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Risk of gastric cancer in asymptomatic, middle-aged Japanese subjects based on serum pepsinogen and *Helicobacter pylori* antibody levels

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A total of 5,209 asymptomatic, middle-aged subjects, whose serum pepsinogen (PG) and *Helicobacter pylori* antibody levels had been assessed, were followed for 10 years. Subjects with positive serum *H. pylori* antibodies (>50 U/mL) had an increased cancer risk (HR = 3.48, 95% CI = 1.26–9.64). Risk of gastric cancer increased as the antibody level increased; the *H. pylori*-positive group with antibody levels >500 U/mL had the highest incidence rate (325/100,000 person-years). Cancer development also increased with a reduced serum PG I level or a reduced PG I/II ratio; the risk was significantly elevated with serum PG I level ≤30 ng/mL (HR = 3.54, 95% CI = 1.95–6.40) or PG I/II ratio ≤3.0 (HR = 4.25, 95% CI = 2.47–7.32). Furthermore, the risk of diffuse-type cancer increased as PG II level increased; it was significantly elevated with PG II level ≥30 ng/mL (HR = 3.81, 95% CI = 1.10–13.21). Using *H. pylori* antibody and PG levels, subgroups with an especially high or low cancer incidence rate could be identified. *H. pylori*-negative or indeterminate subjects with low PG level (PG I ≤30 ng/mL or PG I/II ratio ≤2.0) or *H. pylori*-positive subjects with antibody levels >500 U/mL and a low PG level were among the subgroups with a high cancer incidence rate (over 400/100,000 person-years). In contrast, *H. pylori*-negative subjects with a PG I level >70 ng/mL or a PG I/II ratio >3.0 had the lowest risk; none of these subjects developed cancer. Thus, serum PG levels and/or *H. pylori* antibody levels can be used to predict the risk of cancer in individuals with *H. pylori*-related gastritis from the general population.

Key words: gastric cancer; pepsinogen; *Helicobacter pylori*; chronic atrophic gastritis; intestinal metaplasia

Stomach carcinogenesis in high-risk populations, including the Japanese population, is believed to begin with chronic active inflammation of the stomach mucosa, proceeding to extensive atrophy together with intestinal metaplasia, then to dysplasia, and finally to cancer.[1] Currently, *Helicobacter pylori* (*H. pylori*) is considered a major factor in the establishment of the carcinogenic sequence in the stomach.[2–12] *H. pylori*-related gastritis usually starts in the antrum and expands proximally towards the body of the stomach.[13–15] During this process, both multifocal atrophy and intestinal metaplasia develop, eventually leading to chronic atrophic gastritis (CAG) with extensive intestinal metaplasia. Several studies dealing with endoscopic biopsies or chromoendoscopic testing found that the progression of CAG increases the risk for cancer.[12,16–20] Thus, an accurate and reliable evaluation of the extent of CAG is considered to be important for identifying individuals at high risk of cancer. The diagnosis of CAG is based on histopathology of the stomach mucosa. However, gastric endoscopy with biopsy is an invasive test and inappropriate for mass population screening. In addition, since CAG together with intestinal metaplasia is a multifocal process, it is difficult to accurately diagnose the extent of CAG based on a few biopsy samples. Furthermore, histological diagnosis of gastric atrophy depends on subjective judgment without a gold standard.[21,22] Thus, a test for CAG progression that is more convenient, free of discomfort or risk, economical and based on objective parameters is needed.

There is general agreement that serum pepsinogen (PG) levels reflect the morphological and functional status of the stomach mucosa.[23,24] We have previously shown that a reduction in the area of fundic gland mucosa with CAG progression was well correlated with a stepwise reduction in the serum PG I level or PG I/II ratio, and that the PG levels are considered reliable markers for CAG progression.[25] Therefore, it is highly probable that these serum tests can effectively identify a cancer-susceptible population. Indeed, previous seroepidemiological studies revealed that the risk of gastric cancer was significantly increased in subjects with extensive CAG diagnosed by PG levels.[26–30] These results strongly indicate that the serum PG levels reflect an individual's risk for gastric cancer. However, there have been few reports describing the relationship between PG levels and the cancer incidence rate. Meanwhile, the extent and severity of mucosal inflammation is believed to be involved in a series of molecular events that lead to cancer and that depend on multiple bacterial and host factors, including the immune response.[31,32] To date, several animal model and human studies have indicated that serum *H. pylori* antibody levels reflect the severity of *H. pylori*-related gastritis.[33–36] Thus, cancer development may be related to the serum antibody level. In the present study, the correlation between the gastric cancer incidence rate and serum PG levels and/or *H. pylori* antibody levels was assessed in a middle-aged, general male population based on the results of a 10-year longitudinal cohort study.

Material and methods

Study population

The subjects were 5,706 male employees (mean age [SD], 50.4 [5.4] years; range, 40–60 years) who had an annual multiphasic health checkup at their workplace in Wakayama City, located in the western part of Japan. In 2005, the gastric cancer mortality

Abbreviations: CAG, chronic atrophic gastritis; CI, confidence interval; DR, digital radiography; ELISA, enzyme-linked immunosorbent assay; *H. pylori*, *Helicobacter pylori*; HR, hazard ratio; IgG, immunoglobulin G; PG, pepsinogen; SD, standard deviation.

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TABLE I – BASELINE CHARACTERISTICS OF THE SUBJECTS STRATIFIED BY SERUM *H. pylori* ANTIBODY LEVEL

	Total	*H. pylori* antibody level (U/ml)					p (trend)[1]
		Negative <30	Indeterminate ≧30 and ≦50	Positive			
				Total	>50 and ≦500	>500	
Total Subjects	5209	999	554	3656	2817	839	
Person-years	50426	9784	5540.5	35101.5	27092	8009.5	
Age [mean (SD)]	49.2 (4.7)	48.3 (4.5)	46.9 (5.1)	49.8 (4.6)[2]	49.8 (4.6)[2]	49.7 (4.6)[2]	
Follow-up years [mean (SD)]	9.7 (0.9)	9.8 (0.7)	10.0 (0.2)	9.6 (1.0)	9.6 (0.9)	9.6 (1.1)	
PG I [mean (SD)]	60.6 (30.4)	58.2 (20.7)	55.9 (30.0)[2]	62.0 (32.5)[2]	61.2 (32.3)[2]	64.9 (33.2)[2]	
PG II [mean (SD)]	17.0 (10.6)	9.6 (4.4)	11.0 (6.6)[2]	20.0 (11.0)[2]	19.3 (10.6)[2]	22.5 (11.8)[2]	
PG I/II [mean (SD)]	4.2 (2.1)	6.4 (1.7)	5.6 (1.9)	3.4 (1.6)[2]	3.5 (1.7)[2]	3.1 (1.4)[2]	
Total gastric cancer							
Age [mean (SD)]	50.8 (3.7)	51.0 (3.5)	51.8 (2.1)	50.8 (3.9)	51.6 (3.7)	49.7 (3.9)	
Follow-up years [mean (SD)]	6.0 (2.6)	6.4 (3.6)	7.8 (1.0)	5.9 (2.5)	5.4 (2.6)	6.4 (2.5)	
Cases/incidence rate[3]	63/125	4/41	4/72	55/157	29/107	26/325	
HR (95%CI)		1	3.16 (0.75–13.22)	3.48 (1.26–9.64)	3.10 (0.94–10.21)	9.56 (2.89–31.65)	<0.0001
PG I [mean (SD)]	53.1 (43.3)[4]	35.5 (12.5)	35.7 (13.2)	55.6 (45.7)[2,4]	48.4 (28.3)[2,4]	64.3 (59.9)[2]	
PG II [mean (SD)]	21.6 (18.1)[4]	19.0 (3.9)	18.3 (9.7)	22.0 (19.2)	17.6 (9.2)	26.9 (25.5)	
PG I/II [mean (SD)]	2.7 (1.5)[4]	1.9 (0.7)	3.0 (3.0)[2]	2.7 (1.4)[2,4]	2.8 (1.4)[2,4]	2.6 (1.5)[2]	
Intestinal-type gastric cancer							
Cases/incidence rate[3]	42/83	3/31	3/54	36/103	19/70	17/212	
HR (95%CI)		1	3.84 (0.70–20.97)	2.98 (0.91–9.72)	2.98 (0.69–12.82)	9.16 (2.11–39.78)	0.0009
PG I [mean (SD)]	47.5 (47.2)[4]	34.6 (15.1)	37.7 (5.4)	49.4 (50.6)[2,4]	39.6 (21.3)[4]	60.2 (69.6)[2]	
PG II [mean (SD)]	20.1 (20.9)[4]	20.6 (2.7)	14.5 (7.4)[2]	20.6 (22.5)	14.1 (4.7)[2,4]	27.8 (31.3)[2]	
PG I/II [mean (SD)]	2.6 (1.7)[4]	1.7 (0.8)	3.7 (3.2)[2]	2.7 (1.6)[2,4]	2.9 (1.5)[2]	2.4 (1.7)	
Diffuse-type gastric cancer							
Cases/incidence rate[3]	21/42	1/10	1/18	19/54	10/37	9/112	
HR (95%CI)		1	1.85 (0.12–29.54)	4.99 (0.66–37.45)	3.36 (0.43–26.39)	10.37 (1.31–82.14)	0.019
PG I [mean (SD)]	64.3 (32.4)[4]	38.2	29.7	67.5 (32.4)[2,4]	62.8 (34.7)[2]	72.8 (30.8)[2,4]	
PG II [mean (SD)]	24.4 (10.0)[4]	14.1	29.7[2]	24.7 (10.2)[2]	24.2 (12.0)[2]	25.2 (8.6)[2]	
PG I/II [mean (SD)]	2.7 (1.1)[4]	2.7	1.1	2.8 (1.1)	2.6 (1.5)[4]	2.9 (0.7)	
Lung cancer							
Age [mean (SD)]	49.0 (4.7)	47.3 (5.4)	45.0 (7.1)	50.0 (4.2)	50.0 (4.2)	0	
Follow-up years [mean (SD)]	7.6 (1.1)	7.8 (0.5)	7.0 (1.4)	7.5 (1.3)	7.5 (1.3)	0	
Cases/incidence rate[3]	20/40	4/41	2/36	14/40	14/52	0	
HR (95%CI)		1	0.81 (0.26–2.62)	0.94 (0.33–3.25)	1.57 (0.62–3.93)	0	
PG I [mean (SD)]	74.7 (45.9)[4]	55.0 (16.9)	78.3 (18.7)	79.9 (53.3)[2]	79.9 (53.3)	0	
PG II [mean (SD)]	17.5 (10.8)	8.8 (2.8)	12.5 (1.1)	20.6 (11.5)[2]	20.6 (11.5)	0	
PG I/II [mean (SD)]	4.8 (2.0)	6.5 (1.6)	6.3 (0.9)	4.1 (1.8)[2]	4.1 (1.8)	0	

[1]Trend for the increase in HR with increase in serum *H. pylori* antibody level.–[2]Significantly different from the negative subjects ($p < 0.05$).–[3]Per 10.000 person-years.–[4]Significantly different from the total subjects in each subgroup stratified by serum anti-*H. pylori* antibody level ($p < 0.05$).

rate for the area was 53.0/100,000 person-years, compared with 39.9/100,000 person-years for the whole of Japan. In fact, in the same year, Wakayama ranked fourth in terms of gastric cancer mortality among the 47 administrative divisions of Japan. Between April 1994 and March 1995, fasting blood samples were collected as part of the routine laboratory tests for a general health checkup (baseline). Aliquots of the separated sera were stored below −20°C until they were used. In Japan, health checkup programs are done to identify selected diseases in their early stage of development. Therefore, subjects who had specific symptoms, required prompt medical care or had a previous gastric resection were excluded from the study. Symptom-free subjects took part in the following tests and procedures: an interview to determine their general health status, a physical examination, chest X-rays, an electrocardiogram, blood laboratory tests, urinalysis and a fecal occult blood test.

Serologic analysis

Serum PG levels were measured using PG I/PG II RIA-Bead Kits (Dainabbot Co., Tokyo, Japan), which involve a modified radioimmunoassay method that we previously established.[37] Subjects with extensive CAG were diagnosed on the basis of the previously described PG test-positive criteria (PG I $\leq$70 μg/L and PG I/II ratio $\leq$3.0).[38,39] These criteria have a sensitivity of 70.5% and a specificity of 97%.[38] Subjects who had been prescribed medications that might affect gastrointestinal function, such as proton pump inhibitors or nonsteroidal antiinflammatory drugs, prior to the examination, and subjects who had undergone *H. pylori* eradication therapy were excluded from the analysis of the PG test results. Anti-*H. pylori* IgG titers were measured using ELISA (MBL, Nagoya, Japan). Subjects with antibody titers >50 U/mL were classified as *H. pylori*-infected; those with antibody titers <30 U/mL were regarded as infection negative; and subjects with a titer level that was $\geq$30 and $\leq$50 U/mL were considered indeterminate. The sensitivity and specificity of the ELISA used in the present study was 93.5% and 92.5%, respectively.[40] The *H. pylori*-infected group was further divided into 2 subgroups depending on the antibody titer: a low-titer group and a high-titer group, which included subjects with titers >500 U/mL.

Surveillance method

The subjects were screened annually to identify incident gastric cancer cases during the 10-year period between April 1994 and March 2004. Gastric cancer surveys were conducted using a combination of screening methods; all subjects were screened using the PG test and double contrast barium digital radiography (DR). The DR system includes remote-controlled X-ray fluoroscopy (TU-230XB, Hitachi Medico, Tokyo, Japan) and real-time digital radiography (DR-2000H, Hitachi Medico). A total of 150 mL of high-concentration barium (200 w/v %) was used for the double contrast upper-gastrointestinal X-ray series; 11 films were taken for each subject. This system is superior to conventional barium X-ray in image quality and diagnostic accuracy; the cancer detection rate by DR was 1.9 times higher than by conventional screening.[41] Those with positive X-ray findings and/or a positive PG test based on the aforementioned criteria were further examined by panendoscopy (Types XQ200, Olympus, Tokyo, Japan). On the basis of their location, the detected cancers were classified into cardia or non-cardia. The resected specimens obtained by endoscopy or surgery were assessed histopathologically and classified using Lauren's classification into intestinal-type or diffuse-type.[42] The incident day of gastric cancer was defined as the day of the health checkup when the cancer was detected. The length of the observation period was calculated for each subject from the time of the baseline survey to the diagnosis of gastric cancer. The incidence of various cancers (including gastric cancer) and resultant death in the cohort was determined and analyzed yearly and was confirmed by workplace clinic and health insurance records. The ethics committee of Wakayama Medical University approved the protocol, and informed consent was obtained from all participating subjects.

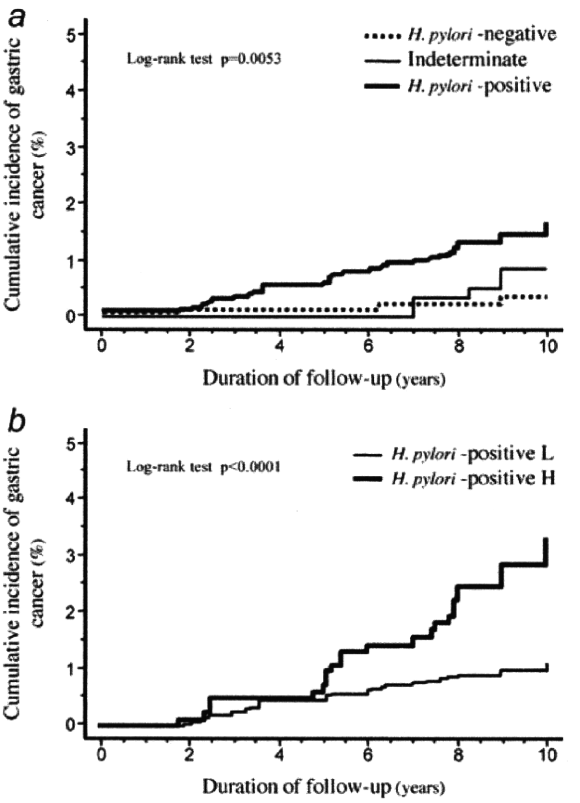


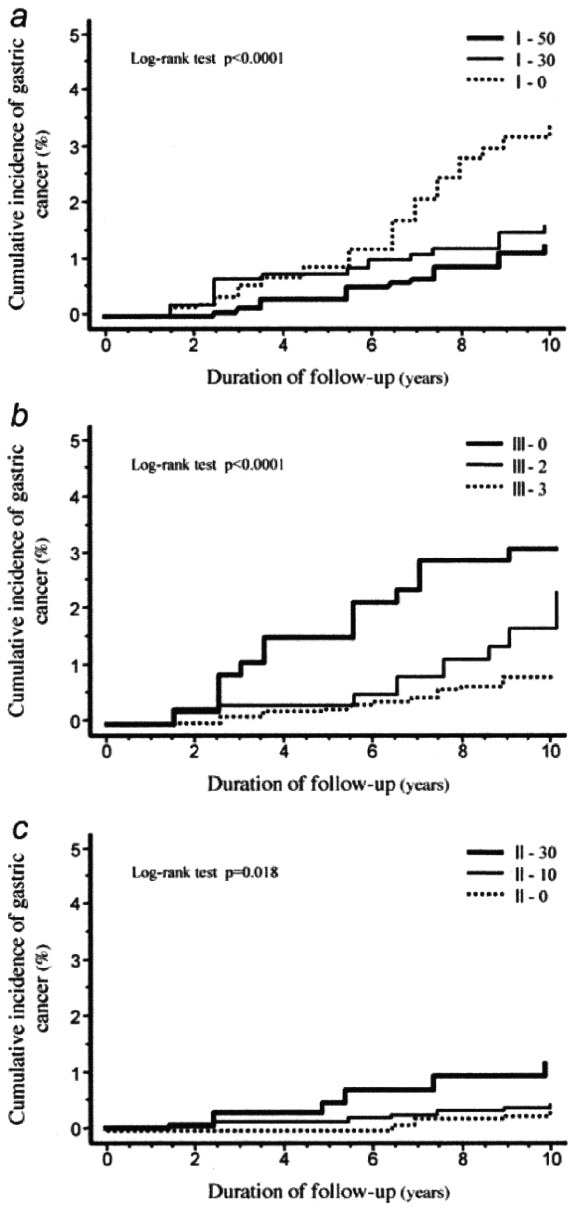
FIGURE 1 – Risk of gastric cancer and *H. pylori* infection. (*a*) The development of gastric cancer and serum *H. pylori* antibody levels. Subjects were classified into 3 groups (*H. pylori*-negative, indeterminate, and *H. pylori*-positive) based on the serum *H. pylori* antibody level as described in the text. The cumulative incidence of gastric cancer in the 3 groups was plotted using the Kaplan-Meier analysis; the differences between the groups were assessed using the log-rank test. The cancer incidence rates for the *H. pylori*-negative, indeterminate, and *H. pylori*-positive groups were 41/100,000 person-years, 72/100,000 person-years, and 157/100,000 person-years, respectively. The differences between the *H. pylori*-positive group and the other 2 groups were significant ($p = 0.0053$). (*b*) The development of gastric cancer in the *H. pylori*-positive group based on antibody levels. The *H. pylori*-positive group was divided into 2 subgroups: the *H. pylori*-positive L (low-titer) subgroup and the *H. pylori*-positive H (high-titer) subgroup based on the titer levels as described in the text. The cumulative gastric cancer incidence was analyzed using the Kaplan-Meier analysis. The cancer incidence rates for the *H. pylori*-positive L and H subgroups were 107/100,000 person-years and 325/100,000 person-years, respectively. The difference between the 2 subgroups was significant ($p < 0.0001$).

Statistical analysis

The data were analyzed using SPSS 11.0 (SPSS, Chicago, IL) and STATA (STATA Corp., College Station, TX). The differences were tested for significance using the *t* test for comparisons between 2 groups, analysis of variance (ANOVA) for comparisons among multiple groups, and Scheffe's LSD test for comparisons of pairs of groups. The χ^2 test was used to compare categorical variables. The long-term effects of the serum PG levels and the *H. pylori* antibody titer levels on gastric cancer development were evaluated using Cox proportional hazards models.

Results

Among the 5,706 eligible subjects, those who met the exclusion criteria were not included in the study. The 8 cases of gastric cancer that developed within the first year of the study were also excluded. Table I shows the baseline characteristics of the remaining 5,209 study subjects. The mean (SD) age of the subjects at the start of the study was 49.2 (4.7) years, and the mean (SD) follow-up period was 9.7 (0.9) years. During the study period, 63 cases of gastric cancer developed (incidence rate = 125/100,000 person-years) and 7 patients died from the cancer. To date, nearly 4 years after the study, there has not been a single cancer case that is believed to have escaped detection by the annual screening during the study period. Macroscopically, only 2 cancers were located in the cardia; most (96.8%) were non-cardia cancers. Histopathologically, 66.7% (42/63) of the detected cancers were intestinal type, and the remaining 33.3% (21/63) were diffuse type (Table I).



H. pylori *antibody level and cancer risk*

Irrespective of their histopathology, the majority (87.3% [55/63]) of cancers developed in the *H. pylori*-positive group (Table I). *H. pylori* infection was associated with a significantly increased risk of gastric cancer (HR = 3.48, 95% CI: 1.26–9.64). Figure 1 shows the Kaplan-Meier analysis of the subjects stratified by serum *H. pylori* antibody level. After 7 years of follow-up, there was a stepwise increase in the cumulative incidence of gastric cancer as the antibody level increased from negative to positive. The incidence rates of gastric cancer in the *H. pylori*-negative, the indeterminate, and the positive groups were 41/100,000, 72/100,000 and 157/100,000 person-years, respectively (Fig. 1*a*, Table I). In the *H. pylori*-positive group, the cancer development was significantly higher in the high-titer subgroup than in the low-titer subgroup (Fig. 1*b*, Table I). In addition, there was also a significant stepwise increase in the HR (95% CI) with an increase in the antibody level; in the high-titer subgroup, the ratio was 9.56 (2.89–31.65), and the p value for the linear trend was 0.0001. This dose-dependent positive association between antibody level and cancer risk was observed in both histological cancer types.

PG I levels and cancer risk

Next, the relationship between the serum PG I level and cancer development during the study period was investigated. The study subjects were divided into 3 groups based on their PG I levels, as follows: group I-50 with PG I >50 ng/mL, group I-30 with PG I >30 ng/mL and ≤50 ng/mL, and group I-0 with PG I ≤30 ng/mL. The Kaplan-Meier analysis of the subjects in the 3 groups showed that after 5 years of follow-up, cancer development increased in a stepwise manner as the PG I level decreased (Fig. 2*a*); the incidence rate of gastric cancer ranged from 85/100,000 person-years in group I-50 to 341/100,000 person-years in group I-0 (Table II). Likewise, there was a stepwise increase in the HR (95%CI) for cancer with reduction in PG I level, showing a significant elevation in group I-0 (3.54 [1.95–6.40], p = 0.0001). With respect to the histopathological cancer type, only in the intestinal-type cancer was a significant stepwise increase in the incidence rate and HR noted with reduction in the PG I level. Next, the subjects were

FIGURE 2 – Risk of gastric cancer and serum pepsinogen (PG) level. (*a*) Gastric cancer development and the serum PG I level. Subjects were classified into 3 groups (I-50, I-30, and I-0) based on the serum PG I level as described in the text. The cumulative incidence of gastric cancer in the 3 groups was plotted using the Kaplan-Meier analysis; the differences among the groups were assessed using the log-rank test. The cancer incidence rates for groups I-50, I-30, and I-0 were 85/100,000 person-years, 130/100,000 person-years, and 341/100,000 person-years, respectively. The difference between group I-50 and the other 2 groups was significant ($p < 0.0001$). (*b*) Gastric cancer development and the serum PG I/II ratio. Subjects were classified into 3 groups (III-3, III-2 and III-0) based on the serum PG I/II ratio as described in the text. The cumulative incidence of gastric cancer in the 3 groups was plotted using the Kaplan-Meier analysis; the differences among the groups were assessed using the log-rank test. The cancer incidence rates for groups III-3, III-2 and III-0 were 60/100,000 person-years, 209/100,000 person-years, and 302/100,000 person-years, respectively. The difference between group III-3 and the other 2 groups was significant ($p < 0.0001$). (*c*) Diffuse-type gastric cancer development and the serum PG II level. Subjects were classified into 3 groups (II-30, II-10 and II-0) based on the serum PG II level as described in the text. The cumulative incidence of diffuse-type gastric cancer in the 3 groups was plotted using the Kaplan-Meier analysis; the differences among the groups were assessed using the log-rank test. The cancer incidence rates for groups II-30, II-10 and II-0 were 119/100,000 person-years, 35/100,000 person-years, and 7/100,000 person-years, respectively. The difference between group II-30 and the other 2 groups was significant ($p < 0.018$).

TABLE II – GASTRIC CANCER INCIDENCE RATES ACCORDING TO SERUM PG I LEVEL

Group	I-50	I-30	I-0	p (trend)
Serum PG I level (ng/mL)	>50	≦50 and >30	≦30	
Subjects	3267	1352	590	
Person-years	31748.0	13099.0	5579.0	
Age [mean (SD)]	49.1 (4.7)	49.1 (4.8)	49.3 (4.9)	
Follow-up years [mean (SD)]	9.7 (0.8)	9.7 (0.9)	9.5 (1.1)	
Total gastric cancer				
Age [mean (SD)]	50.1 (4.4)	51.0 (3.1)	51.7 (3.3)[1]	
Follow-up years [mean (SD)]	6.6 (2.3)	5.2 (3.0)	6.2 (2.3)	
Cases/incidence rate[2]	27/85	17/130	19/341	
HR (95%CI)	1	1.51 (0.83–2.78)	3.54 (1.95–6.40)	<0.0001
Intestinal-type gastric cancer				
Age [mean (SD)]	50.0 (3.8)	50.7 (3.2)	52.3 (3.4)[1]	
Follow-up years [mean (SD)]	6.3 (2.5)	5.7 (2.3)	5.7 (2.3)	
Cases/incidence rate[2]	12/38	15/115	15/269	
HR (95%CI)	1	3.01 (1.41–6.42)	6.19 (2.88–13.32)	<0.0001
Diffuse-type gastric cancer				
Age [mean (SD)]	50.2 (4.9)	53.0 (1.4)	49.5 (1.3)	
Follow-up years [mean (SD)]	6.8 (2.2)	2.0 (0.7)	8.0 (1.6)	
Cases/incidence rate[2]	15/47	2/15	4/72	
HR (95%CI)	1	0.32 (0.07–1.40)	1.38 (0.45–4.20)	0.23
Lung cancer				
Age [mean (SD)]	48.0 (4.6)	51.0 (4.6)	55	
Follow-up years [mean (SD)]	7.9 (0.6)	6.7 (2.1)	7	
Cases/incidence rate[2]	15/47	4/31	1/18	
HR (95%CI)	1	0.65 (0.21–1.95)	0.38 (0.50–2.89)	0.72

[1]*vs.* I-50 group, $p < 0.05$.–[2]Per 100,000 person-years.

TABLE III – GASTRIC CANCER INCIDENCE RATES ACCORDING TO SERUM PG I AND *H. pylori* ANTIBODY LEVELS

Group			I-50	I-30	I-0	p (trend)
Serum PG I level (ng/mL)			>50	≦50 and >30	≦30	
H. pylori-positive	Total	Case/subjects	26/2302	14/855	15/499	
		Incidence Rate[1]	117	170	320	
		HR (95%CI)	1	1.43 (0.75–2.74)	2.52 (1.33–4.80)	0.0059
	High titer	Case/subjects	14/557	6/173	6/109	
		Incidence Rate[1]	262	363	596	
		HR (95%CI)	1	1.39 (0.53–3.62)	2.27 (0.86–5.98)	0.3755
	Low titer	Case/subjects	12/1745	8/682	9/390	
		Incidence Rate[1]	71	122	244	
		HR (95%CI)	1	1.65 (0.67–4.03)	2.97 (1.24–7.10)	0.0077
Indeterminate		Case/subjects	0/328	2/179	2/47	
		Incidence Rate[1]	0	112	430	
		HR (95%CI)	(1)[2]	3.88 (0.35–42.83)	11.92 (1.06–133.57)	0.0942
H. pylori-negative		Case/subjects	1/637	1/318	2/44	
		Incidence Rate[1]	16	32	472	
		HR (95%CI)	1	1.99 (0.12–31.80)	27.34 (2.4–302.42)	0.0127

[1]Per 100,000 person-years.–[2]In reality, the cancer incidence in the subgroup was null; thus, comparison of the cancer risk was impossible. Therefore we tentatively presumed that a single cancer case derived from the subgroup during the study period and the HR was calculated in each subgroup of the same antibody level according to Cox proportional-hazards model.

stratified by serum *H. pylori* antibody levels and the same analysis was applied (Table III). The cancer incidence rate and HR in the same antibody level group increased as the PG I level decreased, reaching the highest level in group I-0. Despite the small number of *H. pylori*-negative and indeterminate subjects in group I-0, the cancer incidence rate was quite high in these groups, that is 472/100,000 and 430/100,000 person-years, respectively. The overall incidence rate of these 91 subjects with an antibody level ≤50 U/mL (*H. pylori*-negative and indeterminate groups) in group I-0 was 439/100,000 person-years, which was higher than that of the *H. pylori*-positive subjects in group I-0. When the *H. pylori*-positive group was divided into high- and low-titer subgroups, the cancer incidence rate of group I-0 in the high-titer subgroup was the highest, and that in the low-titer subgroup was the lowest of the 4 subgroups of I-0 (Table III). In contrast, the cancer incidence rate was low in the antibody-negative subjects with a PG I level >30 ng/mL. Furthermore, among the antibody-negative subjects with a PG I >70 ng/mL, who accounted for 4.5% (n = 233) of the cohort, no one developed cancer (not shown in Table III).

PG I/II ratio and cancer development

Table IV shows the relationship between the serum PG I/II ratio and cancer development. Subjects were classified into 3 groups according to the PG I/II ratio; group III-3 with a PG I/II ratio >3.0, group III-2 with a PG I/II ratio >2.0 and ≤3.0, and group III-0 with a PG I/II ratio ≤2.0. The Kaplan-Meier analysis showed that after 3 years of follow-up there was a stepwise increase in cancer development with reduction in the PG I/II ratio (Fig. 2*b*). The cancer incidence rates were 60/100,000 person-years in group III-3, 209/100,000 person-years in group III-2, and 302/100,000 person-years in group III-0; the highest and most significant HR was observed in group III-0 (HR = 4.89, 95% CI: 2.66–8.99). The observed significant negative correlation between cancer incidence rate and the PG I/II ratio was noted to be unrelated to histopathological cancer type. Next, the same analysis was used to assess the groups stratified by *H. pylori* antibody level. As shown in Table V, in all of the groups except the indeterminate group there was a stepwise increase in the cancer incidence rate and HR with a reduction in the PG I/II ratio, reaching the highest level in

TABLE IV – GASTRIC CANCER INCIDENCE RATES ACCORDING TO PG I/II RATIO

Group	III-3	III-2	III-0	*p* (trend)
PG I/II ratio	>3.0	≦3.0 and >2.0	≦2.0	
Subjects	3453	939	817	
Person-years	33353.0	9112.5	7960.5	
Age [mean (SD]	48.7 (4.8)	49.8 (4.5)[1]	50.7 (4.3)[1]	
Follow-up years [mean (SD)]	9.7 (0.9)	9.7 (0.8)	9.5 (1.1)	
Total gastric cancer				
Age [mean (SD)]	51.3 (3.9)	50.5 (4.3)	50.8 (3.2)	
Follow-up years [mean (SD)]	5.7 (2.3)	6.0 (2.7)	6.3 (2.7)	
Cases/incidence rate[2]	20/60	19/209	24/302	
HR (95%CI)	1	3.70 (1.97–6.95)	4.89 (2.66–8.99)	<0.0001
Intestinal-type gastric cancer				
Age [mean (SD)]	51.8 (3.4)	50.1 (3.8)[1]	51.4 (3.3)	
Follow-up years [mean (SD)]	5.3 (2.5)	6.4 (2.7)	5.8 (2.5)	
Cases/incidence rate[2]	13/39	12/132	17/214	
HR (95%CI)	1	3.76 (1.71–8.27)	5.36 (2.54–11.33)	<0.0001
Diffuse-type gastric cancer				
Age [mean (SD)]	50.6 (4.8)	51.1 (5.3)	49.3 (2.7)	
Follow-up years [mean (SD)]	6.5 (1.8)	5.4 (2.7)	7.8 (2.8)	
Cases/incidence rate[2]	7/21	7/77	7/88	
HR (95%CI)	1	3.60 (1.26–10.30)	4.04 (1.40–11.67)	0.016
Lung cancer				
Age [mean (SD)]	48.6 (4.7)	52.3 (4.6)	45	
Follow-up years [mean (SD)]	7.8 (0.6)	6.2 (2.3)	8.1	
Cases/incidence rate[2]	16/48	3/33	1/13	
HR (95%CI)	1	0.70 (0.20–2.41)	0.27 (0.04–2.05)	0.61

[1]*vs.* III-3 group, $p < 0.05$.–[2]Per 100,000 person-years.

TABLE V – GASTRIC INCIDENCE RATES ACCORDING TO PG I/II AND *H. pylori* ANTIBODY LEVELS

Group			III-3	III-2	III-0	*p* (trend)
PG I/II ratio			>3.0	≦3.0 and >2.0	≦2.0	
H. pylori-positive	Total	Case/subjects	18/1991	17/903	20/762	
		Incidence Rate[1]	94	197	277	
		HR (95%CI)	1	2.05 (1.06–3.99)	2.77 (1.46–5.26)	0.0025
	High titer	Case/subjects	8/400	7/241	11/198	
		Incidence Rate[1]	208	304	591	
		HR (95%CI)	1	1.47 (0.53–4.04)[4]	2.83 (1.13–7.07)	0.0139
	Low titer	Case/subjects	10/1591	10/662	9/589	
		Incidence Rate[1]	55	158	168	
		HR (95%CI)	1	2.26 (0.91–5.59)	2.28 (0.95–5.48)	0.0187
Indeterminate		Case/subjects	2/500	1/22	1/32	
		Incidence Rate[1]	40	457	317	
		HR (95%CI)	1	13.30 (1.81–97.80)	11.59 (1.05–127.90)	0.182
H. pylori-negative		Case/subjects	0/962	1/14	3/23	
		Incidence Rate[1]	0	744	1448	
		HR (95%CI)	(1)[2]	83.39 (7.47–931.40)	131.98 (11.95–1457.36)	0.0001

[1]Per 100,000 person-years.–[2]In reality, the cancer incidence in the subgroup was null; thus, comparison of the cancer risk was impossible. Therefore we tentatively presumed that a single cancer case derived from the subgroup during the study period and the HR was calculated in each subgroup of the same antibody level according to Cox proportional-hazards model.

group III-0. Despite the small number, the cancer incidence rate of subjects with a PG I/II ratio ≤3.0 were quite high in the *H. pylori*-negative and indeterminate groups. The overall cancer incidence rate in the 91 subjects with a PG I/II ratio ≤3.0 (groups III-2 and III-0) and an antibody titer ≤50 U/mL (*H. pylori*-negative and indeterminate groups) was 659/100,000 person-years, which was higher than that of *H. pylori*-positive subjects in group III-2 or III-0. Within the *H. pylori*-positive group, the cancer incidence rate in group III-0 was higher in the high-titer subgroup than in the low-titer subgroup. Meanwhile, the cancer incidence rate among subjects with a PG I/II ratio >3.0 (group III-3) in the *H. pylori*-negative or indeterminate group was quite low.

PG II levels and cancer risk

The subjects were also divided into 3 groups according to PG II level: group II-0 with a PG II ≤10 ng/mL, group II-10 with a PG II >10 ng/mL and ≤30 ng/mL, and group II-30 with a PG II >30 ng/mL, and the relationship between serum PG II level and cancer development was analyzed. As shown in Table VI, there was a stepwise increase in the cancer incidence rate and the HR with an increase in the PG II level ($p = 0.025$). This significant, dose-dependent, positive association between cancer development and PG II level was observed only in diffuse-type cancer; a significant HR increase was noted with a PG II >30 ng/mL (HR = 15.67, 95% CI: 1.88–130.64). The Kaplan-Meier analysis showed that after 3 years of follow-up, the diffuse-type cancer development was the highest in group II-30, followed by group II-10, then group II-0; the incidence rates were 119/100,000 person-years, 35/100,000 person-years and 7/100,000 person-years, respectively (Fig. 2*c*). The subjects stratified by serum *H. pylori* antibody level were analyzed in the same manner (Table VII). In the *H. pylori*-positive group, the development of diffuse-type cancer tended to increase with an increase in the PG II level, reflecting cancer development in the high-titer subgroup. About 42.9% (9/21) of diffuse-type cancers developed in the high-titer subgroup. There was

TABLE VI – GASTRIC CANCER INCIDENCE RATES ACCORDING TO SERUM PG II LEVEL

Group	II-0	II-10	II-30	*p* (trend)
Serum PG II level (ng/mL)	≦10	≦30 and >10	>30	
Subjects	1435	3247	527	
Person-years	14068.5	31302.0	5055.5	
Age [mean (SD)]	48.2 (4.8)	49.6 (4.6)[1]	49.8 (4.6)[1]	
Follow up-years [mean (SD)]	9.7 (0.9)	9.6 (0.9)	9.6 (0.9)	
Total gastric cancer				
Age [mean (SD)]	51.6 (3.4)	51.2 (3.4)	47.9 (4.7)[1]	
Follow-up years [mean (SD)]	5.0 (2.5)	6.2 (2.4)	7.7 (2.5)	
Cases/incidence rate[2]	7/50	45/147	10/198	
HR (95%CI)	1	2.67 (1.21–5.97)	3.57 (1.36–9.40)	0.025
Intestinal-type gastric cancer				
Age [mean (SD)]	51.5 (3.0)	51.6 (3.6)	46.8 (1.5)[1]	
Follow up-years [mean (SD)]	4.7 (2.8)	6.1 (2.3)	8.1 (1.9)	
Cases/incidence rate[2]	6/43	32/102	4/79	
HR (95%CI)	1	2.14 (0.89–5.14)	1.63 (0.46–5.78)	0.224
Diffuse-type gastric cancer				
Age [mean (SD)]	51.8 (2.4)	50.4 (3.9)	48.8 (6.4)	
Follow up-years [mean (SD)]	5.1 (2.5)	6.9 (2.2)	7.3 (3.1)	
Cases/incidence rate[2]	1/7	14/35	6/119	
HR (95%CI)	1	5.95 (0.78–45.39)	15.67 (1.88–130.64)	0.018
Lung cancer				
Age [mean (SD)]	51.0 (4.6)	48.5 (5.0)	48.3 (4.2)	
Follow up-years [mean (SD)]	7.5 (0.6)	7.5 (1.3)	7.8 (0.6)	
Cases/incidence rate[2]	4/284	13/42	3/59	
HR (95%CI)	1	1.48 (0.48–4.56)	2.12 (0.47–9.52)	0.805

[1]*vs.* II-0 group, $p < 0.05$.–[2]Per 100,000 person-years.

TABLE VII – DIFFUSE-TYPE GASTRIC CANCER INCIDENCE RATES ACCORDING TO SERUM PG II AND *H. pylori* ANTIBODY LEVELS

Group			II-0	II-10	II-30	*p* (trend)
Serum PG II level (ng/mL)			≦10	≦30 and >10	>30	
H. pylori-positive	Total	Case/subjects	3/691	11/2533	5/432	
		Incidence Rate[1]	45	44	123	
		HR (95%CI)	1	0.96 (0.25–13.05)	3.26 (0.63–43.72)	0.147
	High titer	Case/subjects	0/67	6/647	3/125	
		Incidence Rate[1]	0	99	258	
		HR (95%CI)	(1)[2]	0.43 (0.05–3.59)	0.83 (0.09–7.97)	0.752
	Low titer	Case/subjects	3/624	5/1886	2/307	
		Incidence Rate[1]	50	28	69	
		HR (95%CI)	1	0.61 (0.11–9.26)	1.16 (0.25–13.05)	0.176
Indeterminate		Case/subjects	1/323	0/215	0/16	
		Incidence Rate[1]	31	0	0	
		HR (95%CI)	1	0	0	
H. pylori-negative		Case/subjects	1/668	0/325	0/6	
		Incidence Rate[1]	15	0	0	
		HR (95%CI)	1	0	0	

[1]Per 100,000 person-years.–[2]In reality, the cancer incidence in the subgroup was null; thus, comparison of the cancer risk was impossible. Therefore we tentatively presumed that a single cancer case derived from the subgroup during the study period and the HR was calculated in each subgroup of the same antibody level according to Cox proportional-hazards model.

a marked stepwise increase in the incidence rate with an increase in the PG II level, reaching a high rate of 258/100,000 person-years in groupII-30. This incidence rate was the highest among the subgroups stratified by serum PG II and antibody levels. Conversely, the cancer incidence rate tend to be low in subjects with low serum levels of both PG II and *H. pylori* antibodies.

Other cancer and non-neoplastic disorders developed during the study

During the study period of 10 years, 71 cases of newly developed cancers other than gastric cancer were detected; they were cancer of the bladder ($n = 6$), colon ($n = 14$), esophagus ($n = 5$), head and neck ($n = 12$), kidney ($n = 4$), liver ($n = 4$), lung ($n = 22$), pancreas ($n = 1$), prostate ($n = 2$) and testis ($n = 1$). In addition, there were 2 cases of non-Hodgkin's lymphoma. Fifty-six of these 73 subjects (76.7%) died from these malignant disorders. There was no significant correlation between the development of any of these neoplasms and serum PG or *H. pylori* antibody level. As a reference, the incidence of lung cancer according to serum PG or *H. pylori* antibody level is shown in Tables I, II, IV and VI. In addition, 51 subjects died during the study from various non-neoplastic disorders such as heart disease ($n = 11$), cerebral vascular accident ($n = 10$), chronic liver disease ($n = 4$), traumatic injuries due to accident in the workplace ($n = 6$), or suicide ($n = 9$).

Discussion

In the present study, a cohort of 5,209 healthy, asymptomatic, middle-aged subjects, in whom serum *H. pylori* antibody titer and PG levels had been assessed, was followed for a mean of 9.7 years, and the incidence rate of gastric cancer was estimated in the groups stratified by the levels of each of these serologic markers. It was found that *H. pylori*-infected subjects had a high risk of stomach cancer regardless of histological type, in good agreement with the results of previous studies dealing with the role of *H. pylori* in stomach carcinogenesis.[3–12] Furthermore, there was a stepwise increase in cancer development with an increase in the

antibody level. Previous studies analyzing the association between gastric cancer risk and serum *H. pylori* antibody level have reported contradictory results.[2,4,43,44] While it has been reported that there is no association between a high antibody level and cancer risk,[43] Yamaji *et al.* indicated that there is a possible association between low antibody levels and cancer risk in elderly subjects.[44] Moreover, a positive association between antibody levels and cancer risk has been suggested by 2 nested case-control studies.[2,4] The present longitudinal cohort study clearly demonstrated that there is a positive, dose-dependent association between the two in asymptomatic, middle-aged, male subjects. In general, the *H. pylori* antibody level is considered to be correlated with the severity of inflammation in stomachs infected with *H. pylori*[35,36]; the persistence of severe gastritis appears to lead to the rapid progression to atrophy and cancer. Indeed, in previous studies involving an *in vivo* carcinogenesis model using Mongolian gerbils, the *H. pylori* antibody level was higher in tumor-bearing animals than in tumor-free animals under similar conditions.[33,34,45] The results of the present study, together with those in the experimental animal model, strongly indicate the possibility that an enhancement of the host-immune response contributes to *H. pylori*-induced stomach carcinogenesis. The importance of these results lies in the potential use of *H. pylori* antibody levels or other markers for genetic predisposition affecting inflammation, such as proinflammatory cytokine gene polymorphisms, as indicators of risk for cancer.[46,47] Further studies are required to look for a link between the immune factors and host genetic cancer susceptibility.

The results of this study demonstrated that an increase in risk of gastric cancer occurred with a reduction in the serum PG I level or the PG I/II ratio. The risk of cancer was significantly elevated in subjects with a serum PG I level $\leq$30 ng/mL (HR = 3.54, 95% CI: 1.95–6.40) or with a PG I/II ratio $\leq$3.0 (HR = 4.25, 95% CI: 2.47–7.32). This negative, dose-dependent association between cancer risk and these serologic markers was observed mainly in intestinal-type cancer. Previous studies have indicated that a reduction in the serum PG I level or the PG I/II ratio is closely correlated with the progression of gastric atrophy.[24,25,39] Thus, the present results are in agreement with the clinicopathological and epidemiological studies that have indicated that many gastric cancers, especially the intestinal-type, develop in stomach mucosa affected by severe and extensive CAG, and that subjects with extensive CAG are at high risk of gastric cancer.[1,16–20] Using the combination of the *H. pylori* antibody level and the PG I level or the PG I/II ratio, a subgroup with an especially high cancer incidence rate could be identified. These results are in line with the results of previous nested case-control studies that showed that subjects with elevated *H. pylori* antibody and low PG I had the highest risk of cancer.[27,30] Furthermore, in the *H. pylori*-positive group, the cancer incidence rate was higher in subjects with a lower PG I level or a lower PG I/II ratio. In fact, as the antibody level increased, the incidence rate increased, so that the high-titer subgroup with PG I $\leq$30 ng/mL had the highest incidence rate (596/100,000 person-years), which was similar to that in the high antibody titer subgroup with PG I/II $\leq$2.0 (591/100,000 person-years). These results strongly suggest that the presence of *H. pylori*-related gastritis, which is associated with severe inflammation as indicated by a high antibody level, together with the consequent extensive atrophy as indicated by a low serum PG I level or PG I/II ratio, is associated with a particularly high risk of gastric cancer. Although only 12.7% (8/63) of gastric cancers developed in the *H. pylori*-negative and indeterminate groups during the observation period, the cancer incidence rate in these subgroups was considerably elevated with a low PG I level or a low PG I/II ratio. In the *H. pylori*-negative and indeterminate groups, there were 91 subjects (1.7% of the cohort) with a PG I $\leq$30 ng/mL (group I-0), and the same number of subjects with a PG I/II ratio $\leq$3.0; the cancer incidence rates of these subjects were 439/100,000 person-years and 659/100,000 person-years, respectively. The incidence rate increased with lower serum PG and/or antibody levels, reaching the highest incidence rate of 1,448/100,000 person-years in the *H. pylori*-negative group III-0 and 472/100,000 person-years in the *H. pylori*-negative group I-0. It is widely accepted that in the *H. pylori*-infected stomach, chronic inflammation induces mucosal atrophy together with intestinal metaplasia. With the extension of intestinal metaplasia the serum PG levels are reduced, and spontaneous eradication of the bacterium is induced, showing a low or null specific antibody level.[48,49] Thus, the 1.7% of subjects in the cohort with a low serum PG I level or a low PG I/II ratio who had negative or indeterminate *H. pylori* antibody levels were considered to have metaplastic gastritis. The results strongly support the hypothesis that the presence of metaplastic gastritis is associated with a high risk of gastric cancer and that *H. pylori* infection is not directly involved in stomach carcinogenesis but has an indirect relationship as a driving force of the atrophy-metaplasia-dysplasia-cancer sequence.[1]

In contrast, the subjects with a high PG I or a high PG I/II ratio in the *H. pylori*-negative group or the indeterminate group were at low risk. In particular, cancer development was not observed during the study period in the *H. pylori*-negative group among subjects with a PG I >70 ng/mL or a PG I/II ratio >3.0. These subgroups are considered to consist primarily of *H. pylori*-free subjects with nonatrophic stomach. The present results strongly indicate the possibility that in the current epidemiological environment of Japan it is quite rare for gastric cancer to develop in *H. pylori*-free healthy stomachs.

Previous studies have indicated that *H. pylori* infection alters the expression of PG II in the stomach mucosa[50]; serum PG II levels are higher in *H. pylori*-related nonatrophic gastritis and lower in atrophic gastritis. The increase in serum PG II levels is reported to be correlated with histological changes reflecting the severity of mucosal inflammation,[51,52] and the eradication of *H. pylori* reverses serum PG II elevation.[53–55] Therefore, the PG II level is considered an index of *H. pylori*-induced gastric inflammation in the nonatrophic stomach. In the present study, the development of diffuse-type cancer, but not intestinal-type, significantly increased with the PG II level; the risk of cancer was significantly elevated in subjects with a PG II level >30 ng/mL (HR = 3.81, 95% CI: 1.10–13.21). These results are in agreement with the previously proposed hypothesis that chronic active inflammation directly induces diffuse-type cancer without passing through atrophic gastritis with intestinal metaplasia.[6,31,56,57] Moreover, stratification using the combination of serum *H. pylori* antibody and PG II levels showed that this type of cancer tends to develop in subjects with high serum levels in both of the tests. *H. pylori*-induced, severe, chronic inflammation is believed to trigger a series of molecular intracellular events that lead to various genetic alterations in the stomach mucosa.[31,32] In addition, there is an increasing body of evidence, including ours, that CpG island methylation is induced by *H. pylori* infection in the stomach mucosa,[58–60] and inactivation of the E-cadherin gene by DNA methylation is reported to be highly prevalent in diffuse-type cancer.[61] The present results strongly support the notion that severe *H. pylori*-induced inflammation, together with a strong host immune response, induces a series of genetic and epigenetic events that directly lead to the development of diffuse-type cancer.

In conclusion, our results show that the serum PG and/or *H. pylori* antibody levels provide an index of gastric cancer development, and that based on these markers the risk for gastric cancer can be objectively determined in each individual with *H. pylori*-related gastritis from the general population. The Japanese anti-gastric cancer strategy has given priority to secondary prevention, based on mass screening using barium X-ray examination as a filter test, over primary prevention. To improve the efficiency of the screening programs, it is necessary to establish a new test for high-risk subjects, and various trials have been conducted in a number of countries including Japan.[62–66] Our results clearly indicate that serum PG and/or *H. pylori* antibody levels can be used as objective markers to differentiate individuals at high and low risk for gastric cancer, and they can provide data that could be a basis for cancer control. The measurement of these serum markers is

simple, reproducible, easy to accept, relatively inexpensive, and can be used to screen a large population.[39] Therefore, by adding these serum tests to a mass screening program greater efficacy can be expected. Since the atrophy-metaplasia-dysplasia-cancer sequence caused by *H. pylori* infection is the main route of stomach carcinogenesis not only in Japan but also in China, Korea, Eastern Europe, Central America, and South America, the detection and strict follow-up of the high-risk population using these serum markers can be considered an effective strategy for the control of gastric cancer worldwide.

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Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation

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Chronic inflammation is deeply involved in induction of aberrant DNA methylation, but it is unclear whether any type of persistent inflammation can induce methylation and how induction of cell proliferation is involved. In this study, Mongolian gerbils were treated with five kinds of inflammation inducers [*Helicobacter pylori* with cytotoxin-associated gene A (CagA), *H.pylori* without CagA, *Helicobacter felis*, 50% ethanol (EtOH) and saturated sodium chloride (NaCl) solution]. Two control groups were treated with a mutagenic carcinogen that induces little inflammation (20 p.p.m. of *N*-methyl-*N*-nitrosourea) and without any treatment. After 20 weeks, chronic inflammation with lymphocyte and macrophage infiltration was prominent in the three *Helicobacter* groups, whereas neutrophil infiltration was mainly observed in the EtOH and NaCl groups. Methylation levels of eight CpG islands significantly increased only in the three *Helicobacter* groups. By Ki-67 staining, cell proliferation was most strongly induced in the NaCl group, demonstrating that induction of cell proliferation is not sufficient for methylation induction. Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* showed increased expression specifically in the three *Helicobacter* groups. In human gastric mucosae infected by *H.pylori*, *NOS2* and *TNF* were also increased. These data showed that inflammation due to infection of the three *Helicobacter* strains has a strong potential to induce methylation, regardless of their CagA statuses, and increased cell proliferation was not sufficient for methylation induction. It was suggested that specific types of inflammation characterized by expression of specific inflammation-related genes, along with increased cell proliferation, are necessary for methylation induction.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is deeply involved in human carcinogenesis (1,2). As inducers of aberrant DNA methylation, aging and chronic inflammation have been suggested because methylation was present in colonic tissues of the aged (3) and patients with long-standing ulcerative colitis (4–6), in the liver with chronic hepatitis (7) and in gastric tissues with *Helicobacter pylori* (*H.pylori*)-induced gastritis (8,9). Especially in the stomach, accumulation levels of aberrant methylation correlate with risk of gastric cancers (8,10–12). Chronic inflammation is characterized by transition of inflammatory cell types from polymorphonuclear cells (mainly neutrophils) to mononuclear cells (lymphocytes and macrophages) and persistent cell proliferation (13). However, it is still unclear whether chronic inflammation with infiltration of mononuclear cells and expression of specific genes or simply persistent inflammation is important for methylation induction and how cell proliferation is involved in it.

As an animal model for methylation induction, we recently demonstrated that inflammation triggered by *H.pylori* infection induces aberrant methylation in the stomach of Mongolian gerbils (*Meriones unguiculatus*) (14). In the gerbil stomach, *H.pylori* with a bacterial virulence factor, cytotoxin-associated gene A (CagA), which is associated with a high risk of human gastric cancers (15), can induce more severe inflammation than that without (16). *Helicobacter felis*, which does not possess CagA (17), can induce chronic gastritis without direct damage of epithelial cells (18,19). High concentrations of ethanol (EtOH) and sodium chloride (NaCl) can induce gastric erosion associated with inflammation (20–22). Their repeated administration can induce persistent inflammation with cell proliferation without transition of inflammatory cell types. In contrast, little inflammation is induced by *N*-methyl-*N*-nitrosourea (MNU), a mutagenic gastric carcinogen (23).

Regarding inflammation-related genes, high expression of *IFNG*, *IL1B*, *TNF*, *NOS2* and *COX2* has been reported in human gastritis induced by *H.pylori* infection (24,25). Also in gerbils, high expression of *Ifng*, *Il1b*, *Cox2* and *Nos2* has been observed (26,27). Our previous time-course study after *H.pylori* infection and eradication in gerbils showed that expression levels of *Cxcl2*, *Il1b*, *Nos2* and *Tnf* were correlated with methylation levels in gastric epithelial cells (GECs) (14). In humans, a polymorphism of *IL1B* is associated with gastric cancer risk (28) and with methylation of multiple genes in gastric cancers (29).

In this study, using five inducers of inflammation (*H.pylori* with CagA, *H.pylori* without CagA, *H.felis*, EtOH and NaCl) and a carcinogen control (MNU), we aimed to clarify the roles of transition of inflammatory cell types, induction of cell proliferation and specific inflammation-related genes in methylation induction.

Materials and methods

Preparation of Helicobacter strains

Helicobacter pylori with CagA (ATCC 43504, also known as NCTC 11637) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Helicobacter pylori* without CagA, SS1, was kindly provided by Professor Takashi Joh at Nagoya City University (30). *Helicobacter felis* (ATCC 49179) was also obtained from ATCC. Each strain was inoculated in Brucella broth (Becton Dickson, Cockeysville, MD) with 7% vol/vol heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an AnaeroPack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 h. For the culture of *H.felis*, 0.1% wt/vol of BactoAgar (Becton Dickson) was supplemented. Before harvesting bacteria, their mobility and shape were confirmed under phase contrast microscopy.

Animal experiments and sample preparation

Five-week-old male Mongolian gerbils (MGS/Sea; Kyudo, Tosu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for *Helicobacter* treatment were inoculated with $\sim 10^8$ CFU/gerbil of *H.pylori* ATCC 43504 (ATCC group), *H.pylori* SS1 (SS1 group) or *H.felis* (HF group) and were kept without further treatment. Gerbils in groups of EtOH and NaCl treatment were administered with 5 ml/kg body wt of 50% EtOH group and saturated NaCl group, respectively, by gavage twice a week from 5 to 25 weeks of age. Gerbils in the group of MNU treatment (MNU group) were administered with 20 p.p.m. of MNU (Sigma–Aldrich, St Louis, MO) in drinking water from 5 to 25 weeks of age. A control group was kept without any treatment.

Abbreviations: CagA, cytotoxin-associated gene A; CGI, CpG island; Dnmt, DNA methyltransferase; EtOH, ethanol; GEC, gastric epithelial cell; MNU, *N*-methyl-*N*-nitrosourea; NaCl, sodium chloride; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcriptase–polymerase chain reaction.

At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

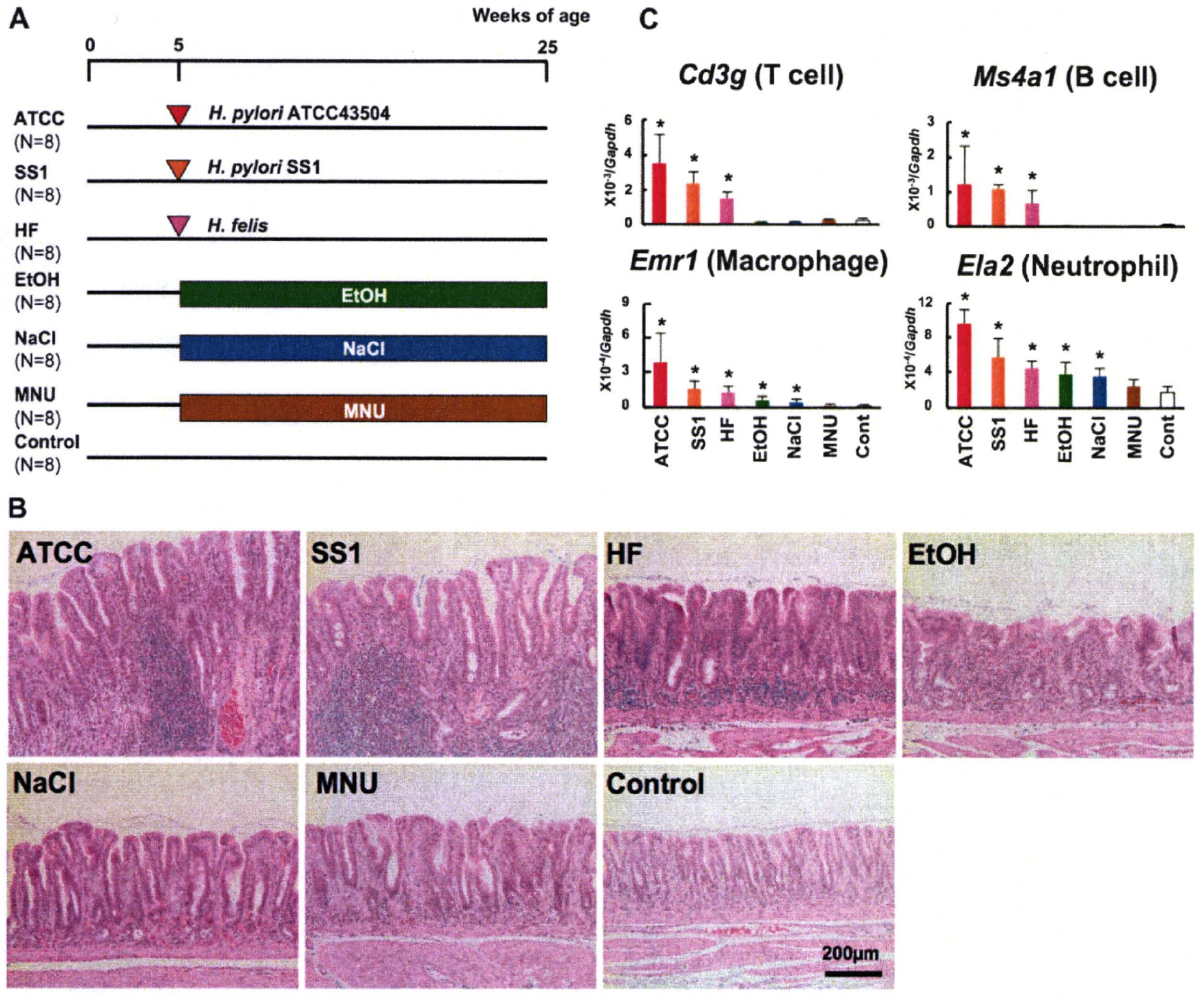

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^*P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
$^*P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.

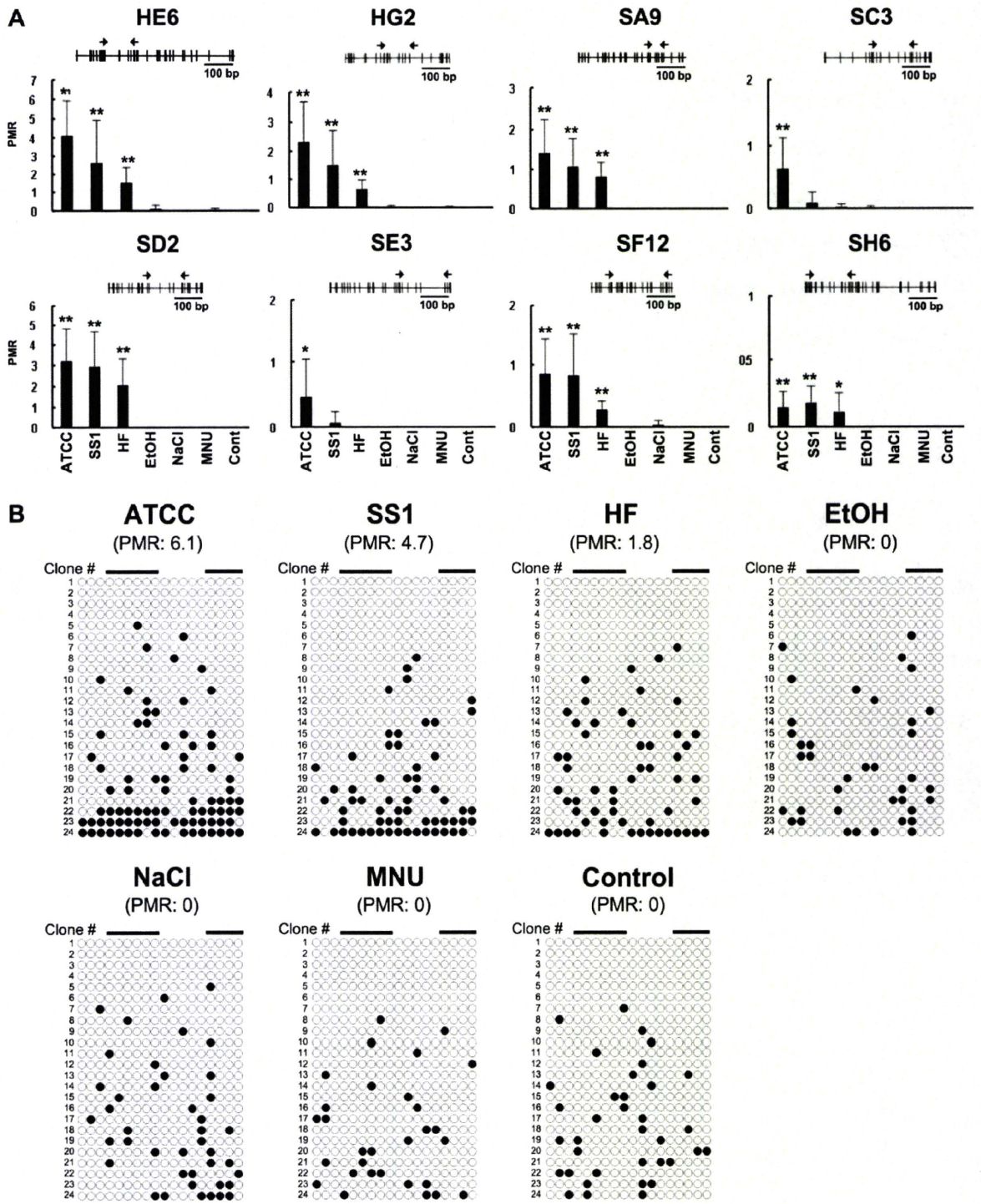

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

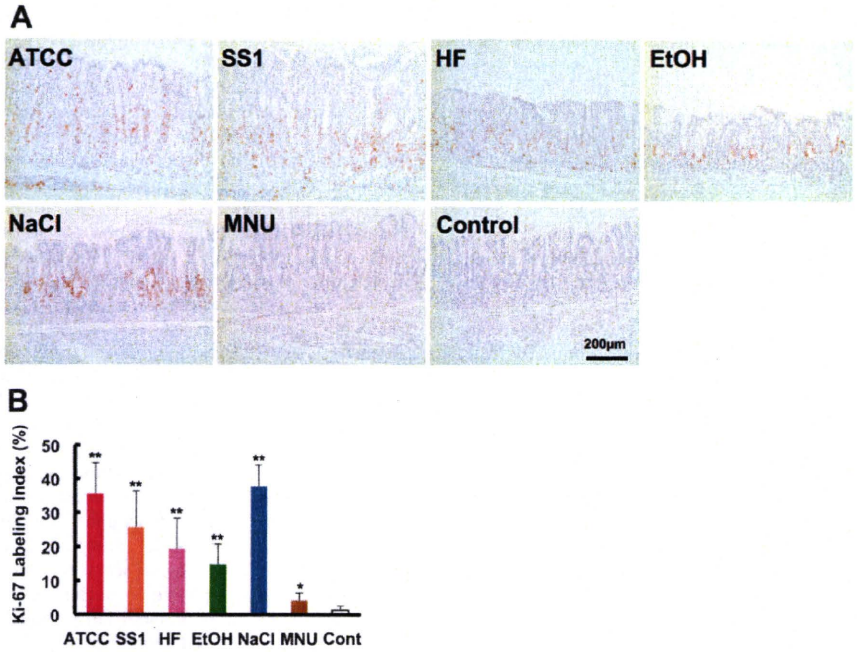

Fig. 3. Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase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

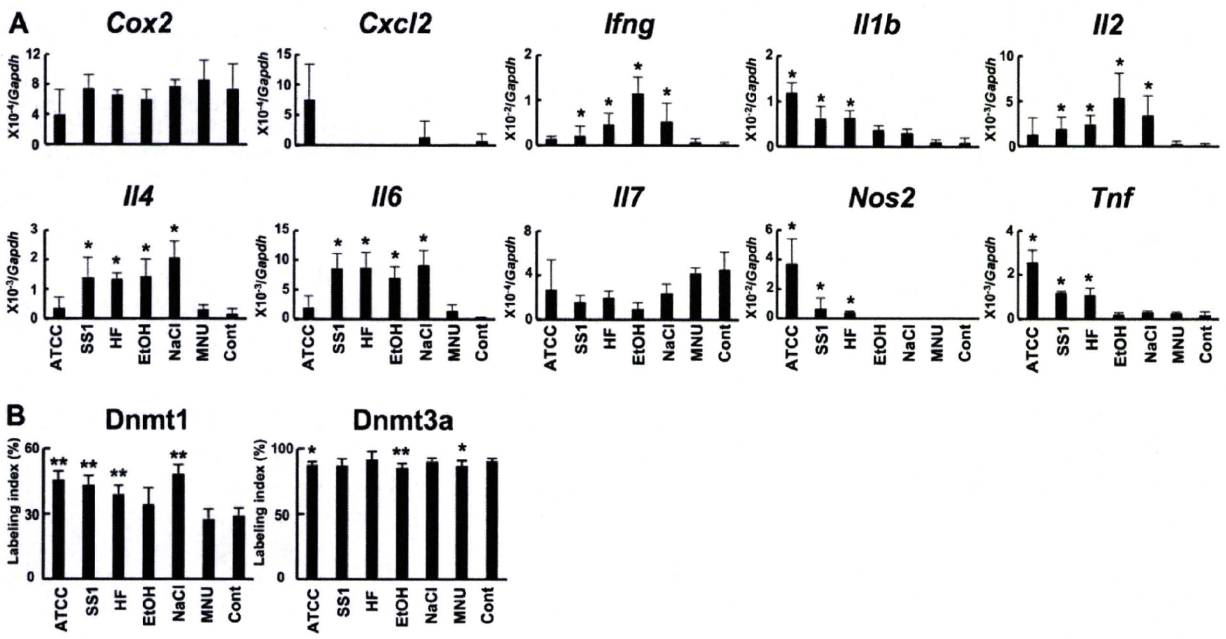

Fig. 4. Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* 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,

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

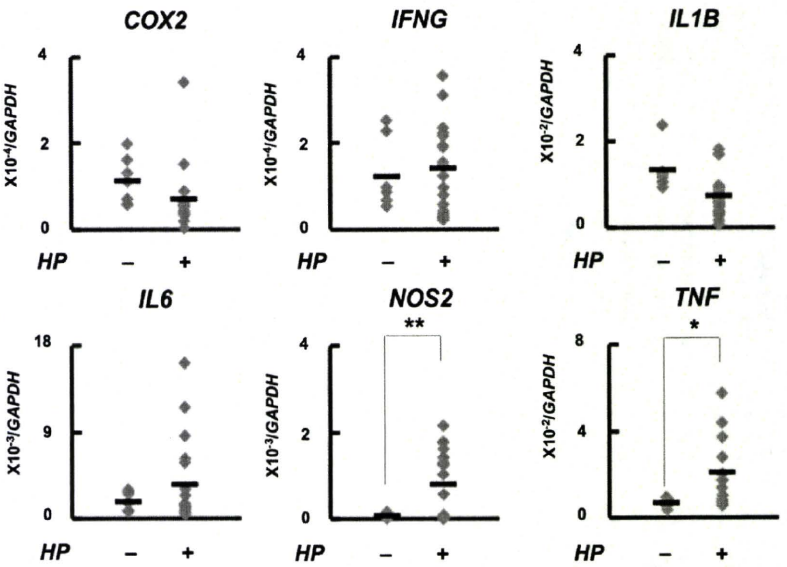

Fig. 5. Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

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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Review Article

Aberrant DNA methylation in contrast with mutations

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Aberrant DNA methylation is known as an important cause of human cancers, along with mutations. Although aberrant methylation was initially speculated to be similar to mutations, it is now recognized that methylation is quite unlike mutations. Whereas the number of mutations in individual cancer cells is estimated to be ~80, that of aberrant methylation of promoter CpG islands reaches several hundred to 1000. Although mutations of a specific gene are very few in non-cancerous (thus polyclonal) tissues (usually at 1×10^{-5}/cell), aberrant methylation of a specific gene can be present up to several 10% of cells. Mutagenic chemicals and radiation are well-known inducers of mutations, whereas chronic inflammation is deeply involved in methylation induction. Although mutations are induced in mostly random genes, methylation is induced in specific genes depending on tissues and inducers. Methylation is potentially reversible, unlike mutations. These characteristics of methylation are opening up new fields of application and research. (*Cancer Sci* 2010; 101: 300–305)

Aberrant DNA methylation is deeply involved in human carcinogenesis,[1–3] and is often described as ''genome-overall hypomethylation and regional hypermethylation''. Genome-overall hypomethylation was discovered in the early 1980s[4,5] and has been shown to induce genomic instability and promote carcinogenesis.[6–8] Regional hypermethylation denotes methylation of normally unmethylated CpG islands (CGI) and, in particular, methylation of a promoter CGI is known to silence its downstream gene by multiple mechanisms, including aberrant nucleosome formation.[9,10] Inactivation of a tumor-suppressor gene was first discovered for *RB* in 1993,[5,11] and now a wide variety of tumor-suppressor genes, including *CDKN2A* (p16), *MLH1*, and *CDH1* (E-cadherin), are known to be inactivated by aberrant methylation.[2] In many types of cancers, aberrant promoter methylation is frequently observed and in some types of cancers, such as gastric cancers, aberrant methylation is more frequent than mutations in inactivating mechanisms of specific tumor-suppressor genes.[12]

In the 1990s, investigators found that tumor-suppressor genes can be inactivated by aberrant methylation of promoter CGI, and that most CGI analyzed by conventional methods were kept unmethylated, even in cancers. This made them think that genes with aberrant methylation of promoter CGI were tumor-suppressor genes. Some investigators were inspired that they could identify tumor-suppressor genes if they could identify aberrant methylation by genome-wide screening methods.[13–16] Actually, these methods contributed to the identification of important CGI in diagnostic purposes and isolation of tumor-suppressor genes.[3] In addition, the fact that aberrant methylation of promoter CGI is an alternative to a mutation for inactivation of tumor-suppressor genes made many investigators think that epigenetic alterations would share similar features with mutations in other aspects, such as their frequencies in cancer and non-cancerous tissues, inducers, and target genes.

However, recent findings by high-resolution genome-wide analysis of DNA methylation and by many other approaches have shown that aberrant DNA methylation has many unique features different from mutations (here, point mutations and small base deletions) (Table 1). In this review, we will summarize the contrasts between these two kinds of alterations: aberrant DNA methylation and mutations.

Number of alterations in a cancer cell

Recent use of high-throughput sequencing and high-resolution microarray technologies has illuminated detailed genetic and epigenetic alterations in cancer cells.

Assessment of the role of genetic alterations in carcinogenesis. The assessment of whether a specific sequence alteration is a mutation and what the role of a mutation is in carcinogenesis is relatively straightforward. If a possible sequence change is specifically present in cancer tissues but not in non-cancerous tissues, it is a somatic mutation. If the mutation alters the amino acid sequence of an encoded protein, it is a candidate for a driver mutation.[17,18] Comparison between the incidence of mutations with amino acid alteration and that of silent mutations can provide information on whether there is a selection bias for cells with a mutation of the gene in carcinogenesis. Mutations that drive the initiation, progression, or maintenance of a cancer are classified as driver mutations, and mutations that simply accompany carcinogenesis or are produced as a result of transformation are classified as passenger mutations.

Number of driver and passenger mutations in cancers. As high-throughput sequencing becomes more powerful, a wider selection of genes has been analyzed for broader ranges of cancers. By sequencing more than 20 000 transcripts in breast and colon cancers, it was estimated that approximately 80 non-silent mutations are present in a typical cancer, and that <15 genes are likely to be driver mutations.[18] By sequencing of a wide variety of cancers for selected genes (518 protein kinases), it was shown that lung cancers harbor more mutations than colon and gastric cancers, and that one-third of cancers did not have any somatic mutations in these kinases.[17] The presence of a limited number of driver mutations and a large number of passenger mutations was confirmed in these studies.

Assessment of the role of "aberrant" methylation in carcinogenesis. In contrast to mutations, assessment of the biological significance of ''aberrant'' DNA methylation is very difficult. At least, the effect of methylation on gene silencing and the role

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Table 1. Comparison between mutations and DNA methylation

	Mutation	DNA methylation	References
Number of alterations per cancer cell	~80	Several hundred to 1000	(18,23,27–30)
Frequency of alterations of a specific gene in non-cancerous tissues	10^{-5}/cell, up to 10^{-3}/cell	0.1 to several % up to several 10% of cells	(44,46)
Inducers	Mutagenic chemicals, radiation, oxygen radical	Chronic inflammation, aging	(45,56)
Target gene	Random	Specific	(18,27,37,61)
Reversibility	Irreversible	Reversible	(18,61,70–73)

Detailed explanations are in individual sections.

of the silencing in carcinogenesis need to be assessed separately and precisely.

To assess the effect on gene silencing, the location of a methylated region and the CpG density of the region are critically important.[19,20] The methylation status of promoters with high CpG density, namely promoter CGI, has a clear association with decreased transcription whereas that of promoters with low CpG density are unclear. Depending on the relative position against a transcription start site (TSS), the degree of association between DNA methylation and decreased gene expression is different. Methylation of a 200–300-bp upstream region of a TSS has been known to be consistently associated with repressed transcription.[1–3,21] The region is now known as a "nucleosome-free region" (NFR), which lacks a nucleosome[9] and whose DNA methylation leads to formation of nucleosome(s) and represses transcription.[10] Recent genome-wide studies also support the idea that methylation of NFR is consistently associated with low gene transcription.[19,20,22,23] At the same time, methylation of a far upstream region and exon 1 can also be associated with decreased transcription via methylation of the NFR. On the other hand, methylation of a gene body is occasionally associated with increased gene expression.[22–25] It is noteworthy that, even within a CGI, the methylation status of different regions is occasionally heterogeneous and investigators should analyze an appropriate region.[3]

Even if limited to DNA methylation that causes gene silencing, the role of the DNA methylation in carcinogenesis needs to be carefully assessed. As described below, there are hundreds to 1000 genes with methylation of their NFR in cancer cells, and it is likely that most of them are passengers. Also as described below, genes without expression in normal cells tend to become methylated in cancers, and such genes without expression are unlikely to be tumor-suppressor genes. To establish a gene with methylation of its NFR in cancers as a tumor-suppressor gene, we need mutation analysis of the gene in cancers and functional analysis of the gene after its transduction into cancer cells and expression at a physiological level and after its knock down in normal cells. Most tumor-suppressor genes are known to be inactivated by homozygous mutation, by combination of methylation and mutation, or by methylation of all copies, and methylation is more frequent than mutations.[26]

Number of methylation of CGI in NFR in cancers. Detailed pictures of CGI aberrantly methylated in cancers are becoming clear by microarray analysis combined with methylated DNA immunoprecipitation or methylated-CpG island recovery assay using methylated-DNA binding domain proteins.[23,27–30] As normalization of signals obtained by microarray is still under development[23,31–33] and CGI in various positions against TSS and various regions within CGI have been analyzed so far, it is difficult to compare different reports at this time.

According to our previous studies focusing on methylation of NFR in promoter CGI,[23,34] large fractions of them were methylated in gastric cancer cell lines (Table 2). Although there is controversy about how methylation in cell lines reflects that in primary cancers,[35,36] it seems safe to estimate that one-third to one-half of CGI methylated in cell lines are also methylated in primary cancers. We currently estimate that several hundred to 1000 NFR in promoter CGI are methylated in a primary cancer cell. If not limited to NFR, 216–848 of 27 800 CGI are reported to be methylated in primary lung squamous cell cancers.[30] If limited to methylation of NFR that can be detected by re-expression after treatment with a demethylating agent, the number decreases markedly, such as to less than 1/100.[23] These show that a large number of NFR and other CGI are methylated in cancers, which is in line with pioneering studies.[37,38] The large number is in sharp contrast to the number of mutations in a cancer.

Methylation of a specific gene in a large fraction of cells in non-cancerous tissues

DNA methylation shows a sharp contrast to mutations also in the fraction of cells with an alteration of a specific gene in non-cancerous tissues. Moreover, the degree of accumulation of aberrant DNA methylation can be associated with cancer risk.

Meaning of the fraction of cells with an alteration in cancer and non-cancerous tissues. The fraction of cells with an alteration (mutation or methylation) of a specific gene is often compared between cancer and non-cancerous tissues. However, the meaning of the fraction is entirely different in the two kinds of tissues.

Not to mention, a cancer develops after multiple processes of clonal selection (Fig. 1). In non-cancerous tissues, no selection for a cell with an alteration has been imposed yet, and thus the fraction of cells with the alteration is mainly determined by the frequency with which the alteration is induced. The frequency

Table 2. Estimated number of methylated CpG islands (CGI)

Cell lines	Nucleosome-free region	CGI (not restricted to promoters)
Stomach cancer	641–1205 of 9624 (6.6–12.5%)	3768–7310 of 30 533 (12.3–23.9%)
Prostate cancer	501–800 of 8930 (5.6–8.6%)	5593–7638 of 34 405 (16.3–22.2%)
Breast cancer	480–673 of 8866 (5.4–7.6%)	4118–4755 of 34 424 (12.0–13.8%)

The number of nucleosome-free regions and CGI analyzed are different in individual experiments because the number of probes assessed as functional was different in each experiment.

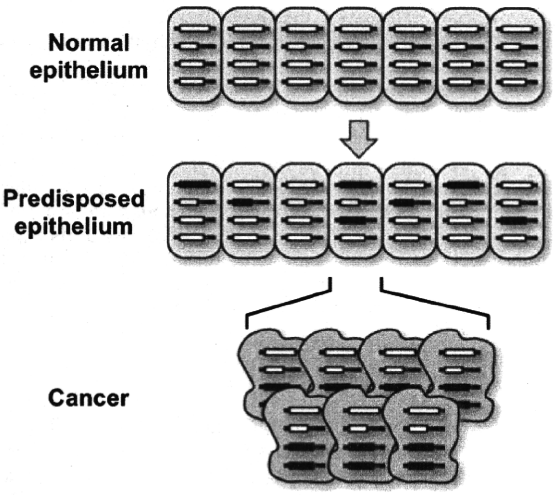

Fig. 1. Epigenetic field for cancerization and clonal selection in cancer. Normal epithelium consists of cells with little aberrant methylation. By exposure to inducers of methylation, specific genes are methylated in minor fractions of cells. A cancer develops from one of the cells that has already accumulated silencing of driver genes. From the viewpoint of assessment of an effect of an inducer, analysis of non-cancerous tissues provides overall information on the genes methylated, and that of a cancer provides information on the genes stochastically methylated in the very precursor cell and driver genes.

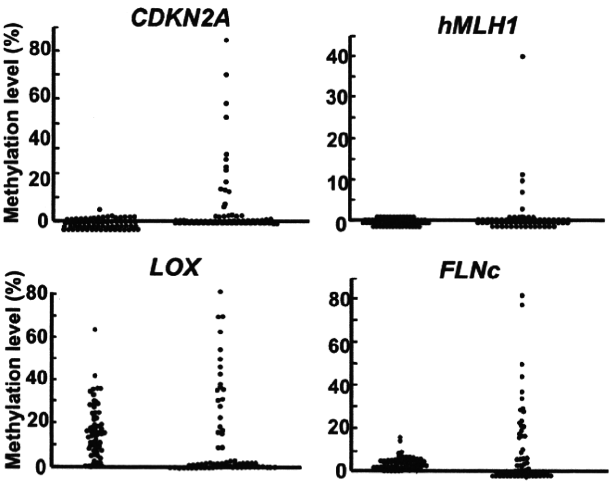

Fig. 2. Distribution patterns of methylation in non-cancerous and cancer tissues. Methylation levels, which reflect fractions of cells with the methylation, were quantified in 66 paired samples of non-cancerous and cancer tissues of gastric cancer patients (modified from Enomoto *et al.*[39]). They showed a unimodal distribution in non-cancerous tissues, and a "bimodal" distribution, namely zero or positive, in cancer tissues. This finding supports the idea that methylation in a non-cancerous tissue reflects events in many cells in the tissue whereas that in a cancer tissue mostly reflects only events in its single precursor cell.

can be affected by the overall exposure level to its inducers and by the susceptibility of individual genes to undergo an alteration. In actual analysis, the proportion of target cells, such as content of epithelial cells in a sample with epithelial and stromal cells, also affects the fraction of cells with an alteration.

In contrast, in cancer tissues, an alteration responsible for clonal growth (driver) is present in all the cancer cells. Even if an alteration is not a driver, if the alteration has taken place before the clonal growth started, it is present in all the cancer cells. In actual analysis, cancer samples contain a large contamination of non-cancer cells, and the fraction of cells with the alteration is mainly determined by the fraction of cancer cells in a sample. If an alteration is induced after initiation of clonal growth, it can be present in a fraction of cancer cells, and its overall fraction is determined by the fraction within cancer cells and by the fraction of cancer cells within a sample.

These theoretical considerations were substantiated by actual measurement of cells with methylation of specific genes in non-cancerous and cancer tissues of gastric cancer patients (Fig. 2) and esophageal cancer patients.[39,40] The methylation level, which reflects the fraction of DNA molecules with methylation and thus the fraction of cells with the methylation, shows a unimodal distribution in non-cancerous tissues, especially for the weak tumor-suppressor gene *LOX* and the marker gene *FLNc*.[41] It shows a "bimodal" distribution, namely zero or positive, in cancer tissues, especially for the tumor-suppressor genes *CDKN2A* and *MLH1*.

Rare presence of mutations in non-cancerous tissues. Adjacent non-cancerous tissues are often used as a control for cancer tissues, and are regarded not to have detectable levels of mutations. To detect accurately such low levels of mutations in non-cancerous tissues, transgenic animals in which rare mutations can be quantified by selectable mutations of a marker gene have been developed.[42,43] Using these transgenic animals and various carcinogenic factors, mutation frequencies of a specific marker gene in non-cancerous tissues have been shown to be $\sim 10^{-5}$/cell, and to be 10^{-3}/cell, even in a tissue heavily exposed to a mutagenic compound.[44] This very low frequency of mutations in non-cancerous tissues gives a rationale for the routine use of such tissues as a control.

DNA methylation in non-cancerous tissues and aging. Once the situation goes to DNA methylation, many investigators noticed that trace amounts of DNA with methylation are present in non-cancerous tissues of cancer patients. However, it is usually difficult to distinguish whether such methylation is a simple drift or fluctuation without any biological or pathological meaning or something associated with cancer development. A pioneering work by Issa *et al.* analyzed the correlation between age and levels of methylation, and convincingly showed that aging is one factor that induces DNA methylation.[45]

Association between methylation accumulation and cancer risk: Epigenetic field for cancerization. We systematically collected gastric tissue samples from healthy individuals and gastric cancer patients (non-cancerous part) in an age-matched manner.[46] Methylation levels of eight CGI in various positions against TSS were accurately quantified. Methylation levels in non-cancerous gastric tissues of gastric cancer patients were in the range 0.2–8.2%, and were much higher than those in gastric mucosae of healthy individuals. This showed that very high levels of methylation can be present in non-cancerous tissues, different from mutations. The finding also suggested that accumulation of methylation is related to gastric cancer risk. Subsequently, gastric mucosae of patients with multiple gastric cancers were shown to have higher methylation levels than those of patients with a single gastric cancer (Fig. 3).[47] These discoveries clearly demonstrated that methylation levels in gastric mucosae correlate with gastric cancer risk.

A higher incidence or level of methylation in non-cancerous tissues of cancer patients than that in the corresponding tissues of healthy individuals was also observed for liver,[48] colon,[49] esophageal,[50] and renal[51] cancers. In these types of cancers, accumulation of methylation is likely to be involved in the formation of a field for cancerization (Fig. 1).[52] The gene inactivated by methylation of its promoter CGI in non-cancerous

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