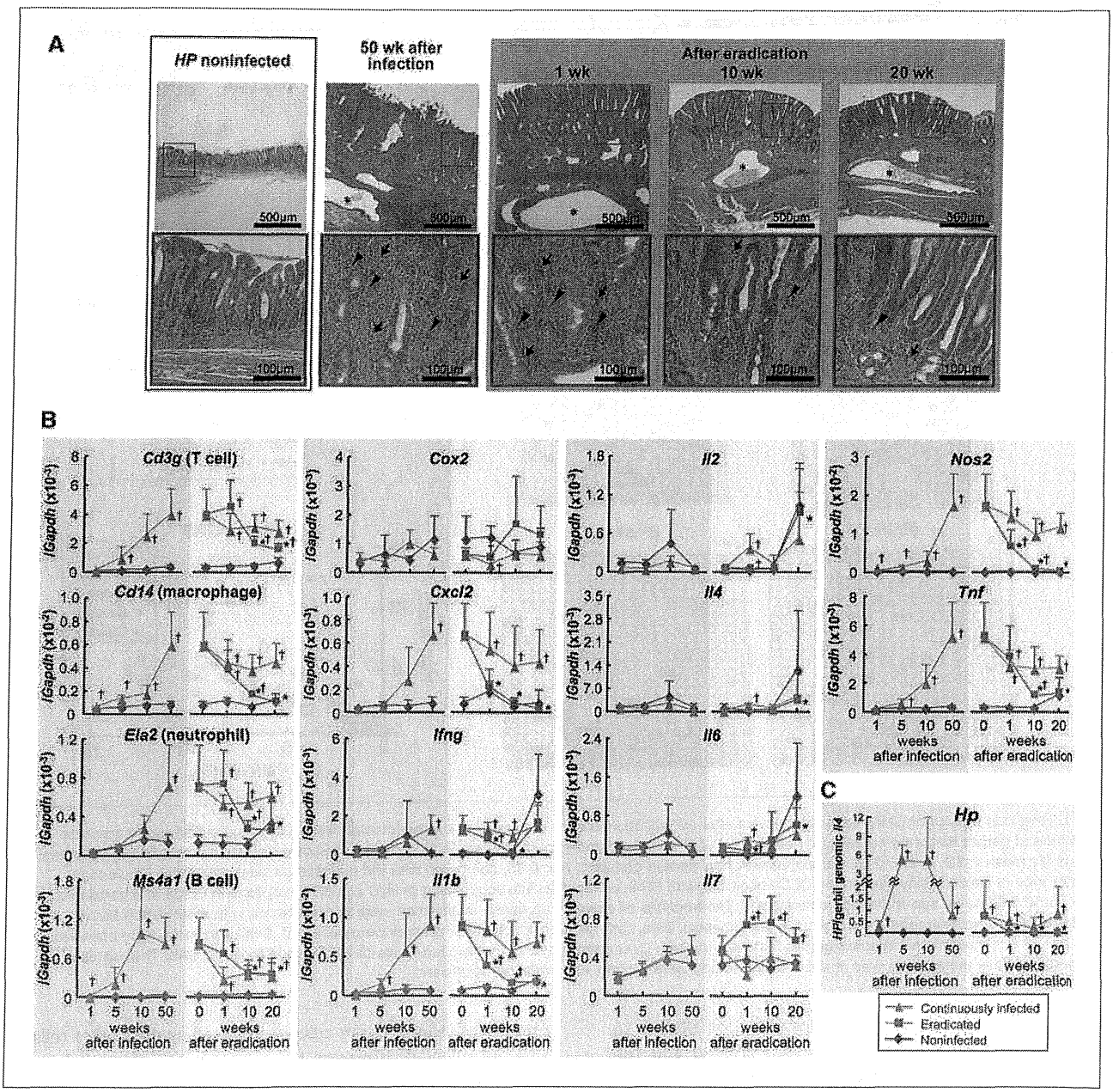


Figure 4. Changes in inflammation after *HP* infection and its eradication. A, histologic changes in gastric mucosa before and after *HP* eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) did not change at 1 wk after eradication but markedly decreased at 10 and 20 wk. However, the presence of fibrosis and heterotopic proliferative glands (*) did not differ. B, temporal profiles of expression of inflammatory cell markers and inflammation-related genes. Red, green, and blue lines, gerbils with continued infection, gerbils with eradication, and those without any *HP* infection, respectively. C, numbers of *HP* in the gerbil stomach. Real-time PCR of *HP*-specific DNA using DNA extracted from gastric tissues containing mucus was done. Values are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the expression level before eradication.

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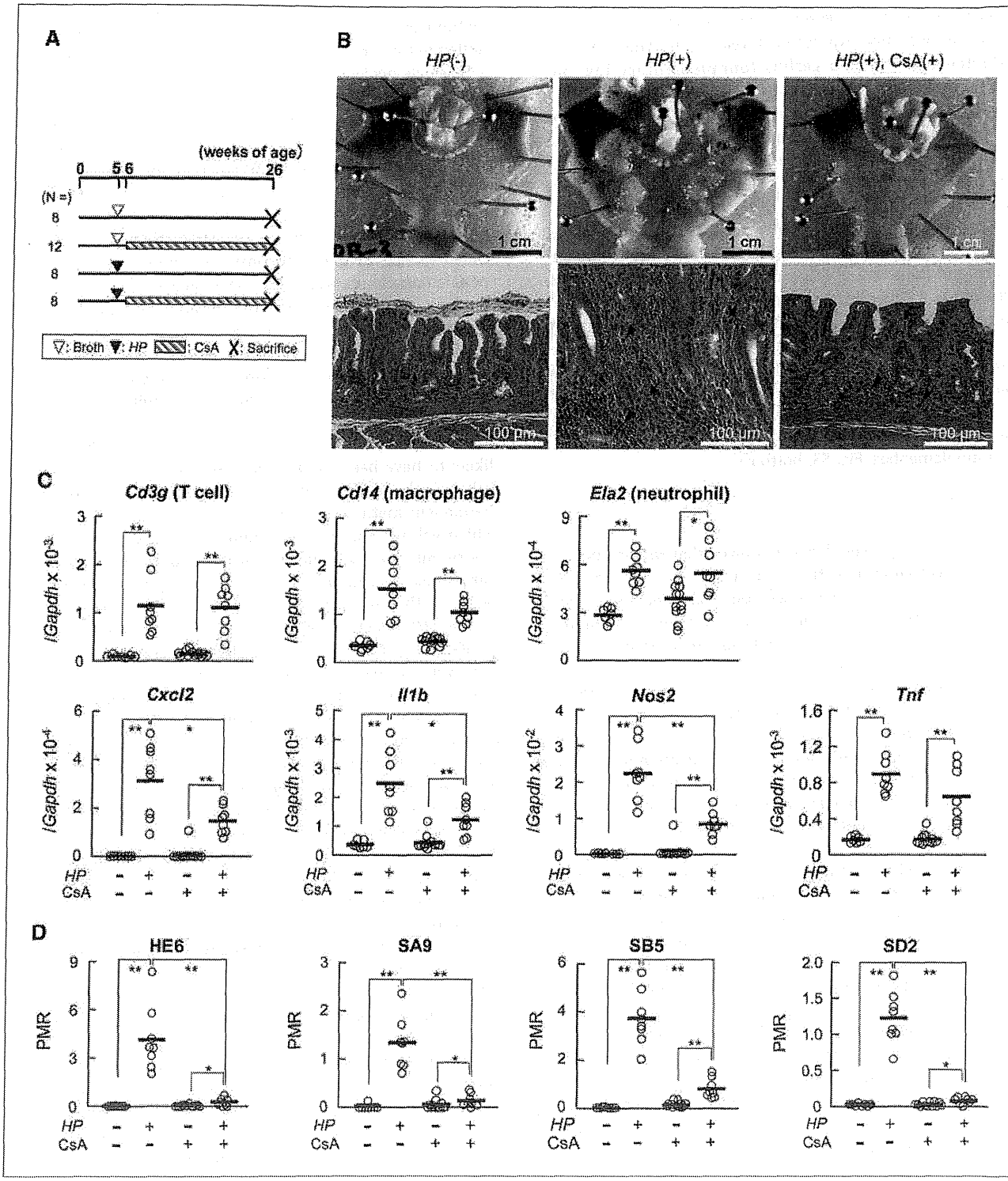


Figure 5. Suppression of inflammation and methylation induction by CsA treatment. A, experimental design for CsA treatment and *HP* infection. B, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in *HP*-infected gerbils without the CsA treatment and were markedly suppressed by the CsA treatment. Infiltration of mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) was also severe in *HP*-infected gerbils without the CsA treatment and was repressed in CsA-treated animals. Gastric mucosae of *HP*-negative gerbils with CsA treatment showed no abnormal changes (data not shown). C, expression of inflammatory cell markers and inflammation-related genes. The expression of inflammatory cell markers normalized to *Gapdh* expression was not reduced. However, the expression of three inflammation-related genes (*Cxcl2, Il1b*, and *Nos2*) was significantly reduced by the CsA treatment. D, methylation levels in GECs. The CsA treatment markedly suppressed methylation induction by *HP* infection. Bold horizontal bar, average. *, $P < 0.05$; **, $P < 0.01$.

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after infection) and in gastric cancer cell lines. All the three genes showed low expression levels in the GECs of noninfected and infected gerbils (Supplementary Fig. S8). *Rnf152* expression was significantly decreased in *HP*-infected gerbils compared with noninfected gerbils (44% and 25% at 10 and 50 weeks, respectively, after infection; $P < 0.001$). None of the three genes were expressed in cancer cell lines with complete methylation of these CGIs (Fig. 1B; Supplementary Fig. S8, top).

***The absence of DNA methyltransferase upregulation.*** DNA methyltransferases (Dnmt) are final effectors of maintenance and induction of DNA methylation, and their overexpression is frequently observed in various types of human cancers (33). To analyze possible upregulation of Dnmts by *HP* infection, expression levels of *Dnmt1, Dnmt3a,* and *Dnmt3b* mRNAs were quantified in GECs of gerbils with and without *HP* infection. Contrary to our initial expectation, the expression levels of the three Dnmts were significantly lower in GECs with *HP* infection (1/2 to 1/3) than those without (Supplementary Fig. S8, bottom).

## Discussion

Our study using a gerbil model showed that *HP* infection is causally involved in induction of aberrant DNA methylation in GECs. Thus far, a strong association has been shown between the presence of *HP* infection and high methylation levels or high incidence of methylation in human gastric mucosae (5, 10–12). Taking advantage of an animal model, we were able to conduct an experiment by infecting gerbils with *HP* and showed that *HP* infection was the cause of methylation induction.

The critical role of inflammation in methylation induction was shown. Temporal analysis showed that methylation levels were closely associated with infiltration of inflammatory cells, and suppression of inflammation by CsA markedly repressed methylation induction even in the presence of *HP*. These results indicated that *HP* itself was not necessary for methylation induction once inflammation was induced by it. This finding is important because a direct role of *HP* is suggested by the facts that the SHP2 oncoprotein is deregulated by injection of virulent factors such as CagA into GECs (34) and *HP* possesses multiple DNA (cytosine-5) methyltransferases (35).

Among the inflammation-related genes analyzed, the expression levels of *Cxcl2, Il1b, Nos2,* and *Tnf* were upregulated in the stomach with *HP* infection and decreased after eradication, almost paralleling those of methylation levels. In the CsA treatment, in which methylation induction was markedly suppressed, upregulation of *Cxcl2, Il1b,* and *Nos2* by *HP* infection was significantly suppressed and that of *Tnf* also had a tendency to be suppressed. These results suggest that some specific inflammation-related genes are cooperatively involved in methylation induction by *HP* infection. In human ulcerative colitis and hepatitis (cirrhosis), where aberrant methylation is believed to be induced, increased expression of *IL8* (human functional homolog of *Cxcl2*), *IL1B, NOS2,* and *TNF* was also observed (36–39), suggesting that upregulation of these genes is a common feature of methylation-associated inflammation. Especially for human *IL1B*, its allele with a specific single nucleotide polymorphism is known to be associated with increased gastric cancer risk and increased incidence of *CDH1* promoter methylation in gastric cancers (40, 41). Also, increased production of nitric oxide, due to upregulation of a nitric oxide synthase (*NOS2*) by IL1B or administration of nitric oxide donors, induced methylation of *FMR1* and *HPRT* genes *in vitro* (42).

This study also clearly shows that methylation in gastric mucosae with *HP* infection consists of temporary and permanent components, which has been suggested by studies in humans (5, 10). Methylation that disappeared after eradication corresponds to the temporary component, and methylation that did not disappear corresponds to the permanent component. A pyloric gland (mucosal epithelia) is known to be composed of one or a few stem cells, multiple progenitor cells, and a large number of differentiated cells, and it is renewed within 3 to 14 days (43, 44). Temporary methylation is likely to have been induced in progenitor or differentiated cells, which will finally drop off from the gastric epithelium. Permanent methylation is likely to be induced in stem cells, which will remain for life. In humans, methylation levels in gastric mucosae without *HP* infection correlate with gastric cancer risk (5, 10), and this fact is also in line with the hypothesis that permanent methylation in gastric mucosae without *HP* infection reflects methylation in stem cells.

HG2, SC3, and SD2 were methylated in GECs, although they were located in promoter CGIs, which are generally resistant to DNA methylation (29). Among promoter CGIs, those of genes with low transcription are known to be susceptible to methylation (30, 31, 45), and as expected, all the three genes had low transcription levels in GECs. Transcription levels at $10^{-4}$ to $10^{-3}$/*Gapdh* (*GAPDH*) correspond to 1 to 10 copies of mRNA per cell and are less than 35% of the average expression level of all the genes analyzed by expression microarray (46). Because their methylation levels in GECs of gerbils infected with *HP* for 10 and 50 weeks were less than a few percent, their methylation was unlikely to have affected the overall expression levels in gastric mucosae. As a response to *HP* infection, *Rnf152* was downregulated whereas *Gpr37* and *Nptx2* were not.

Promoter CGIs of *GPR37* and *NPTX2* were highly methylated in human gastric mucosae with *HP* infection and were frequently methylated in human gastric cancers. Because their tumor-suppressive functions have not been reported and they are not expressed in normal gastric mucosae (RefExA database[4]), their silencing is unlikely to be causally involved in gastric carcinogenesis, and they are considered to be passengers. Likewise, methylated CGIs that were not associated with genes were likely to be passengers. However, it is now known that a lot of passengers and limited number of drivers are methylated to high and small degrees, respectively, in human gastric mucosae with *HP* infection (5, 45). Therefore, although most methylation identified here was

[4] http://157.82.78.238/refexa/main_search.jsp

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considered to be passenger, it is likely that tumor-suppressor genes are also methylated in association with their methylation. Gastric mucosa with accumulation of silencing of various genes, including both drivers and passengers, is considered to form a field where cancers will develop (epigenetic field for cancerization; refs. 7, 10, 47).

As a final effector of methylation induction, we examined overexpression of *Dnmts*, which are implicated in methylation induction in various human cancers (33). Unexpectedly, all the three *Dnmts* were downregulated by *HP* infection. Our recent data in humans also showed that mRNA levels of Dnmts had decreasing tendencies in *HP*-infected gastric mucosae (45). These results indicate that overexpression of *Dnmts* is not involved in *HP*-induced methylation induction, and suggest that local distribution of Dnmts and/or protective factors, such as the presence of RNA polymerase II (48), might be disturbed by inflammation.

Genome-wide screening to isolate DNA fragments methylated by *HP* infection was done by MS-RDA, which is applicable to any species without genome information. We used cell lines as the driver so that we could avoid heterogeneity of primary samples and aberrant methylation will be present in all the DNA molecules in the driver. This was considered to be essential for a genome-wide screening because most methods cannot detect small differences. Although cell lines might have artificial methylation, we confirmed the presence of specific methylation in GECs, and a high-sensitivity method, qMSP, was used for this. As expected, methylation levels of CGIs identified here were small (i.e., a few percent) in GECs with *HP* infection, showing that the strategy was correct.

In summary, *HP* infection was causally involved in induction of aberrant DNA methylation, and a critical role of inflammation in the induction was indicated. This model is expected to be useful in analyzing detailed molecular mechanisms for induction of aberrant DNA methylation.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Preventive effects of etodolac, a selective cyclooxygenase-2 inhibitor, on cancer development in extensive metaplastic gastritis, a *Helicobacter pylori*-negative precancerous lesion

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**The present study investigated the preventive effects of etodolac, a selective cyclo-oxygenase (COX)-2 inhibitor, on metachronous cancer development after endoscopic resection of early gastric cancer. Among 267 early gastric cancer patients who underwent endoscopic resection, 47 patients with extensive metaplastic gastritis were selected based on endoscopic findings and our previously described criteria of serum pepsinogen (PG) test-positive and *Helicobacter pylori* antibody-negative conditions. Nonrandomized etodolac treatment (300 mg/day) was administered to 26 patients (Group A), while the remaining 21 patients were untreated (Group B). No significant differences in age, sex distribution, lifestyle factors or extent of metaplastic gastritis at baseline were identified between groups. Patients were followed for metachronous cancer development with endoscopy every 6–12 months for up to 5 years. Mean (standard deviation) follow-up period was 4.2 (0.9) years. In Group B, 5 cancers developed (incidence rate = 6,266/100,000 person-years), significantly more than the 1 cancer in Group A (incidence rate = 898/100,000 person-years; $p < 0.05$). Long-term etodolac treatment did not influence the extent of metaplastic gastritis as revealed by endoscopic findings or by serum PG levels, but effectively reduced metachronous cancer development in patients with extensive metaplastic gastritis. These results strongly suggest that chemoprevention of cancer in the metaplastic stomach is possible by controlling COX-2 expression.**

**Key words:** gastric cancer, pepsinogen, *Helicobacter pylori*, chronic atrophic gastritis, cancer prevention, chemoprevention, COX-2 inhibitor, intestinal metaplasia

**Abbreviations:** *H. pylori*: *Helicobacter pylori*; CAG: chronic atrophic gastritis; COX: cyclo-oxygenase; ESD: endoscopic submucosal dissection; ELISA: enzyme-linked immunosorbent assay; PG: pepsinogen; NSAIDs: nonsteroidal anti-inflammatory drugs

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*Helicobacter pylori* triggers chronic inflammation of the infected stomach mucosa and is considered a major risk factor for gastric cancer and associated precursor lesions.[1] As postulated in the multistep model of gastric cancer development by Correa, long-lasting inflammation in the stomach mucosa leads to a cascade of molecular and morphological changes of stomach carcinogenesis, representing the gastritis-atrophy-metaplasia-dysplasia-cancer sequence.[2] This sequence of stomach carcinogenesis is now widely accepted to be strongly promoted by *H. pylori* and is affected by a variety of genetic and environmental factors that may act synergistically.[3] *H. pylori* eradication thus appears to be the most promising approach for the control of gastric cancer development, and the results of animal experiments have revealed that eradication of *H. pylori*, especially in the early stage, is effective for preventing stomach carcinogenesis.[4–6] However, current data indicate that *H. pylori* eradication does not lead to complete eradication of gastric cancer[7–12] and might be effective only in subjects without chronic atrophic gastritis (CAG) together with intestinal metaplasia.[7,10] Moreover, patients with extensive intestinal metaplasia—that is, metaplastic gastritis—should not be treated with eradication

therapy, as bacterial load decreases with the progression of intestinal metaplasia, eventually resulting in spontaneous eradication.[13,14] Alternative chemopreventive measures are thus needed for the prevention of stomach cancer in subjects with metaplastic gastritis.

Cyclo-oxygenase (COX)-2 is an inducible enzyme overexpressed in sites of inflammation and neoplastic tissues. This overexpression leads to enhancement of cell proliferation and migration, suppression of apoptosis, stimulation of neovascularization and alteration of intercellular adhesion, all of which are involved in carcinogenesis.[15] In the stomach, *H. pylori* infection triggers mucosal COX-2 upregulation, and this enhanced expression level is maintained throughout the progression of the aforementioned premalignant lesions to cancer.[16,17] Furthermore, selective inhibition of COX-2 has been shown to prevent the progression of premalignant gastric lesions[18] and the development of gastric cancer in *H. pylori*-infected Mongolian gerbils.[19,20] Treatment with COX-2 inhibitors thus appears to have beneficial preventive effects on *H. pylori*-associated stomach carcinogenesis. We conducted a prospective follow-up study in a group of patients with metaplastic gastritis who underwent endoscopic resection for early gastric cancer to determine whether administration of etodolac, a selective COX-2 inhibitor, prevents metachronous gastric cancer development.

## Material and Methods

### Study subjects

Between February 2003 and January 2005, a total of 267 patients with early gastric cancer underwent curative endoscopic resection such as endoscopic mucosal resection or endoscopic submucosal dissection (ESD) in Wakayama Medical University Hospital.[21–26] All patients were inhabitants from around the Wakayama area. In Japan, where the incidence of gastric cancer is high, treatment of mucosal gastric cancer without lymph node metastasis is usually achieved with endoscopic resection, preserving the stomach. Although institutional differences in indications for endoscopic resection exist, lesions with preoperative endoscopic diagnoses of intestinal-type intramucosal cancer without ulcer findings, intestinal-type intramucosal cancer $\leq$3 cm in diameter with ulcer findings and intestinal-type minute invasive submucosal (<500 μm below muscularis mucosa) cancer $\leq$3 cm in diameter are considered to be indicated for endoscopic resection.[25,26] In these 267 patients, the extent of coexisting CAG was evaluated endoscopically and by the results of 2 serum tests, pepsinogen (PG) and *H. pylori* antibody level, as described in the following section.

### Serologic diagnosis of metaplastic gastritis, etodolac treatment and follow-up

Sera for analyses were obtained from fasting blood samples collected from the 267 patients before endoscopy, stored at −20°C and used for the measurement of serum PG levels and *H. pylori* antibody titers. Serum PG (PGI and PGII) levels were measured using a modification (RIAbeads Kit; Dainabott, Tokyo, Japan) of our previously reported radio-immunoassay.[27] Patients with extensive CAG were diagnosed using the previously described "PG test-positive" criteria: PGI $\leq$ 70 ng/mL and PGI/II ratio $\leq$ 3.0.[28,29] These criteria have a sensitivity of 70.5% and a specificity of 97% for the diagnosis of extensive CAG using pathological diagnosis as the gold standard.[28] Serum anti-*H. pylori* immunoglobulin G titers were measured using enzyme-linked immunosorbent assay (ELISA) (MBL, Nagoya, Japan).[30] Subjects with antibody titers >50 U/mL were classified as positive (*H. pylori*-infected) and those with antibody titers $\leq$50 U/mL were regarded as negative. The sensitivity and specificity of the ELISA test used in the present study were 93.5 and 92.5%, respectively.[30]

As described previously,[31,32] the natural course of *H. pylori* infection can be classified into the following 4 groups based on the results of the 2 serum tests for PG and *H. pylori* antibody: (*i*) healthy subjects without *H. pylori* infection are PG test-negative and *H. pylori* antibody-negative; (*ii*) with the establishment of *H. pylori* infection, the antibody test becomes positive; (*iii*) as infection persists, gastric atrophy advances and the PG test also becomes positive; and (*iv*) as gastric atrophy together with intestinal metaplasia becomes extensive, this leads to a reduction in *H. pylori* load and eventually to spontaneous eradication, so the antibody test again becomes negative. Subjects with metaplastic gastritis can thus be diagnosed serologically based on a PG test-positive, *H. pylori* antibody-negative condition.

Among the 267 patients, those with a previous history of gastric cancer or adenoma; severe liver, kidney or cardiopulmonary disease; past history of gastrointestinal bleeding or peptic ulcer disease; or long-term use of adrenocortical steroids or nonsteroidal anti-inflammatory drugs (NSAIDs) were excluded from the study. In addition, patients who had a previous history of *H. pylori* eradication or renal failure and those who had been prescribed proton pump inhibitors were also excluded from the study. The remaining subjects comprised 47 patients with metaplastic gastritis diagnosed both endoscopically and serologically.

All patients received a full explanation of etodolac treatment after endoscopic mucosal resection and were given the option to undergo this treatment. Patients who consented to treatment received etodolac at 300 mg/day. Patients who rejected this option but provided consent to participate in the study were followed as controls. Patients with and without etodolac treatment were endoscopically followed for metachronous cancer development at 1 week, 4 weeks, 8 weeks and 6 months after resection. Thereafter, patients underwent regular follow-up endoscopy every 6 months. Other than endoscopic follow-up, all patients were reviewed regularly every 1–2 months by clinicians for general health condition, and patients receiving etodolac treatment were monitored for adverse events by interview and clinical laboratory evaluations. Compliance was monitored by pill counts at the time

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第3次対がん総合戦略研究事業

# がん化学予防剤の開発に関する基礎及び臨床研究

## 平成22年度～25年度　総合研究報告書

Ⅰ．　総合研究報告

Ⅱ．研究成果の刊行に関する一覧表

（　３　／　５　冊　）

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平成２６（２０１４）年３月

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Glandular stomach adenocarcinomas induced by NIAN treatment plus *H. pylori* infection were located in the pyloric region, similar to MNNG or MNU treatment plus *H. pylori* infection-induced glandular stomach adenocarcinomas in MGs.[26,27] Meanwhile, no glandular stomach cancers were observed in the groups of *H. pylori*-infected MGs without NIAN treatment, which is consistent with previous studies,[26,27] nor in the group treated with only NIAN. These findings indicated that *H. pylori* is a strong promoter of gastric carcinogenesis. Histological examination revealed that the tumors developed by NIAN + *H. pylori* were of well or moderately differentiated adenocarcinomas. Well or poorly differentiated adenocarcinomas and signet ring cell carcinomas were observed in *H. pylori*-infected MGs treated with MNNG or MNU.[26,27] Further studies are required to clarify the histological variety of stomach adenocarcinomas induced by NIAN, MNNG or MNU, since the type of cancer might depend on the genotoxic action of chemical carcinogens, rather than the effects of *H. pylori* infection.[27] In addition, tumors were observed in skin and kidney, which were suspected to spontaneously develop. The MGs have been reported to develop spontaneous skin tumors such as sebaceous and squamous cell carcinoma.[34]

Epidemiological studies have indicated that nitrate intake increases gastric cancer risk, and major sources are vegetables including Chinese cabbage, spinach and parsley.[14] Indole-3-acetonitrile, a precursor of NIAN, is distributed widely in cruciferous vegetables including Chinese cabbage and sprouts.[35] Furthermore, fava beans (*Vicia faba*), which are commonly consumed in Colombia, give rise to a potent mutagen in the presence of nitrite under acidic conditions.[36] The nitrosatable precursor of the mutagen in fava beans and the major product of nitrosation are reported to be an indole compound, 4-chloro-6-methoxyindole and an *N*-nitroso compound, 4-chloro-2-hydroxy-$N^1$-nitroso-indolin-3-one oxime, respectively.[37] Other indole compounds are also reported to produce direct-acting mutagens after nitrite treatment under acidic conditions.[38,39] In general, conversion of indole derivatives to nitrosated forms *in vitro* is known to be rapid and efficient at physiologically feasible nitrite concentrations with the low pH of the human stomach.[37] Thus, it is conceivable that nitrosation of indole compounds such as indole-3-acetonitrile probably occurs in human stomach. On the other hand, nitric oxide is suggested to be produced by activated macrophages in inflamed organs with *H. pylori* infection.[18] Therefore, nitrosation of indole compounds could be mediated by both acid catalysis and inflammatory responses in the human stomach.[18,20,37–40] On the basis of the conversion rate of NIAN from indole-3-acetonitrile under physiological conditions, the dose of NIAN used in the present study appears about 500–1000 fold the expected human exposure to NIAN *via* fresh or pickled Chinese cabbage. However, humans continually consume various kinds of foods containing indole compounds and nitrate during ordinary life. Thus, it is probable that the total amount of nitroso-indole compounds would be much closer to the dose of NIAN used in the present study. Moreover, it has been reported that low doses of chemical carcinogens, such as MNNG and MNU, could induce glandular stomach cancers in rodents under inflammation conditions including NaCl treatment and *H. pylori* infection, but hardly induce glandular stomach cancer without NaCl treatment and *H. pylori* infection. Therefore, the continuous intake of indole compounds and nitrate may play an important role for gastric carcinogenesis in East Asian countries still with a high salt consumption and *H. pylori* infection rate.

Gastric cancer is tending to decline in most countries.[41–43] One of the explanations for this tendency is the reduced prevalence of *H. pylori* infection.[42] Changes in dietary habits, mainly being lower salt consumption, could be also related to reduced gastric cancer incidence. However, the gastric cancer prevalence in East Asian countries, such as Japan and Korea, is still high.[2] At present, we have not succeeded in detecting NIAN in human bodies nor the exposure levels of the precursor, indole compounds for humans. Thus, it is necessary to estimate the human exposure levels to nitroso-indole compounds including NIAN, and to study further animal experiments and epidemiological analyses for clarification of contribution of nitroso-indole compounds under *H. pylori* infection in humans gastric carcinogenesis.

In conclusion, the present study demonstrated that NIAN can induce gastric cancer in *H. pylori*-infected MGs. It is noteworthy that nitrosatable precursors widely exist in foods. Thus, it is suggested that *N*-nitroso indole compounds including NIAN might contribute to the frequent development of gastric cancer in East Asian countries such as Japan and Korea in which the prevalence of *H. pylori* infection is relatively high. Further studies of interaction with other dietary elements appear warranted to promote the prevention of human gastric cancer.

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Table 2. Incidence of glandular stomach adenocarcinoma in MGs

| Group | Treatment | Effective No. | No. of animals with glandular stomach adenocarcinoma (%) | | |
|---|---|---|---|---|---|
| | | | Total | Well dif. | Moderately dif. |
| A | Broth | 15 | 0 (0) | 0 (0) | 0 (0) |
| B | NIAN + Broth | 22 | 0 (0) | 0 (0) | 0 (0) |
| C | *H. pylori* | 18 | 0 (0) | 0 (0) | 0 (0) |
| D | NIAN + *H. pylori* | 26 | 8 (31)* | 7 (27) | 1 (4) |

Well dif., well differentiated adenocarcinoma; Moderately dif., moderately differentiated adenocarcinoma.
*$p < 0.05$ versus group A and C and $p < 0.01$ versus group B.

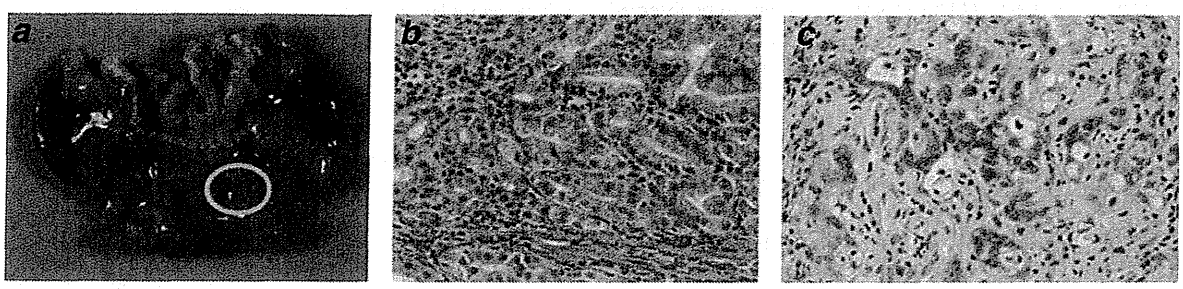


Figure 4. Histological findings of gastric adenocarcinoma in the animals treated with both NIAN and *H. pylori*. (*a*) Typical macrograph of a stomach. The yellow circle shows the suspected lesion of gastric cancer. (*b*) Well differentiated adenocarcinoma. (*c*) Moderately differentiated adenocarcinoma. (*b* and *c*) H&E staining, ×400.

the animals infected with *H. pylori* (groups C and D) (Fig. 3). Heterotopic proliferative glands, whose development is related to severe gastritis in *H. pylori*-infected MGs, were sometimes observed in *H. pylori*-infected groups (groups C and D). No gastritis was found in animals not infected with *H. pylori* (groups A and B). The gastric inflammation score in *H. pylori*-infected animals was significantly increased compared with that of animals uninfected with *H. pylori* ($p < 0.01$). There were no significant differences of gastric inflammation score between groups C and D.

### Development of glandular stomach adenocarcinomas in MGs treated with both NIAN and *H. pylori*

The observed incidences of glandular stomach adenocarcinomas are shown in Table 2. Glandular stomach adenocarcinomas, histologically featuring tubular structures with cellular atypia infiltrating into the muscle layer, were found in eight animals treated with both NIAN and *H. pylori* (8/26 = 31%) at 54–104 weeks. All adenocarcinomas were observed in the pyloric mucosa and located in the lesser curvature of the stomach, where macroscopically severe edematous thickening was also seen (Fig. 4*a*). The observed adenocarcinomas in seven animals were of well differentiated (Fig. 4*b*), and a moderately differentiated lesion was observed in one animal (Fig. 4*c*). In the animals treated with broth alone, broth + NIAN and *H. pylori* alone (groups A, B and C), no glandular stomach adenocarcinomas were observed. The incidence of glandular stomach adenocarcinomas in group D was significantly higher than that in groups A, B and C ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively).

Irrespective of NIAN treatment and *H. pylori* infection, skin tumors, which histologically were well to poor differentiated squamous cell carcinomas, sebaceous carcinomas and melanomas, were found in one animal (1/15 = 7%) in group A, three animals (3/22 = 14%) in group B, two animals (2/18 = 11%) in group C and five animals (5/26 = 19%) in group D. A hemangioma was also observed in a kidney of one animal in group D (1/26 = 4%). No significant differences were apparent in these tumor incidences among groups A–D.

## Discussion

In the present study, NIAN was found to induce glandular stomach adenocarcinomas in MGs in combination with *H. pylori* infection. NIAN-DNA adducts were also detected in the glandular stomach of MGs after treatment with NIAN, although clarification of their chemical structure(s) has yet to be performed. DNA adducts observed in the glandular stomachs of NIAN-treated MGs probably contain an indole-3-acetonitrile moiety. However, it is further likely that NIAN would act as an NO donor under aqueous conditions, thereby causing DNA modifications.[31–33] In fact, Lucas *et al.* demonstrated that NIAN can efficiently transfer nitroso groups to nucleophilic targets in purine nucleotides, causing *N*-nitrosation, deamination and the formation of a novel guanine analog, oxanine.[33]

Table 1. *H. pylori* infection induced-gastritis in MGs

| Group | Treatment | Effective No. | Stomach wet weight (g) | Inflammation score |
|---|---|---|---|---|
| A | Broth | 15 | 0.647 ± 0.097 | 0 |
| B | NIAN + Broth | 22 | 0.631 ± 0.094 | 0 |
| C | *H. pylori* | 18 | 1.432 ± 0.445* | 2.22 ± 0.43* |
| D | NIAN + *H. pylori* | 26 | 1.483 ± 0.445* | 2.38 ± 0.64* |

*$p < 0.01$ versus group A and B.
Values for results are expressed as averages ± SD.



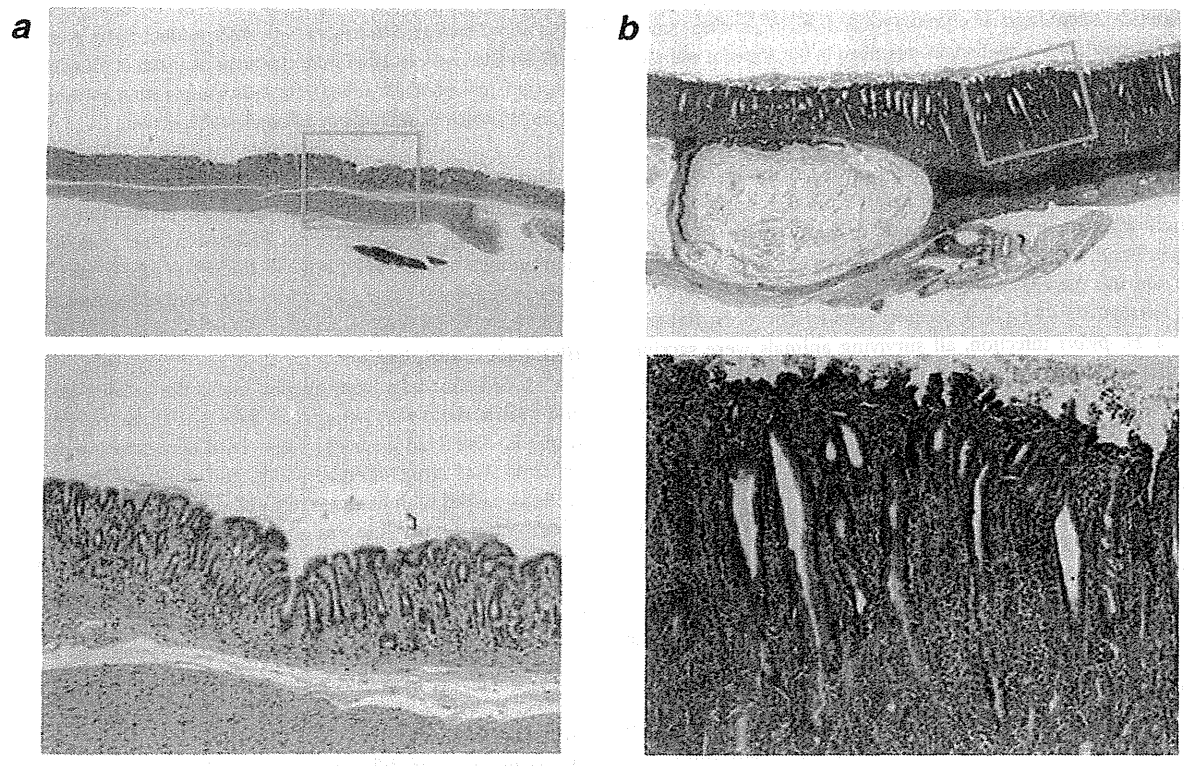


Figure 3. Macroscopic and microscopic views of gastritis in MGs infected or uninfected with *H. pylori*. (*a*) Normal gastric mucosa in group A. (*b*) Severe infiltration of many inflammatory cells with development of heterophilic proliferative glands in group C; H&E staining, ×40. Yellow boxes are shown at greater magnification below, ×200.

in total. This TLC pattern was similar to that in the *in vitro* reaction of calf thymus DNA with NIAN (total adduct level of 4.8 adducts/$10^7$ nucleotides, Fig. 2*b*). In the case of DNA samples derived from control animals, no adduct spots were seen on the TLC sheets (Fig. 2*c*).

### Macroscopical and microscopical observation of *H. pylori*-induced gastritis in MGs

MGs were sacrificed until 104 weeks after *H. pylori* infection, and gastric disorders were analyzed. Stomach wet weights and gastric inflammation scores are shown in Table 1. Macroscopically, edematous thickening with hemorrhagic spots was apparent in the gastric mucosa in *H. pylori*-infected MGs (groups C and D), but not in animals uninfected with *H. pylori* (groups A and B). The stomach wet weight, reflecting edematous thickening, in animals infected with *H. pylori* (groups C and D) was significantly increased compared with that of animals not infected with *H. pylori* (groups A and B) ($p < 0.01$). No significant differences of stomach wet weight were detected between groups A and B and also between groups C and D.

Microscopically, gastritis, featuring infiltration of many inflammatory cells, and hyperplastic change of glandular epithelium, and erosion were observed in the pyloric regions of

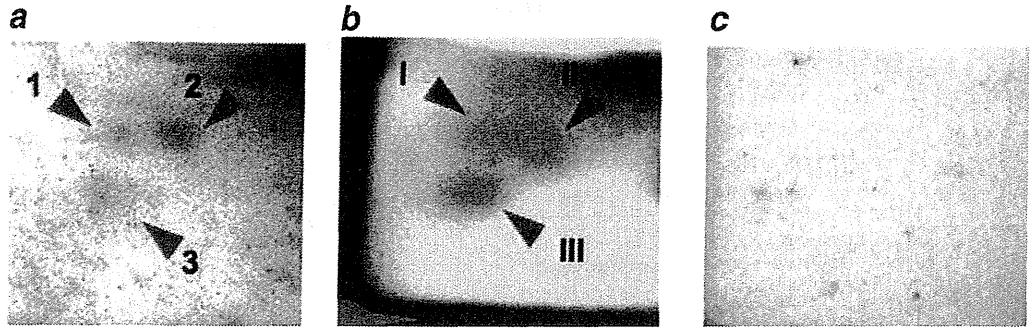


Figure 2. Autoradiograms of NIAN-DNA adducts in glandular stomach of MGs or calf thymus DNA treated with NIAN. Adducts were analyzed by $^{32}$P-postlabeling method, as described in the Material and Methods. DNA samples were isolated from glandular stomach of MGs (*a*) or calf thymus DNA (*b*) after treatment with NIAN. DNA samples were also prepared from glandular stomach of MGs without NIAN treatment (*c*). Arrowheads indicate adducts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

animals of groups C and D were given an intragastric inoculation of *H. pylori* broth culture (0.5 ml, 0.9 × $10^8$ CFU/animal) whereas animals of groups A and B were given sterilized broth alone (0.5 ml).[28]

During the experiments, animals which became moribund or emaciated (<80 g body weight) were sacrificed. At 104 weeks after *H. pylori* infection, all surviving animals were sacrificed under ether anesthesia. At performance of necropsy, all tissues were carefully checked macroscopically and the stomachs and major organs were removed and assessed for macroscopic lesion development. Effective numbers of animals were defined as those surviving until week 54 of the study, when gastric tumors were observed for the first time. In addition, in the *H. pylori*-infected groups, the animals developing gastritis observed on histological examination were regarded as effective. The percentages of gastritis-bearing animals by the single inoculation of *H. pylori* were 62% for group C and 76% for group D, being similar to those previously reported.[27] All animal experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

### Detection of DNA adducts by $^{32}$P-postlabeling method

Calf thymus DNA (0.5 mg, Sigma, St. Louis, MO) treated with NIAN (3 mg) for 12 hr under neutral conditions was used for authentic NIAN-DNA adducts.[23] DNA samples from the glandular stomach of MGs and calf thymus DNA samples were digested with micrococcal nuclease and phosphodiesterase II, and subjected to $^{32}$P-postlabeling analysis using the same procedure as described previously[23] except with solvent systems for two-dimensional development. The solvent system consisted of buffer A (4.0 M lithium formate, 7.7 M urea, pH 3.5) from bottom to top, and buffer B (0.90 M lithium chloride, 0.45 M Tris-HCl, 7.7 M urea, pH 8.0) from left to right, followed by 1.7 M sodium phosphate buffer, pH 6.0, from left to right, with 3.5 cm filter paper. Adducts were detected with a Bio-Image Analyzer (BAS 3000; Fuji Photo Film, Tokyo, Japan) after exposing the TLC sheets to Fuji imaging plates. Relative adduct labeling was determined by the methods of Reddy *et al.*,[29] and values were calculated as averages using data from three assays.

### Histological examination

All excised stomachs were opened along the greater curvature and washed twice with saline, then fixed in 10% neutral-buffered formalin. The fixed stomachs were sliced along the longitudinal axis into 9–12 strips of equal width, and routinely processed to sections stained with hematoxylin and eosin (H&E). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System,[30] by scoring the infiltration of neutrophils and mononuclear cells. Other organs, in which macroscopic lesions were observed, were also fixed in 10% neutral-buffered formalin and routinely processed to sections stained with H&E for histological examination.

### Statistical analysis

The significance of differences in quantitative data for gastric inflammation, gastric adenocarcinoma and tumors of other organs was analyzed by Fisher's exact test. Data for stomach wet weight and inflammation score were examined using Tukey's multiple comparison test. Significance was concluded at $p < 0.05$.

## Results

### DNA adduct formation by NIAN administration in the glandular stomach of MGs

To confirm the formation of NIAN-DNA adducts in the glandular stomach of MGs, NIAN was injected two times a week at a dose of 100 mg/kg by gavage, and then analyzed by $^{32}$P-postlabeling method. Three adduct spots were observed in DNA samples derived from NIAN-treated animals (Fig. 2*a*). The adduct levels were 0.3 for adduct 1, 1.1 for adduct 2, 0.2 for adduct 3 and 1.6 adducts/$10^8$ nucleotides

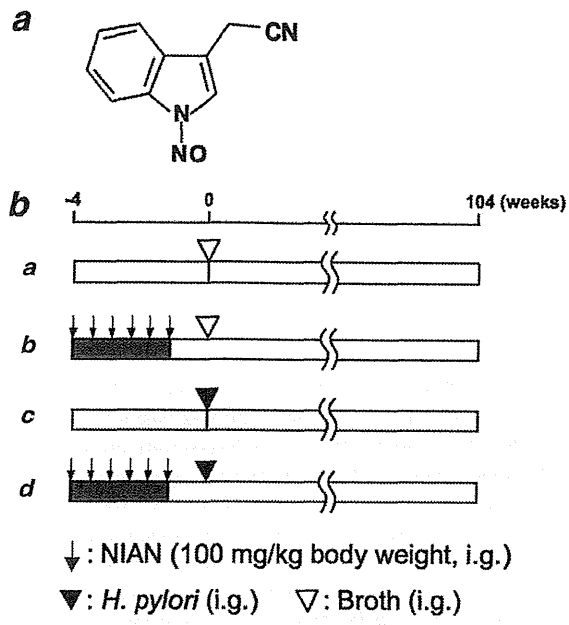


**Figure 1.** Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after the final administration, the animals were inoculated with *H. pylori* (ATCC 43504) (groups C and D) or sterilized broth (groups A and B).

acetonitrile with nitrite under acidic conditions, is a direct-acting mutagen in *S. typhimurium* and Chinese hamster lung cells,[20–22] and it is confirmed to form DNA adducts and to induce DNA single-strand scission in the rat glandular stomach.[23,24] Therefore, NIAN could play some role in gastric cancer development, as in the case of the well-known direct-acting mutagens, MNNG and MNU, in animal experiments.[16,17,25]

The Mongolian gerbil (MG) is reported to be susceptible to colonization by *H. pylori*, and *H. pylori* infection greatly enhances MNNG or MNU-induced gastric carcinogenesis in MGs.[26,27] Therefore, the MG is considered to be a useful animal model for evaluating the gastric cancer risk of direct-acting *N*-nitroso compounds, with or without *H. pylori* infection.

Chinese cabbage, containing nitrate and indole compounds, is commonly consumed in East Asian countries, including Japan, Korea and China, in which gastric cancer mortality is very high. In the present study, DNA adducts were detected with NIAN treatment in the glandular stomach of MGs, and the carcinogenicity of NIAN for gastric cancer *in vivo* was examined. The results clearly demonstrated that gastric cancer developed with a combination of NIAN administration and *H. pylori* infection in MGs. Possible involvement of indole compounds and nitrate derived from various foodstuffs, including Chinese cabbage, in gastric cancer development in humans is discussed.

## Material and Methods

### Materials

Indole-3-acetonitrile was purchased from Tokyo Food Techno (Tokyo, Japan), sodium nitrite from Wako Pure Chemical Industries (Osaka, Japan) and ammonium sulfamate from Kanto Chemical (Tokyo, Japan). Brucella broth was obtained from Becton Dickinson (Cockeysville, MD) and horse serum from Nippon Bio-Supply (Tokyo, Japan).

### Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1*a*. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hr at room temperature in the dark, as previously reported.[21] Nitrosation was stopped by addition of ammonium sulfamate at a final concentration of 50 mM. The reaction solution was filtered and the residue was washed with deionized water, then with *n*-hexane. The residual paste was dried and stored at −80°C until use. The preparation was >93% pure as judged by its UV absorbance on HPLC.

### Bacterial culture

*H. pylori* (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured in brucella broth supplemented with 10% heat-inactivated horse serum for 24 hr at 37°C under microaerobic conditions (5% $O_2$, 10% $CO_2$ and 85% $N_2$), as previously described.[28]

### Animal treatment

Specific pathogen-free male, 6-week-old MGs (MGS/Sea, Kyudo, Fukuoka, Japan) were housed in a biohazard room, air-conditioned at 24°C ± 2°C and 55% humidity, on a 12 hr light–dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and water.

To analyze the formation of DNA adducts in the glandular stomach of MGs by NIAN treatment, NIAN was dissolved in 50% dimethyl sulfoxide (DMSO), and administered to three MGs by gavage of 0.5 ml solution, two times a week at a level of 100 mg/kg body weight. Two further MGs served as a control group receiving the solvent alone (0.5 ml). At 8 hr after administration of NIAN, both groups of animals were sacrificed under ether anesthesia, and their stomachs were resected and stored at −80°C until use. DNA was extracted by a standard procedure with enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

The protocol for long-term gastric carcinogenicity in MGs treated with NIAN + *H. pylori* infection is illustrated in Figure 1*b*. The animals were randomly divided into four groups (groups A–D). Groups A and C were given 50% DMSO without NIAN (0.5 ml) whereas groups B and D were orally administrated NIAN (0.5 ml, 100 mg/kg body weight) dissolved in 50% DMSO by gavage, two times a week for 3 weeks. At one week after the last administration, the

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# Induction of glandular stomach cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-nitrosoindole-3-acetonitrile

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***Helicobacter pylori* (*H. pylori*) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/$10^8$ nucleotides in total), detected in DNA samples from the glandular stomach by $^{32}$P-postlabeling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for 3 weeks, induced well—and moderately—differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under *H. pylori* infection at 54–104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection.**

Carcinogenesis

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia, and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3–5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6,7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8–10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12–14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19,20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde as mutagen precursors. 1-Nitrosoindole-3-acetonitrile (NIAN), an *N*-nitroso-substituted compound formed by treatment of indole-3-

**Key words:** gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile

**Abbreviations:** DMSO: dimethyl sulfoxide; H&E: hematoxylin and eosin; *H. pylori*: *Helicobacter pylori*; MG: Mongolian gerbil; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU: *N*-methyl-*N*-nitrosourea; NIAN: 1-nitrosoindole-3-acetonitrile.

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polymerase II (42) and/or possible overexpression of Dnmt3b might be involved in methylation induction.

In conclusion, inflammation due to infection of *Helicobacter* strains had a high capacity to induce methylation in GECs, regardless of their CagA status. Increased cell proliferation was not sufficient for methylation induction. Therefore, specific types of inflammation, characterized by infiltration of mononuclear cells and expression of specific inflammation-related genes, along with increased cell proliferation were considered to be necessary for methylation induction.

## Supplementary material

Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

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respectively) also in human gastric mucosae (Figure 5). However, *IL1B* expression tended to be lower in gastric mucosae of *H.pylori*-infected individuals.

## Discussion

Among the five groups with inflammation, aberrant methylation was induced only in the three *Helicobacter* groups, which showed inflammation with infiltration of mononuclear cells, increased expression of *Il1b*, *Nos2* and *Tnf* and increased cell proliferation. In the EtOH and NaCl groups, these agents were administered repeatedly for 20 weeks, and increased cell proliferation was present at the end of the experiment. The increased proliferation was considered to have persisted for this period because thickening of lamina propria was observed in these two groups. Nevertheless, aberrant methylation was not induced, at least in the CGIs analyzed here. This showed that cell proliferation alone is not sufficient for methylation induction and suggested that both specific types of inflammation and increased cell proliferation are necessary for induction of aberrant methylation.

The inflammation induced in the *Helicobacter* groups was characterized by infiltration of mononuclear cells (lymphocytes and macrophages). In our previous study, suppression of T-cell activation by cyclosporin A remarkably repressed inflammatory response and methylation induction triggered by *H.pylori* infection (14), showing that T-cell activation is involved in methylation induction in this system. However, our recent study in mouse colon demonstrated that aberrant methylation can be induced even in severe combined immunodeficiency mice, which lack functional T and B cells, by dextran sulfate sodium-induced colitis (Katsurano *et al.*, submitted for publication). It is known that, even in severe combined immunodeficiency mice, colitis with macrophage infiltration can be induced (36). If a common mechanism for methylation induction is present in *H.pylori*-infected gastric mucosae and dextran sulfate sodium-treated colonic mucosae, infiltration of macrophages is a candidate for the proximate effector that transmits signal for methylation induction to epithelial cells. It can be considered that, in *H.pylori*-infected gastric mucosae, activation of T cells is required only for the initiation or maintenance of inflammation capable of inducing aberrant DNA methylation.

Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* were specifically upregulated in the three *Helicobacter* groups. These three genes are reported to be overexpressed also in human chronic inflammation associated with cancers, such as ulcerative colitis and hepatitis (37–40). *IL1B* promoter polymorphism is associated with risk of human gastric cancers (28) and aberrant methylation of multiple genes in gastric cancers (29). The lack of its upregulation in human gastric mucosae infected with *H.pylori* could be because most of them had superficial gastritis and had already increased *IL1B* expression. *NOS2*, which encodes nitric oxide synthase, was upregulated *in vitro* by administration of IL1B and nitric oxide donors induced methylation of *FMR1* and *HPRT* (41). These suggest that IL1B and NOS2 might be involved in methylation induction. On the other hand, *Ifng*, *Il2*, *Il4* and *Il6* were upregulated mainly in the EtOH and NaCl groups, in which no methylation was induced, and also in the SS1 and HF groups, in which methylation induction levels were lower than in the ATCC group. This suggested a possibility that some (one) of the genes could suppress methylation induction.

SS1 and *H.felis*, which lack CagA, were capable of inducing aberrant methylation although the capacity was weaker than the CagA-positive strain (*H.pylori* ATCC 43504). CagA-positive *H.pylori* strains are known to induce severe gastritis in Mongolian gerbils (16) as confirmed in this study, and this explains their stronger capacity to induce methylation. The three inflammation-related genes associated with methylation induction (*Il1b*, *Nos2* and *Tnf*) had the highest expression in the ATCC group among the three *Helicobacter* groups. CagA-positive *H.pylori* seems to promote methylation induction by maximizing expression of such genes and minimizing expression of genes that suppress methylation induction.

Dnmts are the final effectors to methylate DNA, and their overexpression was observed in various human cancers (35). Immunohistochemical analyses here revealed that Dnmt1 was upregulated in gastric mucosae of gerbils in the three *Helicobacter*-infected groups and the NaCl-treated group. However, the highest expression was observed in the NaCl group, where methylation was not induced. This result indicated that expression of Dnmt1 was not associated with methylation induction but with cell proliferation. Expression of Dnmt3a was significantly but slightly decreased in the ATCC group and this also suggested that the expression itself is not involved in aberrant methylation induction. However, due to the lack of an appropriate antibody, we were not able to exclude the possibility that upregulation of Dnmt3b is involved in methylation induction. Therefore, disturbance in the local balance between Dnmts and factors that protect DNA from aberrant methylation, such as the presence of RNA

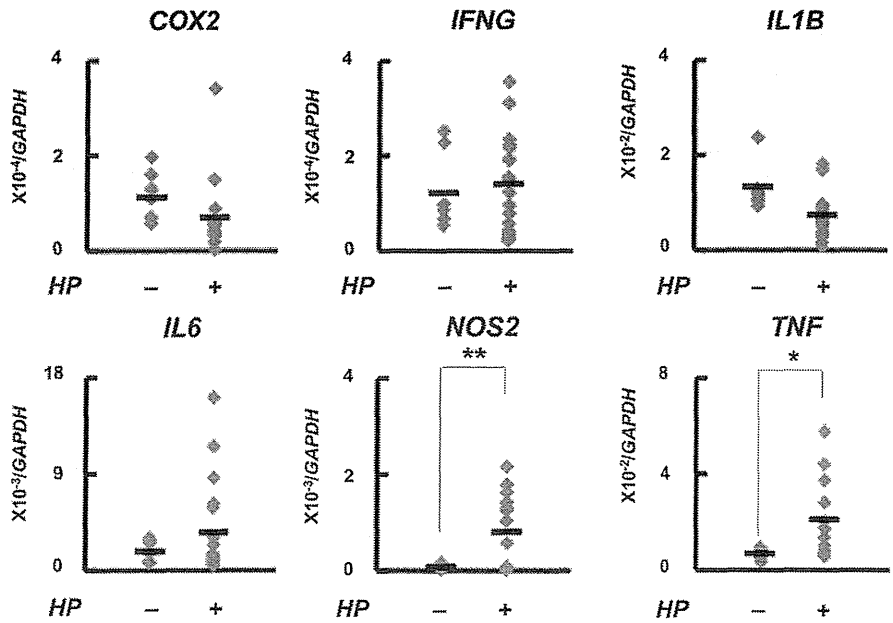


**Fig. 5.** Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

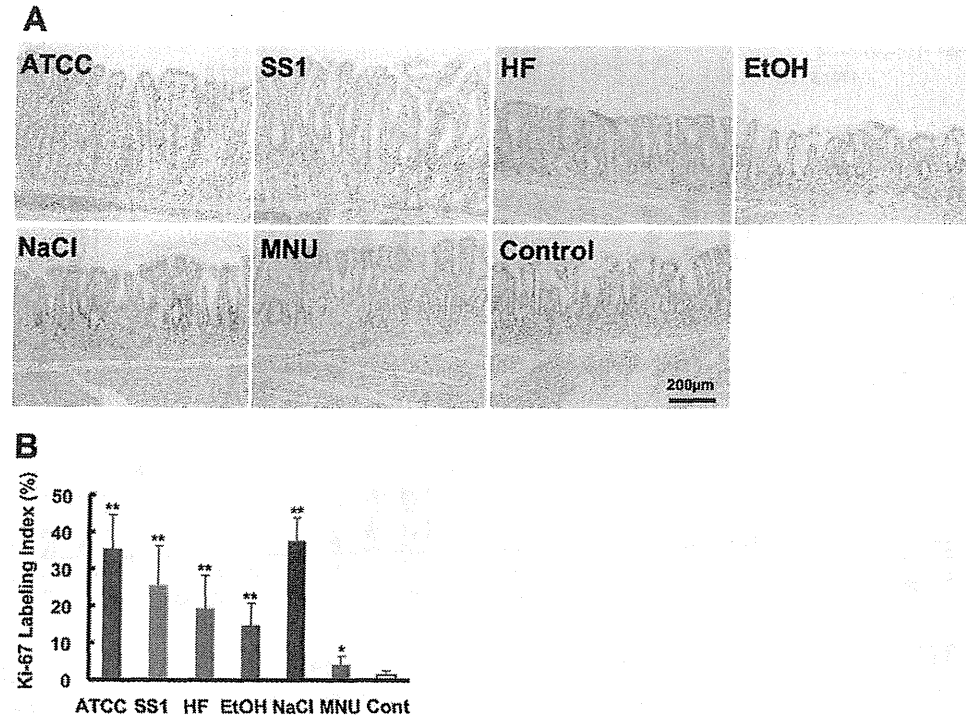


**Fig. 3.** Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase of cell proliferation.

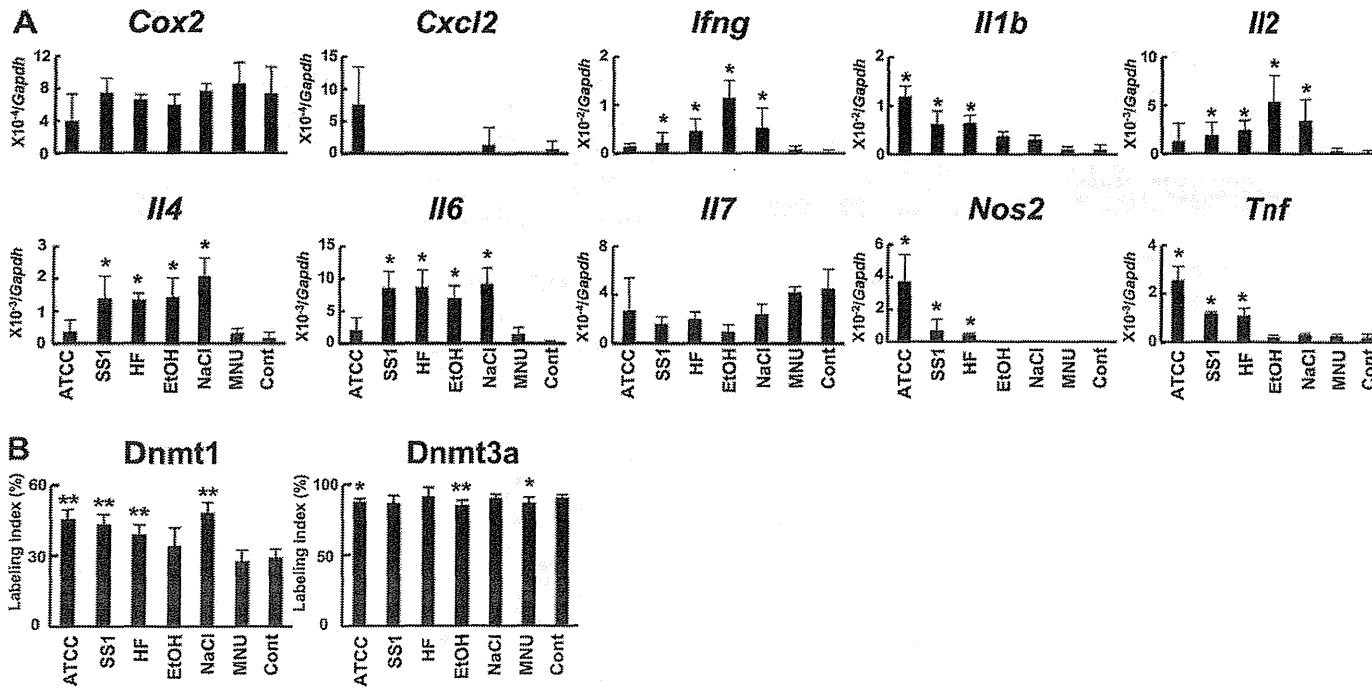


**Fig. 4.** Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* groups. (**B**) The fractions of GECs expressing Dnmt proteins in gastric glands by immunohistochemistry. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

*Human relevance of inflammation-related gene expression*

To address whether upregulation of specific inflammation-related genes are common in the human stomach, we conducted qRT–PCR of *COX2*, *IFNG*, *IL1B*, *IL6*, *NOS2* and *TNF* using human gastric mucosa samples with and without *H.pylori* infection. Expression levels of *NOS2* and *TNF* were markedly upregulated (27- and 3-fold,