is leading to the problem of a high frequency of secondary gastric cancer after ER [4]. Therefore, risk diagnosis in the general population and patients who have undergone ER is indispensable for efficient surveillance of primary and secondary gastric cancers. For accurate risk diagnosis, we have to develop a risk marker that reflects the current accumulation level of genetic and epigenetic alterations in normal-appearing tissues. This is because exposure levels to carcinogenic factors and the degree of response to them are highly variable among individuals [5].

Infection by *Helicobacter pylori* (*H. pylori*) is the major causative agent of gastric cancers in Asian countries, and it is associated with accumulation of epigenetic alterations, namely aberrant DNA methylation, in gastric mucosae [6]. Among individuals without current *H. pylori* infection, a fraction of DNA molecules with methylation (DNA methylation level) of specific genes in gastric mucosae correlates with gastric cancer risk [6, 7]. Methylation levels retain their predictive power, even when adjusted by the extent of gastric atrophy [7]. Based upon these findings, DNA methylation levels in non-cancerous gastric mucosae are expected to provide a useful cancer risk marker that reflects past exposure to carcinogens and the degree of the "field defect" resultantly formed.

However, temporal profiles of DNA methylation levels in gastric mucosae during the course of *H. pylori* infection and its discontinuation have not been clarified yet. Quantification of methylation levels in cross-sectional groups suggested that the presence of *H. pylori* infection induces high levels of aberrant DNA methylation and that the high methylation levels will decrease to certain levels when the infection discontinues [6]. Non-quantitative studies showed that the incidence of methylation of the *CDH1* tumor-suppressor gene in gastric mucosae decreases after eradication of *H. pylori* [8, 9] and that incidences of multiple tumor-suppressor genes also decrease [10]. In a specific type of *H. pylori*-induced gastritis, enlarged fold gastritis, a quantitative study showed that *CDH1* methylation levels decrease at 3 months after eradication in *H. pylori* [11]. The decreased incidence in non-quantitative studies could be due to disappearance of foci with methylation-positive cells in some individuals or due to a decrease of methylation levels to below detection limits.

In this study, to clarify the temporal profiles of aberrant DNA methylation in gastric mucosae, we analyzed time trends of methylation levels in gastric mucosae before, and 6 weeks and 1 year after *H. pylori* eradication by a high-sensitivity quantitative method. To explore the possible role of chronic inflammation in methylation induction, we also analyzed the association between methylation levels and histological findings.

Materials and methods

Subjects, tissue samples and DNA extraction

Thirty-five patients with *H. pylori* infection who had undergone curative endoscopic resection (ER) of a well-differentiated early gastric adenocarcinoma [12, 13] were recruited from June 2006 to November 2006 at the National Cancer Center Hospital (Tokyo, Japan) under approval of the institutional review board and with written informed consents. None of the patients had received *H. pylori* eradication therapy prior to this study or regularly used proton pump inhibitors. The average age of the patients was 65.3 ± 7.4 (range 51–75), and the male-to-female ratio was 4 to 1 (28 men and 7 women). Eleven healthy volunteers with *H. pylori* infection who had no past history of gastric cancer (average age 59.9, male to female 6 to 5) were also recruited.

H. pylori infection status was analyzed by the culture test (Eiken, Tokyo, Japan) and rapid urease test (Otsuka, Tokushima, Japan). *H. pylori* was eradicated by 1-week administration of Lansoprazole (LPZ) 30 mg b.i.d, Amoxicillin (AMPC) 750 mg b.i.d and Clarithromycin (CAM) 200 mg b.i.d. Successful eradication was established by negative results for both the culture and rapid urease tests at multiple time points. *H. pylori* was successfully eradicated in 20 of 26 patients who received eradication therapy. The remaining six patients in whom eradication failed and nine patients who did not want to receive eradication therapy were treated as a group of persistent infection. There was no significant difference in the average age between the group of successful eradication and group of persistent infection (65.2 ± 6.9 and 65.4 ± 8.1, respectively, $P = 0.94$). Among the 11 healthy volunteers who received *H. pylori* eradication therapy, 9 individuals were successfully eradicated (average age 60.4, male to female ratio: 4 to 5).

Endoscopic biopsy materials were collected from three standard sites of non-cancerous gastric mucosae (upper gastric body, middle gastric body and the antrum in the lesser curvature) at three time points: the start point (before the eradication), 6 weeks after eradication and 1 year after eradication. In individuals without eradication therapy, biopsy materials were also collected at corresponding time points. Two biopsy samples were obtained from each site, and used for methylation and histological analyses. High molecular weight DNA was extracted by the standard phenol/chloroform method. Fasting blood samples were collected on the day of endoscopy to measure serum pepsinogen I and II (SRL, Tokyo).

Bisulfite treatment and methylation-specific PCR

Bisulfite treatment was performed as previously described [14]. Briefly, DNA samples (1 μg each) digested by *Bam*HI

were denatured in 0.3 N NaOH at 37°C for 15 min. The samples underwent 15 cycles of 30-s denaturation at 95°C and 15 min incubation at 50°C in 3.6 N sodium bisulfite (pH 5.0) and 0.6 mM hydroquinone. The samples were desalted with a Zymo-Spin Column IC (Zymo Research, Orange, CA) and desulfonated in 0.3 N NaOH. DNA was ethanol-precipitated and dissolved in 40 μl of TE buffer.

For methylation analysis, two promoter CpG islands of the filamin C (*FLNc*) and thrombomodulin (*THBD*) genes were selected from eight CpG island regions previously analyzed because they were closely correlated with the risk of gastric cancer development among individuals without current *H. pylori* infection "epigenetic gastric cancer marker genes" [6, 7]. Methylation levels were quantified by real-time methylation-specific PCR (MSP) as in our previous report [6], and the standard DNA for real-time MSP is available upon request. The methylation level of a sample for a CpG island was calculated as the fraction of methylated molecules among the total DNA molecules (number of methylated molecules + number of unmethylated molecules). The methylation level of a sample was measured in triplicate, and standard deviation of the measurement was confirmed as less than 15% of a methylation level. A methylation level of an individual at a time point was obtained as an average of three samples from the three biopsy sites [7].

For bisulfite sequencing, 1 μl of the sodium bisulfite-treated DNA was used for PCR with the primers common to methylated and unmethylated DNA sequences. The sequences were: *THBD*-forward, 5′-ATTTTGTTGGGGTG TAAGAAGTAT-3′ and *THBD*-reverse, 5′-CTACCCCAT AACTAACCAAAAAC-3′. The PCR products were cloned into a pGEM-T Easy TA Vector (Promega, Madison, WI), and 20–22 clones were cycle-sequenced for each sample.

Histological analysis

Biopsy specimens for histological analysis were fixed in 10% buffered formalin and embedded in paraffin. All tissue sections were stained with hematoxylin-eosin for histological examination. Intensities of acute infiltrates (neutrophil), chronic infiltrates (mononuclear cells), gastric atrophy and intestinal metaplasia [15] were graded according to the updated Sydney System as follows: none (0), mild (1), moderate (2) or marked (3) [16]. Histological review was performed by a single experienced pathologist (YN) who had no prior knowledge of the clinical course of the patients.

Statistical analysis

Methylation levels, patient's age, histological grades and serum pepsinogen value were expressed as an average ± standard deviation. Methylation levels and the histological grades and serum pepsinogen value between two time points were compared by Welch's *t* test (paired samples, two sided).

Results

Effects of *H. pylori* eradication on *FLNc* and *THBD* methylation levels

Methylation levels were measured in patients with successful eradication ($n = 20$) and patients with persistent infection ($n = 15$) at three time points. They were also measured in nine healthy volunteers with successful eradication ($n = 9$) at two time points.

In the patients with successful eradication, the average *FLNc* methylation level decreased from 0.6 ± 0.5 to 0.4 ± 0.3% at 6 weeks after eradication ($P = 0.049$) (Fig. 1a), and it remained at a low level (0.4 ± 0.3%) ($P = 0.50$, compared with 6 weeks after eradication; $P = 0.022$, compared with the start point) at 1 year after eradication. When methylation levels were analyzed separately in three sites of the stomach (Supplemental Figure), those in the middle and upper gastric bodies showed significant decreases ($P = 0.020$ and 0.005, respectively), while that in the antrum did not change. The average *THBD* methylation level (30.1 ± 12.3% before eradication) did not show a significant decrease at 6 weeks after eradication (31.0 ± 9.7%) in the group of successful eradication ($P = 0.32$) (Fig. 1a). However, at 1 year after eradication, it showed a significant decrease to 19.0 ± 11.5% ($P = 0.0032$).

In the patients with persistent infection, the average *FLNc* methylation level (1.1 ± 0.9% before eradication) remained the same at 6 weeks (0.8 ± 1.0%, $P = 0.18$) and 1 year after eradication (0.8 ± 1.0%, $P = 0.27$), compared with the start point. The average *THBD* methylation level (31.2 ± 15.4% before eradication) did not show a significant decrease at 6 weeks after eradication (33.1 ± 14.3%, $P = 0.33$) or at 1 year after eradication (28.9 ± 15.4%, $P = 0.29$), compared with the start point. Patients with persistent infection included those with eradication failure and those who received no eradication therapy, and we analyzed methylation levels separately in these two subgroups. Even in the eradication failure subgroup, there was no significant decrease of the *FLNc* and *THBD* methylation level between the start point and 1 year after eradication therapy ($P = 0.068$ and 0.128, respectively).

In the nine healthy volunteers with successful eradication (Fig. 1b), the average *FLNc* methylation level decreased from 1.6 ± 1.1 to 1.2 ± 0.9%, and the average

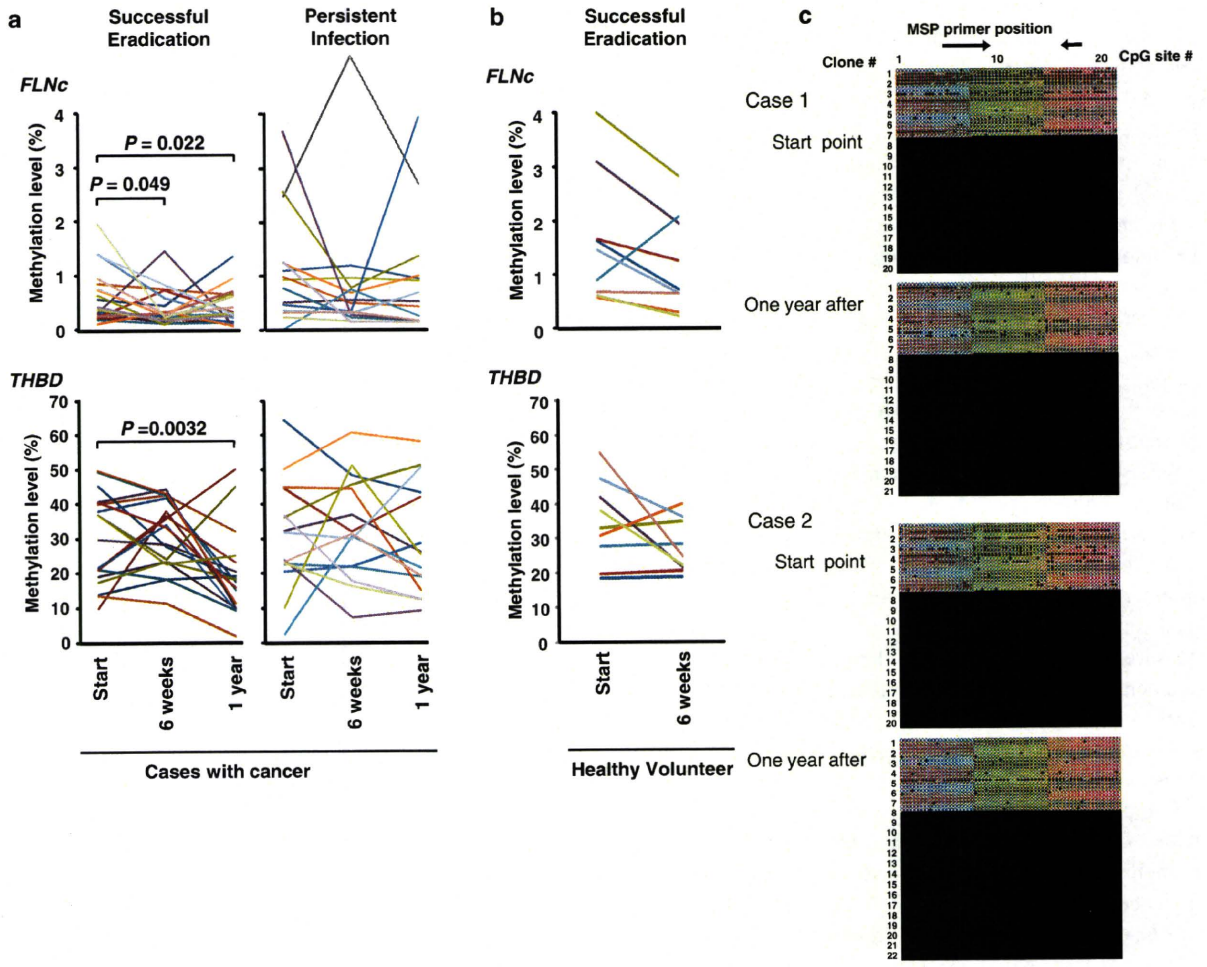

Fig. 1 **a** Effects of *H. pylori* eradication on *FLNc* and *THBD* methylation levels. A methylation level of a sample was measured in triplicate, and an average of three biopsy sites in the stomach was used as a methylation level of an individual. Methylation levels were measured in the group of successful eradication ($n = 20$) and that of persistent infection ($n = 15$) at three time points (before the eradication = start point; 6 weeks after eradication; and 1 year after eradication). Each *color* shows an individual patient. In the group of successful eradication, the *FLNc* methylation level decreased at 6 weeks after eradication ($P = 0.049$). The *THBD* methylation level showed a significant decrease at 1 year after eradication ($P = 0.0032$). In the group of persistent infection, neither the *FLNc* nor the *THBD* methylation level showed a significant decrease 1 year after eradication ($P = 0.27$ and 0.29, respectively). These findings demonstrate the presence of temporary components in DNA methylation. **b** Methylation changes in healthy volunteers. Eleven healthy volunteers received eradication therapy, and methylation levels were measured in nine volunteers with successful eradication before and 6 weeks after the eradication. The average *FLNc* methylation level decreased from 1.6 ± 1.1 to $1.2 \pm 0.9\%$, and the average *THBD* methylation level decreased from 34.7 ± 11.5 to $27.5 \pm 7.4\%$. Although there were tendencies, no significant difference was observed ($P = 0.054$ and 0.066 for *FLNc* and *THBD*, respectively). **c** Decrease of densely methylated DNA molecules shown by bisulfite sequencing. Twenty-one CpG sites of the *THBD* promoter region, covering the CpG sites used for its real-time MSP (shown by *arrows*) were analyzed in two pairs of samples. DNA molecules in which 9 or more of the 14 CpG sites between and on the MSP primers were considered to be densely methylated. Case 1 with a decrease from 54.8 to 6.0% by real-time MSP showed a decrease of densely methylated DNA molecules from 7/20 to 1/21 by bisulfite sequencing. Case 2 with a decrease from 33.7 to 5.1% showed a decrease from 3/20 to 1/22. The fraction of densely methylated DNA molecules by bisulfite sequencing was in accordance with the methylation level by real-time MSP. *Closed* and *open circles* show methylated and unmethylated CpG sites, respectively

THBD methylation level decreased from 34.7 ± 11.5 to $27.5 \pm 7.4\%$. Although both *FLNc* and *THBD* showed decreasing tendencies at 6 weeks, the decrease was not statistically significant ($P = 0.054$ and 0.066, respectively).

To confirm that we detected a decrease of densely methylated DNA molecules, not a decrease of methylated CpG sites, by real-time MSP we performed bisulfite sequencing of two pairs of samples (before and after eradication). The

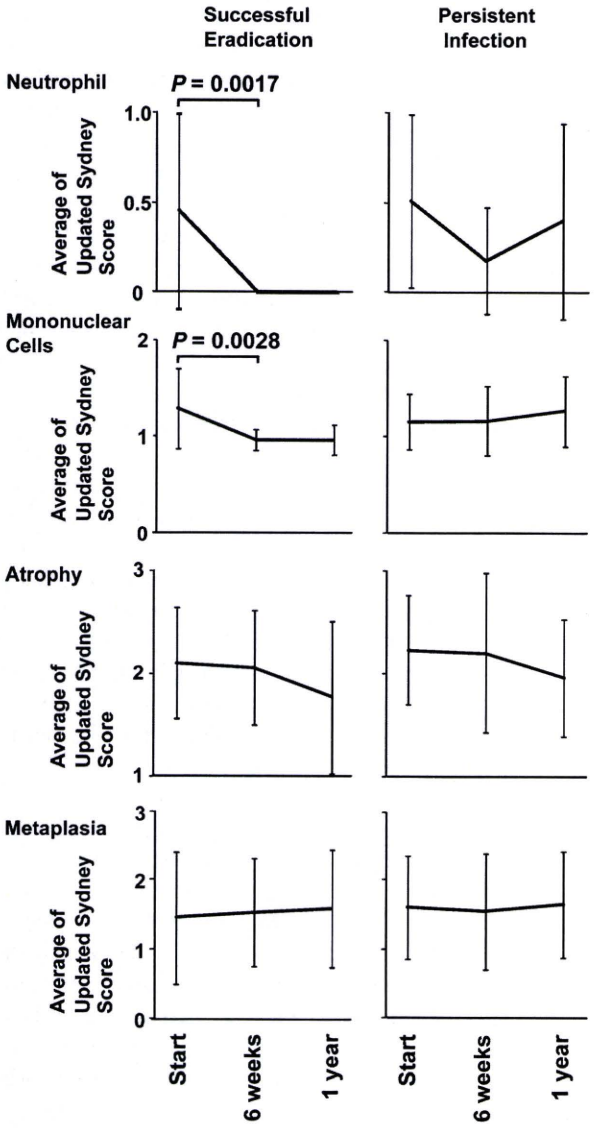

Fig. 2 Histological changes after *H. pylori* eradication. Average scores of histological gastritis of the three biopsy sites in the stomach were measured at three time points. Intensities of acute infiltrates (neutrophil), chronic infiltrates (mononuclear cells), gastric atrophy and intestinal metaplasia were graded according to the updated Sydney System as follows: none (0), mild (1), moderate (2) or marked (3). In the patients with successful eradication, neutrophil and mononuclear cell infiltration decreased at 6 weeks after eradication and remained at the decreased levels at 1 year. On the other hand, in the patients with persistent infection, neutrophil and mononuclear cell infiltration did not show any significant changes. The degree of gastric atrophy and intestinal metaplasia did not show any significant changes even in the group of successful eradication

number of densely methylated DNA molecules decreased in accordance with the decrease of the methylation level (Fig. 1c), confirming that methylation levels reflect the population of densely methylated DNA molecules, thus the population of cells with dense methylation.

Effects of *H. pylori* eradication on histological gastritis

Histological examination was performed on the infiltration of neutrophil and mononuclear cells, and the degree of atrophy and intestinal metaplasia (Fig. 2). In the patients with successful eradication, neutrophil infiltration (0.5 ± 0.5 before eradication) and mononuclear cell infiltration (1.3 ± 0.4) decreased at 6 weeks (zero, $P = 0.0017$ for neutrophil infiltration; 1.0 ± 0.1, $P = 0.0028$ for mononuclear cell infiltration), and remained at the decreased levels at 1 year (zero for neutrophil infiltration; 1.0 ± 0.1 for mononuclear cell infiltration). On the other hand, in the patients with persistent infection, the average score of neutrophils and mononuclear cells score did not show significant changes at 6 weeks and 1 year after eradication (from 0.5 ± 0.5 to 0.2 ± 0.3 and 0.4 ± 0.6, $P = 0.27$ for neutrophil infiltration; from 1.2 ± 0.3 to 1.2 ± 0.3 and 1.3 ± 0.3, $P = 0.068$ for mononuclear cell infiltration). The degree of gastric atrophy and intestinal metaplasia did not show any significant changes even in the group of successful eradication ($P = 0.062$ and 0.179, respectively). Pepsinogen II showed a clear decrease, leading to an increase of the pepsinogen I/II ratio at 6 weeks after eradication in the patients with successful eradication (Fig. 3), confirming previous reports [17, 18].

Association between the *THBD* methylation level before eradication and infiltration of inflammatory cells

To explore a mechanism for methylation induction, we analyzed the correlation between methylation levels and scores of histological analysis in all patients ($n = 35$). The *THBD* methylation level showed a weak correlation with scores of neutrophil and mononuclear cell infiltration (correlation coefficients = 0.40 and 0.45, $P = 0.017$ and 0.0067, respectively) (Table 1). On the other hand, the *FLNc* methylation level did not show any correlation with the scores of neutrophil or mononuclear cell infiltration. The degree of atrophic gastritis had no correlation with the *FLNc* and *THBD* methylation levels (Table 1), but that of intestinal metaplasia had an inverse correlation with the *THBD* methylation level.

Discussion

DNA methylation levels in non-cancerous gastric mucosae of gastric cancer patients were shown to decrease to certain levels after successful eradication of *H. pylori*, in association with the decrease of inflammatory cell infiltration. The same tendency was also observed in healthy volunteers with *H. pylori* infection. The decreased methylation levels after eradication were much higher than those of healthy

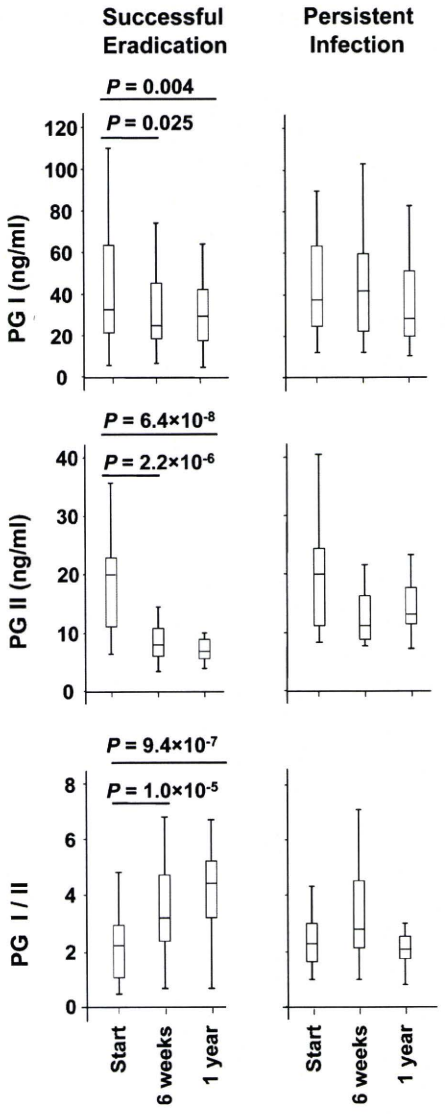

Fig. 3 Changes of serum pepsinogen I and II after *H. pylori* eradication. In the patients with successful eradication, a decrease of pepsinogen I, that of II, and increase of the I/II ratio were observed at 6 weeks after eradication ($P = 0.025$, 2.2×10^{-6}, and 1.0×10^{-5}, respectively) and 1 year ($P = 0.004$, 6.4×10^{-8}, and 9.4×10^{-7}, respectively). In the patients with persistent infection, pepsinogens I and II did not show any significant changes after eradication. The *boxes* represent the 75th and 25th percentiles, and the *line in the box* represents the 50th percentile (the median). *Whiskers* represent the maximum data within [75th percentile + 1.5 × (75th percentile − 25th percentile)] and the minimum data within [25th percentile − 1.5 × (75th percentile − 25th percentile)]

individuals without *H. pylori* infection (0.1% for *FLNc* and 0.8% for *THBD*) [6]. Our previous studies showed that methylation levels in individuals without active *H. pylori* infection correlate with gastric cancer risk [6, 7]. Taken together, methylation in gastric mucosae of individuals with active *H. pylori* infection consists of temporary and permanent components. The temporary component goes away when active *H. pylori* infection discontinues, and the remaining permanent component correlates with gastric cancer risk. The temporary component is likely to be attributed to turnover of cells with methylation, and the permanent component is likely to be methylation induced in long-living cells, possibly stem cells [19]. To use methylation levels in gastric mucosae as a risk marker, removal of the temporary component by *H. pylori* eradication will be important. This will become a fundamental basis for future studies that use DNA methylation levels in gastric mucosae as a risk marker.

FLNc and *THBD* methylation did not disappear in any of the 20 patients and 9 healthy volunteers with successful eradication. The most prominent decrease of the *THBD* methylation level was from 13.4 to 1.8%. Along with the finding by Miyazaki et al. [11], it was suggested that a decreased incidence ("disappearance" in some individuals) of *CDH1* methylation [8, 9] and that of multiple tumor-suppressor genes [10] was likely to be due to a decrease of their methylation levels below detection limits. However, there remains a possibility that eradication of *H. pylori* imposes negative selection pressure on cells with inactivation of tumor-suppressor genes.

We here analyzed methylation levels of *FLNc* and *THBD*. *FLNc* was selected because, in *H. pylori*-negative individuals, its methylation level had the strongest correlation with gastric cancer risk among the eight CGIs analyzed in previous studies [6, 7]. *THBD* was selected because its methylation level was relatively high among the eight CGIs. It was previously shown that methylation of the *FLNc* and *THBD* promoter CGIs can silence them [20]. As a result, the *FLNc* and *THBD* methylation levels showed different temporal profiles in this study. Recently, it was shown that the *miR-124a-1*, *-2*, and *-3* genes show methylation dynamics different from *FLNc* and *THBD* [21], and that *H. pylori* infection induces methylation of specific genes in gastric mucosae [22] with underlying mechanisms [23]. Taken together, it was suggested that target cells of methylation induction are different among various genes and that, even among the target genes in a specific type of cell, the susceptibility of individual genes are different. It was suggested that *FLNc* is methylated in close association with methylation of tumor-suppressor genes in cancer precursor cells although it is relatively resistant to DNA methylation.

A methylation level of an individual at a time point was obtained as an average of three samples from the three biopsy sites, because an average methylation level of the three sites reflects a risk of an individual [7]. When methylation levels were analyzed separately, those in the middle and upper gastric bodies showed significant

Table 1 Correlation between the methylation level and histological gastritis at the start point

	Neutrophil		Mononuclear cell		Atrophy		Metaplasia	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value
FLNc	−0.02	0.91	0.04	0.82	0.04	0.84	0.01	0.93
THBD	0.40	0.017	0.45	0.0067	−0.30	0.084	−0.42[a]	0.010

[a] Inverse correlation

decreases while that in the antrum did not. This was considered to be because the permanent component consisted of the major part of methylation in the antrum while it consisted of a small part in the middle and upper gastric bodies.

Molecular mechanisms of how *H. pylori* infection induces aberrant DNA methylation in gastric mucosae are still unclear. In this study, we found that, before eradication, *THBD* methylation levels were weakly correlated with the scores of inflammatory cell infiltration. We also observed that, in the group of successful eradication, infiltration of inflammatory cells in gastric mucosae improved at 6 weeks after eradication and that the *FLNc* methylation level decreased significantly at this time point. In the subgroup of patients with eradication failure, there was no significant decrease of the methylation level even at 1 year after eradication therapy, and it was suggested that eradication therapy did not affect the methylation levels in gastric mucosae. These findings suggested that chronic inflammation induced by *H. pylori* infection is responsible for methylation induction.

In summary, a decrease, but not disappearance, of methylation in gastric mucosae after successful *H. pylori* eradication was confirmed, and a possible involvement of chronic inflammation in methylation induction was suggested.

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Adenomatous polyposis coli 1A is likely to be methylated as a passenger in human gastric carcinogenesis

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ABSTRACT

Many promoter CpG islands (CGIs) are methylated as a consequence of or in association with carcinogenesis (passenger), in addition to being a cause of carcinogenesis (driver). In gastric cancers, promoter 1A of the adenomatous polyposis coli (*APC*) gene is frequently methylated, and is often discussed as a driver. However, the actual role of 1A methylation is unclear because the same *APC* protein is coded by two transcripts from two promoters, 1A and 1B, and their relative expression levels in gastric mucosae have not been quantified. To clarify this issue, we first identified detailed transcription start sites of 1A and 1B transcripts. We then confirmed that, among nine gastric cancer cell lines, 1A methylation, if present, could repress 1A transcription while 1B was expressed and not methylated. In primary samples, 1B expression was 15-fold higher than 1A expression in gastric mucosae of healthy volunteers, and was decreased markedly in non-cancerous gastric mucosae of cancer patients. Quantitative methylation analysis showed that promoter 1A was methylated at similar levels (20–40%) in healthy individuals and non-cancerous gastric mucosae of cancer patients, and promoter 1B was never methylated in any samples, including gastric cancers. These findings strongly indicated that methylation of *APC* promoter 1A is a passenger, and suggested that marked down-regulation of 1B expression could be related to formation of a field predisposed to gastric cancers.

1. Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is frequently causally involved in human carcinogenesis by inducing permanent silencing tumor-suppressor genes (driver methylation) [1]. At the same time, recent genome-wide studies have shown that a large number of CGIs are methylated in cancer cells [2–5]. Most of the methylated genes have no or little expression in normal precursor cells, and a significant fraction of them are considered to have been methylated as a consequence of or in association with carcinogenesis (passenger methylation) [2,4,6]. The presence of driver and passenger methylation is also true for gastric cancers, a major cancer in Asian countries and in which *H. pylori* infection is deeply involved [7,8]. It was recently shown that *H. pylori* infection induces methylation of various genes, both driver and passenger, in gastric epithelial cells [9,10], and that accumulation of aberrant DNA methylation is associated with gastric cancer development (an epigenetic field for cancerization) [11–13].

The adenomatous polyposis coli (*APC*) tumor suppressor gene, a negative regulator of WNT signaling [14–16],

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is known to be methylated in 34–83% of gastric cancers [17–21] while its mutations are very rare [22]. "*APC* methylation" in most studies deals with methylation of one of its two promoters, 1A and 1B, although transcripts from both promoters encode the same *APC* protein [23]. Promoter 1A is reported to be methylated not only in gastric cancers, but also in the normal mucosae with *H. pylori* infection [21]. On the other hand, promoter 1B is never methylated in gastric cancers and cancer cell lines, and neither in normal gastric tissue [17]. These points indicate that, if 1A is the major transcript in gastric mucosae, its methylation can be involved in gastric carcinogenesis as a driver. However, expression levels of 1A and 1B have not been quantified, and which of 1A and 1B is dominant has not been clarified yet.

In this study, we aimed to clarify the role of promoter 1A methylation in gastric carcinogenesis. To this end, we first confirmed transcription start sites (TSSs) of *APC* 1A and 1B, and analyzed the effect of promoter 1A methylation on 1A expression. We then quantified expression and methylation levels of 1A and 1B in gastric mucosae of healthy volunteers, non-cancerous gastric mucosae of cancer patients, and gastric cancer tissues.

2. Materials and methods

2.1. Cell lines and their 5-aza-2′-deoxycytidine and/or trichostatin A treatment

Four human gastric cancer cell lines, KATOIII, MKN28, MKN74, and NUGC3 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and AGS was obtained from the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44, and HSC57 were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan.

AGS and KATOIII cells were seeded on day 0, and media containing 0.3 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO) added on days 1 and 3, followed by addition of 1 μM trichostatin A (TSA, Sigma) on day 4, and harvested on day 5. Cells were also treated with mock, 5-aza-dC alone, and TSA alone. This dose of 5-aza-dC suppressed cellular growth to approximately half of non-treated cells. High molecular weight DNA was extracted by the phenol/chloroform method. RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) and purified with an RNeasy Mini kit (QIAGEN, Valencia, CA).

2.2. Tissue samples

Normal gastric mucosae were obtained by endoscopic biopsy from 43 healthy volunteers (32 males and 11 females; 20 with *H. pylori* infection and 23 without; average age = 47.9). Eleven and 32 samples were used for expression and methylation analysis, respectively. Non-cancerous gastric mucosae were obtained by endoscopic biopsy from 45 gastric cancer patients (35 males and 10 females; 29 with *H. pylori* infection and 16 without; average age = 66.5), and were used for methylation analysis. All of the biopsy specimens were obtained with informed consents. *H. pylori* infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan), or culture test (Eiken, Tokyo, Japan). Gastric epithelial cells were separated from stromal cells by the gland isolation technique using non-cancerous gastric mucosae of 10 gastric cancer patients (10 males; average age = 59.8) who underwent gastrectomy due to gastric cancers. Peripheral leukocytes were obtained from eight healthy volunteers (seven males and one female; average age = 35.5).

Gastric cancer tissues were obtained from 47 gastric cancer patients (40 males and seven females; average age = 64.3) who underwent gastrectomy due to gastric cancers. All cancers were histologically diagnosed according to the Japanese classification of gastric carcinoma, and classified according to the Lauren classification system [24]. Genomic DNA and total RNA were isolated in the same way as the cell lines.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

cDNA was synthesized from 1 μg of total RNA using a Superscript III kit (Invitrogen, Carlsbad, CA) with a random primer. qRT-PCR was performed by real-time PCR using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA samples that contained known numbers of molecules (10^1–10^6 molecules). The standard samples were prepared by serial dilution of PCR products quantified after purification using Zymo-Spin I™ Columns (Zymo Research, Orange, CA). The amount of the standard samples was measured by the QIAxcel system (QIAGEN). The mRNA quantity of each gene was normalized to that of β2-microglobulin. The primers and PCR conditions are shown in Supplemental Table 1.

2.4. Bisulfite treatment, methylation-specific PCR (MSP) and quantitative MSP (qMSP)

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described [25], and the modified DNA was suspended in 30 μl of TE buffer. MSP was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), using 0.5 μL of the sodium bisulfite-treated DNA. DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, MA) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences, Buckinghamshire, England) were used as fully methylated and unmethylated control DNA, respectively.

qMSP was performed by real-time PCR, using 1 μL of the sodium bisulfite-treated DNA. Although the same primer set as MSP was used for qMSP, a specific annealing temperature in the presence of SYBR® Green I was re-determined using the fully methylated and unmethylated DNA. The primers and PCR conditions are shown in Supplemental Table 2. The standard samples for real-time PCR were pro-

duced by serial dilution of PCR products quantified after purification. Based on the numbers of molecules measured by the M and U primers, a methylation level was calculated as a fraction of methylated molecules in the total number of DNA molecules.

2.5. Rapid amplification of 5′ complementary DNA ends (5′ RACE)

5′ RACE was performed using a GeneRacer™ kit (Invitrogen) on RNA from KATOIII, which is known to have no genetic alteration of *APC* [26]. The PCR product was cloned into a pGEM-T Easy Vector (Promega, Madison, WI), and a total of 31 clones were sequenced using an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA). The TSSs derived from multiple clones and located at the upstream of the *APC* translational start site were searched.

3. Results

3.1. Determination of APC transcriptional start sites by 5′ RACE

DNA methylation of the nucleosome-free region immediately upstream of a TSS is critical for gene silencing, and accurate determination of TSSs is important to evaluate involvement of DNA methylation in gene silencing [1,2,27]. The TSS of *APC* 1A in a database of TSSs (DBTSS) is located 2 bp upstream of the TSS in NCBI (described as +1 here), and no other TSSs of 1A are known. In contrast, *APC* 1B is reported to have three variants, B1 B2 and B3 (Fig. 1A) [23]. The TSS of 1B in DBTSS is located 2 bp downstream of the TSS in NCBI (described as +1 here) based on the report by Horii et al. [23].

To determine TSSs of *APC*, we performed 5′ RACE, and identified five novel TSSs, all of which were in exon 1B (Fig. 1B). Therefore, we analyzed the methylation status of the immediate upstream regions of the TSSs of 1A and 1B in NCBI (200 bp or less) as promoter 1A and 1B. The activity of promoter 1A was assessed by quantification of *APC* 1A using PCR primers on exons 1A and 2. The activity of promoter 1B was assessed by quantification of *APC* B1 plus B2 using primers on the 3′ region of exon 1B (not transcribed in B3) and exon 2 since expression levels of B1 plus B2 paralleled that of B3 among 28 samples of various origins (Fig. 1C).

3.2. Effect of APC 1A methylation on its silencing

To examine the effect of methylation of promoters 1A and 1B on their silencing, their methylation was first analyzed in nine human gastric cancer cell lines. Promoter 1A was completely methylated in six cell lines, completely unmethylated in two, and in a mixed status in one (Fig. 2A). Promoter 1B was completely unmethylated in all the nine cell lines analyzed. By quantitative mRNA expression analysis of individual 1A and 1B transcripts, it was found that 1A was consistently repressed in the six cell lines with 1A methylation (Fig. 2A). 1B was expressed in all of the nine cell lines.

When 1A methylation was removed by a demethylating agent, 5-aza-dC, in two cell lines with its complete methylation (AGS and KATOIII), 1A expression was restored (Fig. 2B). Addition of TSA significantly (48-fold in AGS and 17-fold in KATOIII) enhanced 1A restoration by 5-aza-dC in both cells. In contrast, 1B expression was not restored by treatment with 5-aza-dC alone or TSA alone. Only in AGS, slight (2.7-fold) up-regulation of 1B was observed by the combined treatment with 5-aza-dC and TSA. This showed that, if promoter 1A is methylated, it leads to 1A silencing, but that promoter 1B was consistently unmethylated and expressed.

3.3. APC 1B is the major transcript in normal human gastric mucosae

To examine which of *APC* 1A and 1B is the major transcript in gastric mucosae, we quantified their expression levels using the primers described above in 11 gastric mucosae of healthy volunteers (five with *H. pylori* infection and six without), 10 non-cancerous gastric mucosae of gastric cancer patients, and 19 gastric cancers from which high-grade RNA was isolated (Fig. 3A). In the gastric mucosae of *H. pylori*-negative healthy individuals, the average 1B expression level was 15-fold higher than that of 1A. In the gastric mucosae of *H. pylori*-positive healthy individuals, the average 1B expression level decreased to 52% of that of *H. pylori*-negative individuals, but was still 11-fold higher than that of 1A. In the non-cancerous gastric mucosae of cancer patients, the average 1B expression level further decreased to 9% of that of *H. pylori*-negative individuals. In the 19 gastric cancers, the average 1B expression level was 5% of that of *H. pylori*-negative healthy individuals. The 1A expression level was consistently low among these four groups.

To exclude the possibility that the abundant 1B expression was derived from gastric stromal cells, gastric epithelial cells and stromal cells were separated by the gland isolation technique. For this technique, several cm^2 areas of gastric mucosae were necessary, and we were able to analyze only non-cancerous gastric mucosae of cancer patients (surgical specimens). Isolation of gastric epithelial cells was confirmed by the shape of the glands obtained (Fig. 3B). 1B showed similar expression levels between the isolated gastric epithelial cells and the remaining stromal cells (Fig. 3C). 1A showed lower expression levels in the epithelial cells than in the remaining stromal cells. These findings supported that the abundant 1B expression in gastric mucosae was not due to contamination of stromal cells.

3.4. High methylation level of promoter 1A irrespective of H. pylori infection status in gastric mucosae, and its presence in gastric cancers

In non-cancerous tissues, which are polyclonal, quantification of the DNA methylation level is essential to assess the fraction of cells with methylation. Therefore, methylation levels of promoters 1A and 1B were quantified in gastric mucosae of 32 healthy volunteers (normal mucosae, 14 with *H. pylori* infection and 18 without), and 45 non-cancerous mucosae of gastric cancer patients (29 with *H. pylori* infection and 16 without) obtained by endoscopic biopsy. Methylation levels were also quantified in 47 gastric cancers. The methylation level of promoter 1A was 20–40% in the normal mucosae and non-cancerous mucosae, regardless of *H. pylori* infection statuses (Fig. 4A). In cancer tissues, it ranged from 0% to 73%, reflecting the monoclonal nature of cancer tissues. In sharp contrast, promoter 1B was not methylated at all in any of the samples (Fig. 4A).

To examine in what cell types promoter 1A was methylated, we quantified 1A methylation levels in gastric epithelial cells and stromal cells prepared from non-cancerous gastric mucosae of three gastric cancer patients and in peripheral leukocytes of eight healthy volunteers. Promoter 1A was methylated at higher levels in the gastric epithelial cells than in the stromal cells, and was not methylated in peripheral leukocytes (Fig. 4B). This showed that promoter 1A methylation detected in gastric mucosae was due to methylation in gastric epithelial cells, in addition to stromal cells.

The high fraction of cells with 1A methylation among gastric epithelial cells should have affected its overall 1A expression level if its expression in cells without methylation had been high enough. However, in our observation, the 1A expression level was not correlated with 1A methylation levels among 10 samples of gastric epithelial cells (Spearman's rank-order correlation coefficient = 0.44, Fig. 4C). Also, the 1A expression level observed ($0.5–1 \times 10^{-4}$/β2MG) was considered to be $0.5–1 \times 10^{-2}$ mRNA molecules in a cell, on the assumption that 1 μg total RNA can be isolated from 10^5 cells. This also supported that *APC* 1A was expressed only at a trace level, or not expressed with biological significance, even in cells without 1A methylation.

4. Discussion

APC 1B was the major transcript in normal gastric mucosae. Promoter 1A was methylated at similar levels in gastric mucosae of healthy individuals (with and without *H. pylori* infection) and non-cancerous gastric mucosae of gastric cancer patients. Although promoter 1A methylation could silence its expression, the fraction of gastric epithelial cells with methylation did not influence the overall 1A expression level, showing that 1A was expressed only at trace levels in cells without methylation. It is becoming clear that genes with low expression levels are susceptible

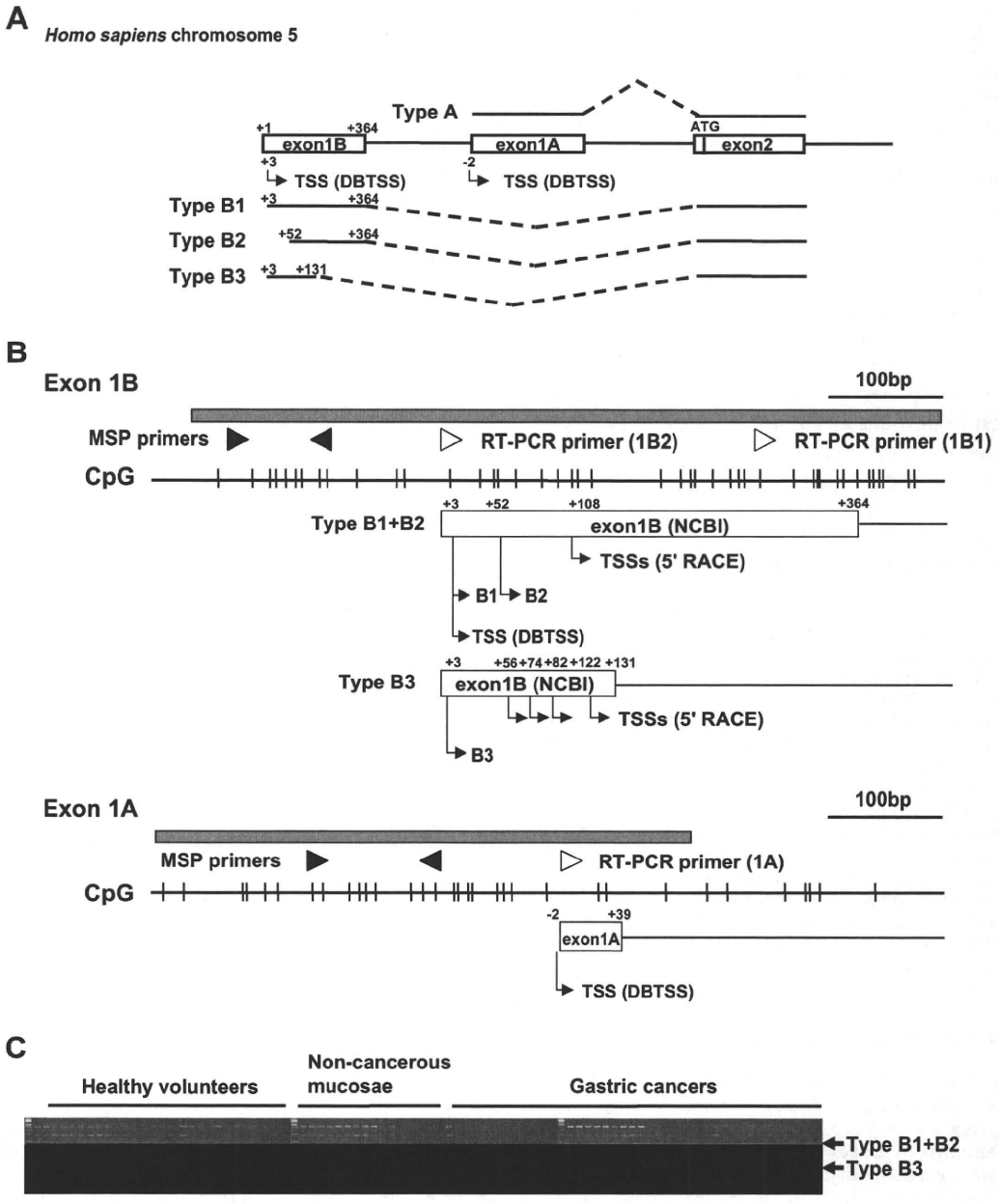

Fig. 1. Genomic and mRNA structures of *APC* 1A and 1B. (A) Splicing forms of 1A and 1B. One form of 1A and three variants of 1B are known to be produced from individual promoters. (B) CpG maps of promoters 1A and 1B and their TSSs. The TSS of *APC* 1A in a database of TSSs (DBTSS) is located 2 bp downstream of the TSS in NCBI (NC_000005.8 112101483, described as +1 here). The TSS of 1B in DBTSS is located 2 bp downstream of the TSS in NCBI (NC_000005.8 112071115, described as +1 here) based on D13981 [23]. Three TSSs, B1 B2 and B3, were based on the report by Horii et al. [23]. Five TSSs were identified in this study by 5′ RACE. *Vertical lines*, individual CpG sites; *Gray boxes*, the CGI regions; *Arrows*, TSSs; and *Arrowheads*, positions of MSP and RT-PCR primers. (C) Similar expression levels of *APC* B1 plus B2 and *APC* B3. Quantitative RT-PCR analysis of all the three variants (B1, B2, and B3) using one set of primers was impossible due to different lengths of PCR products. It was confirmed that the expression level of B1 plus B2 paralleled that of B3 among 28 samples.

to DNA methylation [2,4,6]. Our results and current knowledge strongly indicated that *APC* 1A was methylated as a passenger during gastric carcinogenesis.

This conclusion was not in agreement with many previous reports that discussed *APC* 1A methylation as a driver [17–21]. Our conclusion was attained by accurate quantitative expression and methylation analysis, which has become popular recently, and most previous reports did not adopt quantitative analysis. Quantitative methylation analysis revealed that *APC* 1A was methylated in normal gastric mucosae of healthy individuals, regardless of *H. pylori* infection status, and that the methylation level was not correlated with age ($N = 32$, $r = -0.02$). Therefore, it was considered that *APC* 1A methylation was physiologically present in human gastric mucosae as a simple fluctuation in the methylation status of a non-expressed gene or as a

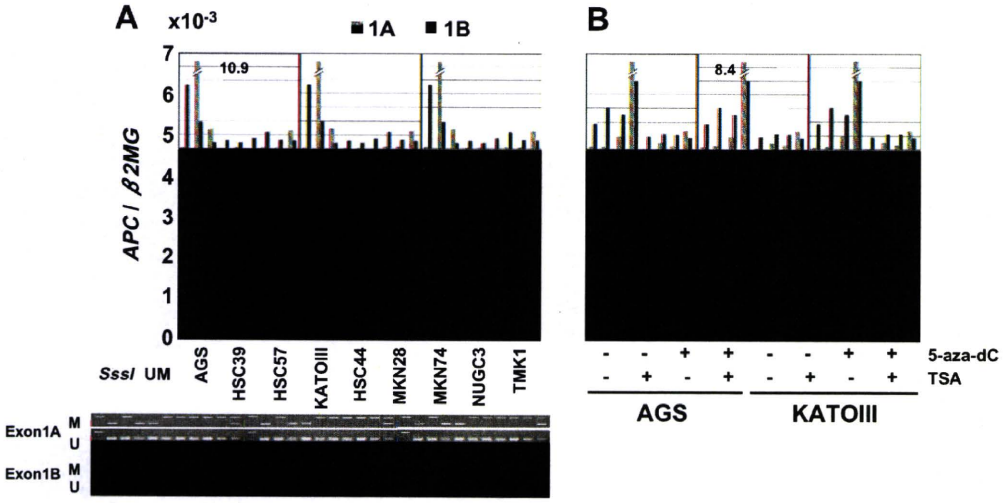

Fig. 2. Methylation-silencing of 1A in human gastric cancer cell lines. (A) qRT-PCR of *APC* 1A and 1B, and MSP of promoters 1A and 1B in nine cell lines. The mRNA quantity of each gene was normalized to that of β2-microgloburin (β2MG). It was found that 1A was consistently repressed in the six cell lines with 1A methylation. *Sss*I, fully methylated DNA (genomic DNA methylated with *Sss*I methylase); and UM, fully unmethylated DNA (DNA amplified by a GenomiPhi DNA amplification kit). (B) Expression changes of *APC* 1A and 1B after 5-aza-dC and/or TSA treatment. Two cell lines with complete methylation of 1A were treated with 5-aza-dC (0.3 μM) and/or TSA (1 μM), and 1A and 1B expression levels were quantified by qRT-PCR. Restoration of the 1A expression in AGS and KATOIII by the 5-aza-dC treatment was observed. Addition of TSA significantly enhanced the 1A restoration by the 5-aza-dC treatment. 1B expression was not restored by treatment with 5-aza-dC alone or TSA alone. Only in AGS, slight up-regulation of 1B was found by combination treatment with 5-aza-dC and TSA.

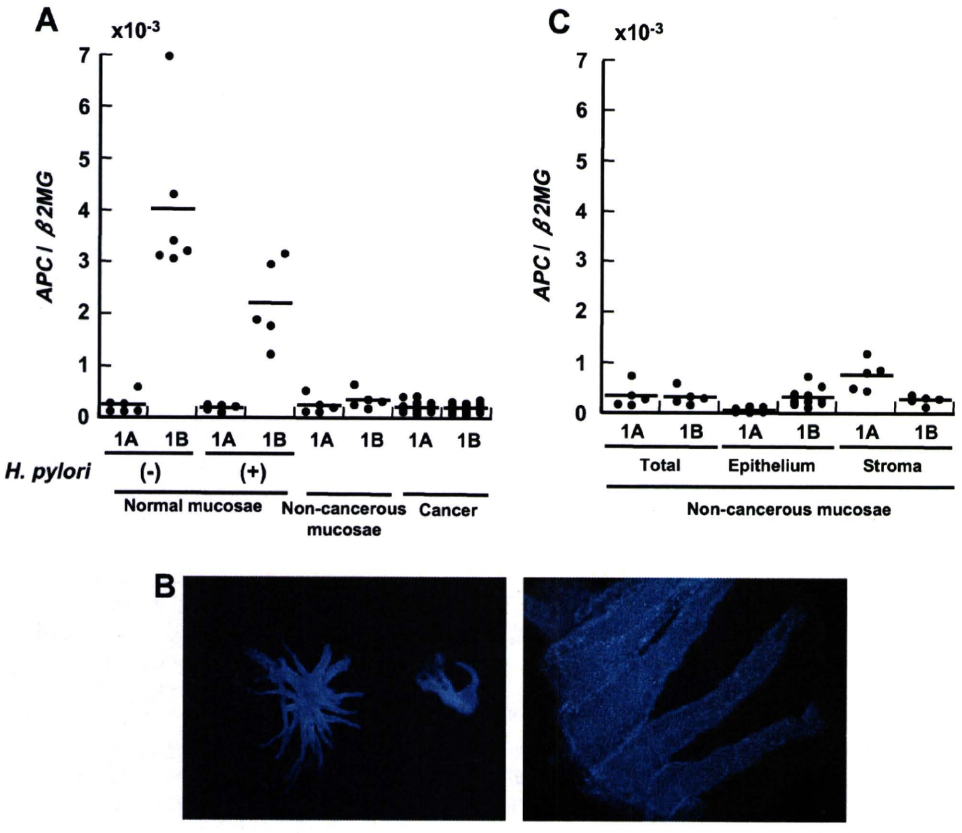

Fig. 3. Expression levels of *APC* 1A and 1B in primary samples. (A) Expression levels of 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers. In *H. pylori*-negative gastric mucosae of healthy volunteers, the average 1B expression level was 15-fold higher than that of 1A. The 1B expression level was down-regulated in *H. pylori*-positive gastric mucosae, and further in non-cancerous gastric mucosae. (B) DAPI staining of isolated gastric glands. (C) Expression levels of 1A and 1B in gastric epithelial and stromal cells obtained from non-cancerous gastric mucosae of cancer patients. 1B showed similar expression levels between the isolated gastric epithelial cells and the remaining stromal cells.

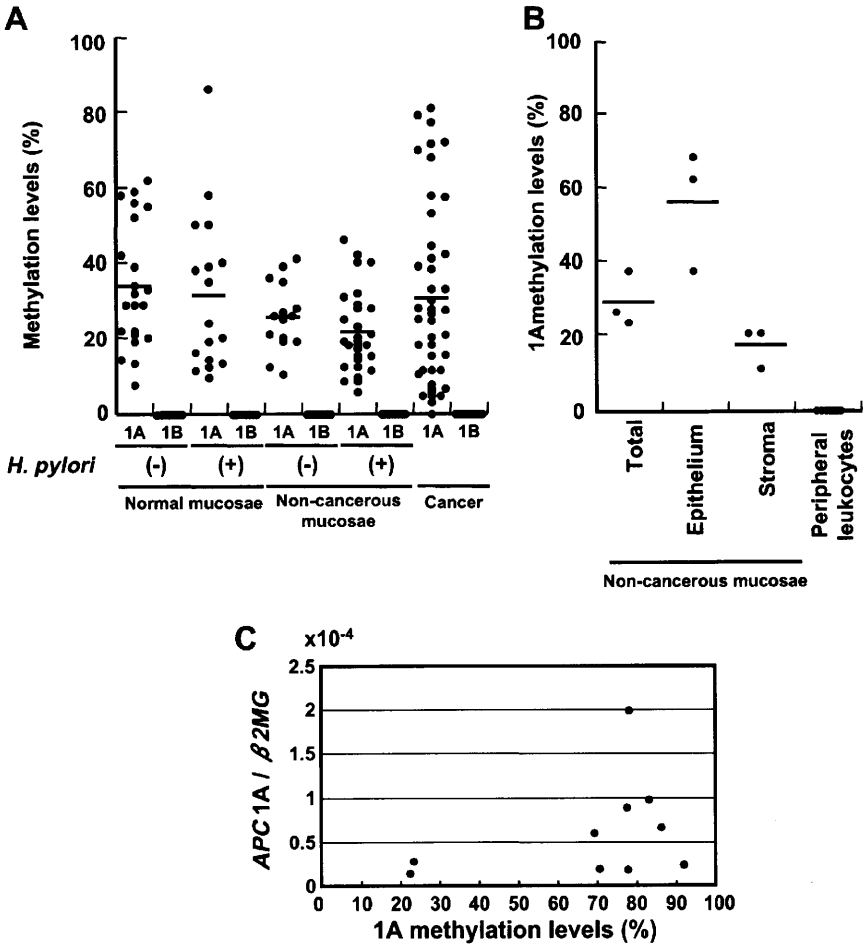

Fig. 4. Methylation levels of promoters 1A and 1B in primary samples. (A) Methylation levels of promoters 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers, analyzed by qMSP. Promoter 1A was methylated in all groups of samples while promoter 1B was never methylated. (B) Methylation levels of promoter 1A in gastric epithelial cells, stromal cells, and peripheral leukocytes. Methylation level of promoter 1A was relatively higher in the gastric epithelial cells than in the stromal cells. (C) Relationship between the 1A methylation level and the 1A expression level in gastric epithelial cells. The 1A methylation level was not correlated with down-regulation of *APC* 1A ($r = 0.44$), suggesting that 1A is expressed only at a trace level even in cells without its methylation.

cell-type-specific methylation, and that the methylation was carried over to gastric cancer cells as a passenger. For promoter 1A methylation to be cell-type-specific, it should be present in 15–60% of cells in normal mucosae and non-cancerous mucosae, based on our quantification (Fig. 4A). Although it was difficult to hypothesize a histologically distinct cell type with this population, there remains the possibility. Quantitative expression analysis enabled us to ascertain that *APC* 1B was dominant in gastric mucosae of healthy individuals.

The expression level of *APC* 1B, the major transcript of *APC* in gastric mucosae, was down-regulated in *H. pylori*-positive gastric mucosae of healthy volunteers and further in non-cancerous mucosae of gastric cancer patients, and the marked down-regulation was carried over to gastric cancers. This suggested that down-regulation of *APC* and activation of the WNT/β-catenin pathway itself could be involved in early stages of human gastric carcinogenesis. In addition to the presence of epigenetic alterations [11], the presence of distinctive expression profiles in early stages of gastric carcinogenesis has been demonstrated [28]. As for the mechanism of 1B down-regulation, we were not able to detect promoter 1B methylation, or involvement of histone deacetylation. Aberrant histone modifications of *APC* 1B other than deacetylation or epigenetic changes of genes that influence 1B transcription could be involved in the down-regulation. It is reported that, although *APC* mutations are rare in gastric cancers, the nuclear accumulation of β-catenin is detected in 39% of human gastric cancers [29]. Also, aged $APC^{Min/+}$ mice spontaneously develop multiple tumors in the stomach, and such tumors consist of adenomatous glands with strong nuclear accumulation of β-catenin [29].

There is a lot of literature on *APC* methylation in cancers of tissues other than the stomach [30–34]. However, at least in some of these tissues, it remains to be clarified which of 1A and 1B is the major transcript. Since genes with low transcription are susceptible to DNA methylation

[2,4,6], the meaning of methylation should be carefully established. To establish that methylation of one of the two *APC* promoters is the driver of carcinogenesis, evidence of low or no expression from the other promoter is necessary.

In summary, the *APC* 1B expression level was significantly and much higher than the *APC* 1A expression level in human normal gastric mucosae. Therefore, methylation of the *APC* promoter 1A is likely to be a passenger in human gastric carcinogenesis.

Conflicts of interest

None declared.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.05.016.

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Original Article

The Presence of Aberrant DNA Methylation in Noncancerous Esophageal Mucosae in Association With Smoking History

A Target for Risk Diagnosis and Prevention of Esophageal Cancers

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BACKGROUND: Esophageal squamous cell carcinomas (ESCCs) tend to have multiple primary lesions, and it is believed that they arise from background mucosae with accumulation of genetic/epigenetic alterations. In this study, the objective was to elucidate the effects of smoking and drinking on the accumulation of epigenetic alterations in background mucosae. **METHODS:** Genes that are silenced in human ESCCs were searched for by treating 3 ESCC cell lines with the demethylating agent, 5-aza-2′-deoxycytidine and performing oligonucleotide microarrays. Methylation levels were analyzed by quantitative methylation-specific polymerase chain reaction analysis of 60 ESCCs and their corresponding background mucosae. **RESULTS:** Forty-seven genes were identified as methylation-silenced in at least 1 of the 3 ESCC cell lines, and 14 of those genes (claudin 6 [*CLDN6*]; G protein-coupled receptor 158 [*GPR158*]; homeobox A9 [*HOXA9*]; metallothionein 1M [*MT1M*]; neurofilament, heavy polypeptide 200 kDa [*NEFH*]; plakophilin 1 [*PKP1*]; protein phosphatase 1, regulatory [inhibitor] subunit 14A [*PPP1R14A*]; pyrin domain and caspase recruitment domain containing [*PYCARD*]; R-spondin family, member 4 [*RSPO4*]; testis-specific protein, Y-encoded-like 5 [*TSPYL5*]; ubiquitin carboxyl-terminal esterase L1 [*UCHL1*]; zinc-finger protein 42 homolog [*ZFP42*]; zinc-finger protein interacting with K protein 1 homolog [*ZIK1*]; and zinc-finger and SCAN domain containing 18 [*ZSCAN18*]) were used as markers. In the background mucosae, methylation levels of 5 genes (*HOXA9*, *MT1M*, *NEFH*, *RSPO4*, and *UCHL1*) had significant correlations with smoking duration ($\rho = .268$; $P = .044$; $\rho = .405$; $P = .002$; $\rho = .285$; $P = .032$; $\rho = .300$; $P = .024$; and $\rho = .437$; $P = .001$, respectively). In contrast, an inverse correlation between *PYCARD* methylation levels and alcohol intake was observed ($\rho = -.334$, $P = .025$) among individuals with the inactive aldehyde dehydrogenase 2 (*ALDH2*) genotype. **CONCLUSIONS:** The current results suggested that ESCCs developed from an epigenetic field for cancerization, which was induced by exposure to carcinogenic factors, such as tobacco smoking. The epigenetic field defect will be a novel target for risk diagnosis and prevention of ESCCs. **Cancer 2009;115:3412–26.**

KEY WORDS: epigenetics, DNA methylation, esophageal cancer, tobacco smoking, alcohol drinking.

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Esophageal cancer is 1 of the most lethal cancers and imposes reduced quality of life even in patients who receive curative treatment.[1] Squamous cell carcinoma (SCC) and adenocarcinoma are 2 major histologic types of esophageal cancer, and SCC is the predominant histologic type in Asian countries.[2] Most patients with esophageal SCC (ESCC) have a history of chronic smoking and/or heavy drinking, and these are established risk factors for ESCC.[1] With regard to the interactions between smoking and drinking, controversial reports are available; however, to our knowledge, to date, the combined risk for ESCC has not been clarified.[3-5]

Patients with ESCC often have multiple primary lesions,[6] and the frequency of multiple occurrence reaches up to 30%.[7,8] In addition, dysplastic lesions frequently are observed in the background mucosae surrounding ESCCs.[9] The incidence of multiple occurrences of ESCC and/or dysplastic lesions reportedly is high in heavy smokers and heavy drinkers.[7,10,11] It is believed that both tobacco smoking and alcohol drinking can induce genetic/epigenetic alterations in esophageal mucosal cells and that genetic/epigenetic alterations already have accumulated in the normal-appearing esophageal mucosae of individuals who have a long history of smoking and drinking, forming a "field for cancerization."[12]

DNA methylation of a CpG island (CGI) in a promoter region causes silencing of its downstream gene and is known as a major epigenetic mechanism for inactivation of tumor-suppressor genes.[13] In ESCCs, various tumor-suppressor genes, such as adenomatous polyposis coli (*APC*), cadherin 1 (*CDH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), retinoic acid receptor β (*RARβ*), Ras association domain family member 1-isoform A (*RASSF1A*), and ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), reportedly are methylated.[14,15] In contrast to the deep involvement of aberrant DNA methylation in ESCCs, its inducers in the esophagus have been poorly clarified, except for induction methylation of the fragile histidine triad gene *FHIT* by nicotine, a tobacco component, in human esophageal epithelial cell lines.[16] Tobacco smoking reportedly induced methylation of several genes in bronchial and oral epithelium,[17-20] but no information was available in esophageal epithelial cells.

In the current study, our objective was to clarify the effects of tobacco smoking and alcohol drinking on the induction of DNA methylation in esophageal mucosae. The mucosae are not clonal, and the degree of methylation is correlated with the power of methylation induction. Because different genes have different susceptibility to methylation induction,[21] first, we screened genes that were silenced in ESCCs by treating ESCC cell lines with a demethylating agent and analyzing the genes that were up-regulated. Then, we quantified the methylation levels of the "marker" genes and 4 tumor-suppressor genes in ESCCs and in noncancerous esophageal mucosae by using quantitative methylation-specific polymerase chain reaction (PCR) (MSP) analysis.

MATERIALS AND METHODS

Cell Lines and 5-Aza-2′-deoxycitidine Treatment

The ESCC cell lines KYSE30, KYSE220, and KYSE270, which were derived from well differentiated, moderately differentiated, and well differentiated ESCCs, respectively,[22] were purchased from Health Science Research Resources Bank (Osaka, Japan). For 5-aza-2′-deoxycitidine (5-Aza-dC) treatment, 2×10^5 KYSE30 cells per 10-cm dish, 5×10^5 KYSE220 cells per 10-cm dish, and 4×10^5 KYSE270 cells per 10-cm dish were seeded on Day 0 and exposed to freshly prepared 5-Aza-dC (Sigma, St. Louis, MO) for 24 hours on Days 1 and 3. The concentration of 5-Aza-dC was 1 μM for KYSE30 and KYSE220 cells and 0.3 μM for KYSE270. After each treatment, the cells were placed in fresh medium and harvested on Day 4. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) and purified using an RNeasy Mini kit (Qiagen, Valencia, Calif). DNA methyltransferase 1 depletion was confirmed by Western blot analysis of the whole cell extract.[23]

Oligonucleotide Microarray Analysis and Database Search

Oligonucleotide microarray analysis was performed using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, Calif) with 47,400 probe sets from 38,500 genes and GeneChip Operating Software. The signal intensities were normalized so that the average

of all genes on a GeneChip was 500. Database searches were performed at a GenBank website, and we searched for CGIs by using a modified criteria of Takai and Jones[24]: 1) CpG score ≥0.60, 2) guanine (G) and cytosine (C) content ≥50%, and 3) length ≥500 base pairs.

Patients and Tissue Samples

Sixty primary ESCC specimens and their paired noncancerous background mucosae were collected from patients who underwent esophagectomy and who were diagnosed histologically with invasive SCC at the National Cancer Center Hospital, Tokyo, Japan (51 men and 9 women; average age, 63.3 years [range, 41-83 years]). Informed consent was obtained from all patients. Background mucosae were resected from areas stained by iodine that were considered histologically normal.[25] All samples were stored in RNAlater (Applied Biosystems, Foster City, Calif) at −80°C until the extraction of genomic DNA. Genomic DNA was extracted by using the phenol-chloroform method.

History of tobacco smoking and alcohol drinking was obtained from 57 patients and 55 patients, respectively, in interviews with the patients by medical staff. Mean daily alcohol intake was calculated, converting a cup of sake (180 mL), a cup of shochu (180 mL), a single finger of whisky (30 mL), a bottle of wine (750 mL), and a bottle of beer (633 mL) into 27 g, 45 g, 12 g, 105 g, and 25 g of alcohol, respectively.

Aldehyde Dehydrogenase Genotyping

Aldehyde dehydrogenase 2 (*ALDH2*) genotyping of each patient was performed on genomic DNA extracted from background mucosae by PCR-restriction fragment length polymorphism.[26] Exon 12 of the *ALDH2* gene was amplified with forward primer (5′-CAAATTACAGGGTCAACTG CT-3′) and reverse primer (5′-CCACACTCACAGTTTTC TCTT-3′). The PCR products were digested with *Eam*1104I (*Ksp*632I) (Fermentas International Inc., Burlington, Ontario, Canada) and electrophoresed on ethidium bromide-stained 2% NuSieve agarose gels.

Bisulfite Modification and Semiquantitative Methylation-specific PCR

DNA was digested by *Bam*HI, and 1 μg of the digested DNA was denatured in 0.3 N NaOH at 37°C for 15 minutes. The samples underwent 15 cycles of 30-second denaturation at 95°C and a 15-minute incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with Zymo-Spin IC Columns (Zymo Research, Orange, Calif), desulfonated in 0.3 N NaOH, and dissolved in 40 μL to 160 μL of Tris-ethylene diamine tetraacetic acid buffer.

MSP was performed with a primer set specific to the methylated (M) or unmethylated (U) sequence using 12.5 ng of the sodium bisulfite-treated DNA and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif). The primers used are listed in Table 1. DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, Mass) and DNA amplified twice by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences, Buckinghamshire, England) were used as fully methylated and unmethylated control DNA, respectively. For semiquantitative MSP, "10%-methylated DNA" was prepared by mixing 10% of methylated DNA and 90% of fully unmethylated control DNA after bisulfite modification.

Quantitative Methylation-specific PCR and Calculation of Methylation Levels

Real-time MSP was performed using 50 ng of the sodium bisulfite-treated DNA, SYBR Green I (BioWhittaker Molecular Applications, Rockland, Md), and an iCycler Thermal Cycler (Bio-Rad Laboratories). Methylated and unmethylated standard DNA was prepared by cloning PCR products from fully methylated and unmethylated control DNA, respectively, into the pGEM-T Easy vector (Promega, Madison, Wis) or by purifying PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The numbers of methylated and unmethylated molecules in a sample were determined by comparing its amplification with that of methylated and unmethylated standard DNA, respectively, that contained known numbers of molecules (10 to 10^6 molecules).

On the basis of the numbers of methylated and unmethylated molecules for a genomic region in a sample, methylation levels were calculated as the fraction of methylated molecules in the total number of DNA molecules (the number of methylated molecules + the number of

Table 1. Primers for Qualitative or Quantitative Methylation-specific Polymerase Chain Reaction

Gene Symbol	Status	Primer Sequence		Annealing Temperature, °C		No. of Cycles*
		Forward	Reverse	Qualitative	Quantitative	
CLDN6	M	ATAAGTTTGGGATTCGTAC	ATCTTAAAAAAACGATAACG	52	54	35
	U	TTGATAAGTTTGGGATTTGTAT	CAAAAATCTTAAAAAAACAATAACA	52	54	35
GPR158	M	GTAATTTTTAGTCGGTTTTTC	GAATAACTAAACTACCGTCG	56	52	35
	U	TGTAATTTTTAGTTGGTTTTTTGT	CCAAATAACTAAACTACCATCA	56	58	35
HOXA9	M	TCGGATTATTAATAGCGTGC	ATCACCTAATAAATTACCGACG	60	58	33
	U	TAATAGTGTGTGGAGTGATTTATGT	CAATCACCTAATAAATTACCAACA	60	60	33
MT1M	M	GTATAGTTTTTTTCGCGTAAATTC	AACCCAACATAAATACCGAACG	59	55	32
	U	TTATTTGGTGTATAGTTTTTTTTGT	TAAACCCAACATAAATACCAAACA	56	52	38
NEFH	M	TTAAGGGGTTGGATTCGGTC	CGAAACGAAACGAAAAACACTACG	61	58	35
	U	GTTAAGGGGTTGGATTTGGTT	CCAAAACAAAACAAAAAACACTACA	61	61	35
PKP1	M	TTTTGTTTTTAAGAGCGTTGGTTTC	ACCCACTCCACCGAACCG	58	62	35
	U	TTTTGTTTTTAAGAGTGTTGGTTTT	CACTCCACCAAACCACACA	58	61	34
PPP1R14A	M	ATTTCGGTTCGGGAGTTTTC	TCGACTTAAAACACGCAATCG	58	60	35
	U	GGAGTTTTTGATGTAGGGATTT	TCAACTTAAAACACACAATCATACA	58	57	35
PYCARD	M	CGGGGAATCGCGGAGGTTTC	AATAAAACCCGAAAAAAAACCG	55	57	35
	U	GGTTTGGGGAATTGTGGAGGTTTT	ATCACACCCTCCAACTAACCTACA	55	60	33
RSPO4	M	CGTTAGGGTAGTGTTTCGGTTTC	TACTATAAACGCGCCGAACG	57	60	35
	U	TTTTTTTTTGTTAGGGTAGTGTTTT	ATAAACACACCAAACACATCCA	57	58	35
TSPYL5	M	GGGTCGTTTTTTGCGTAGTC	GTCACGAACGTACAACTATACCG	62	60	35
	U	GGTTGTTTTTTGTGTAGTTGTAGT	CATCACAAACATACAACTATACCA	62	62	35
UCHL1	M	TCGTATTTATTTGGTCGCGATC	CTATAAAACGCCGACCAAACG	62	64	35
	U	GGTTTGTATTTATTTGGTTGTGATT	CAACTATAAAACACCAACCAAACA	60	61	33
ZFP42	M	GCGTCGTTTAGGTGTTAGGC	AAAACGTAACCGACCCCG	62	57	35
	U	GTGTTTTAGGGTGGGGTTGGTTAT	AAACCCACCCTCCAAACTAACACA	62	63	35
ZIK1	M	GTTTGAGGTGACGTTGGGC	GACCCTTTTCTCAACGCGA	64	62	35
	U	TTTGAGGTGATGTTGGGTG	AACAACCCTTTTCTCAACACA	60	59	35
ZSCAN18	M	TTTTTTGTTCGTTTCGGTGC	GATAACGACCGACAAACTACG	59	62	35
	U	TGTTATGGTTTTTTTGTTTGTTTT	CTACACACTAAACCTCACCACA	59	60	35
CDH1	M	TAGGTTTTAGTGAGTTATCGGC	AAACGAAACTAACGACCCG		59	
	U	ATTTTAGGTTAGAGGGTTATTGTG	ATAAACCCCAAAAACACCA		59	
CDKN2A	M	TTGGTAGTTAGGAAGGTTGTATCGC	TCCCTACTCCCAACCGCG		62	
	U	GGTAGTTAGGAAGGTTGTATTGT	TCCCTACTCCCAACCACA		61	
MLH1	M	CGTTAAGTATTTTTTTCGTTTTGC	TCCGCTCTTCCTATTAATTCG		59	
	U	AGTGTTAAGTATTTTTTTTGTTTTGT	CTATCCACTCTTCCTATTAATTCA		56	
RASSF1A	M	GTCGTCGTTGTGGTCGTTC	AACCCGAAAACGAAACTAAACG		62	
	U	TGTTGTTGTTGTGGTTGTTT	AAAACAAAACTAAACACACTCTCA		62	

CLDN6 indicates claudin 6; M, specific to methylated DNA; A, adenine; T, thymine; G, guanine; C, cytosine; U, specific to unmethylated DNA; *GPR158*, G protein-coupled receptor 158; *HOXA9*, homeobox A9; *MT1M*, metallothionein 1M; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain and caspase recruitment domain containing; *RSPO4*, R-spondin family, member 4; *TSPYL5*, testis-specific protein, Y-encoded-like 5; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18; *CDH1*, cadherin 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MLH1*, MutL homolog 1; *RASSF1A*, Ras association domain family member 1, isoform A.
* The number of polymerase chain reaction (PCR) cycles for qualitative methylation-specific PCR.

unmethylated molecules). For each gene, the deviation value of a methylation level in the background mucosa of a case was calculated.

Statistical Analysis

Correlations between methylation levels in background mucosae and risk factors of patients were analyzed by Spearman rank-order correlation coefficients (ρ). Methylation levels in groups with different clinicopathologic characters were compared using the Kruskal-Wallis test and the Mann-Whitney *U* test. Correlations between methylation frequencies in ESCCs and methylation levels in background mucosae (mean deviation values) were analyzed using Pearson correlation coefficients. The statistical calculations were conducted with SPSS software (13.0J; SPSS Inc., Chicago, Ill).

RESULTS

Genes Up-regulated by 5-Aza-deoxycitidine Treatment and Their Methylation Analysis

Three ESCC cell lines (KYSE30, KYSE220, and KYSE270) were treated with 5-Aza-dC, and changes in gene expression were analyzed by oligonucleotide microarrays. We searched for genes that 1) were up-regulated above a threshold (8-fold, 16-fold, or 32-fold), 2) had signal intensities of $\leq$100 before the treatment and >100 after the treatment, 3) were not located on chromosome X, and 4) had CGIs 5′ upstream of their putative transcription start sites. The higher threshold we adopted, the fewer genes were up-regulated. Because the objective of the screening was to isolate marker genes for exposure to tobacco smoking and alcohol drinking, we adopted a cutoff value of 16-fold so that a manageable number of candidate genes (72 candidate genes in total) would be obtained.

The methylation status of the CGIs at the putative transcription start sites of the 72 genes was analyzed by MSP in the KYSE30, KYSE220, and KYSE270 cell lines. Forty-seven genes were confirmed as completely methylated at least in 1 of the 3 cell lines and were considered to be methylation silenced. Then, their methylation status was analyzed in 6 primary ESCCs and their background mucosae by using semiquantitative MSP. Thirty-nine of those genes were methylated in at least 1 primary ESCC, but 15 genes were methylated too heavily in the background mucosae ($\geq$10% in all 6 samples). Therefore, the remaining 24 genes were considered the most informative.

Methylation Quantification in ESCCs and Background Mucosae

Among the 24 genes, primers for quantitative MSP were designed successfully for 14 genes: claudin 6 (*CLDN6*); G protein-coupled receptor 158 (*GPR158*); homeobox A9 (*HOXA9*); metallothionein 1M (*MT1M*); neurofilament, heavy polypeptide 200 kDa (*NEFH*); plakophilin 1 (*PKP1*); protein phosphatase 1, regulatory (inhibitor) subunit 14A (*PPP1R14A*); pyrin domain and caspase recruitment domain containing (*PYCARD*); R-spondin family, member 4 (*RSPO4*); testis-specific protein, Y-encoded–like 5 (*TSPYL5*); *UCHL1*; zinc-finger protein 42 homolog (*ZFP42*); zinc-finger protein interacting with K protein 1 homolog (*ZIK1*); and zinc-finger and SCAN domain containing 18 (*ZSCAN18*). Methylation levels of these 14 genes and of 4 tumor-suppressor genes (*CDH1*, *CDKN2A*, mutL homolog 1 [*MLH1*], and *RASSF1A*), 3 of which reportedly are silenced in ESCCs,[27–29] were analyzed in 60 ESCCs and their paired noncancerous background mucosae. Similar to results from an examination of gastric cancers and their background mucosae,[30] distributions of methylation levels revealed different patterns between ESCCs and their background mucosae (Fig. 1).

In the background mucosae, different genes had different methylation distribution from the viewpoints of the fraction of methylation-positive samples and their absolute methylation levels. The first group of genes (*CLDN6*, *CDKN2A*, *MLH1*, and *RASSF1A*) had no methylation. The second group of genes (*PYCARD*, *RSPO4*, *TSPYL5*, *ZIK1*, and *ZSCAN18*) was methylated in a small number of samples, and the levels were low ($\leq$3%). The third group of genes (*UCHL1* only) was methylated also in only a small number of samples, but the methylation level was high in some samples, reaching up to 20%. The fourth group of genes (*GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PKP1*, *PPP1R14A*, *ZFP42*, and *CDH1*) was methylated in a large number of samples, and the methylation levels revealed unimodal distribution with various highest values ranging from 6.8% (*MT1M*) to 25.9% (*ZFP42*).

In the 60 ESCCs, 12 marker genes (*CLDN6*, *GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PPP1R14A*, *RSPO4*, *TSPYL5*, *UCHL1*, *ZIK1*, *ZFP42*, and *ZSCAN18*) and 2 tumor-suppressor genes (*CDH1* and *RASSF1A*) were methylated in 11 to 49 ESCCs and in 3 to 4 ESCCs, respectively, with a cutoff threshold of 6%.[30,31] Two marker genes (*PKP1* and *PYCARD*) and 2 tumor-suppressor genes (*CDKN2A* and *MLH1*) were not methylated. The distribution of methylation levels in methylation-positive ESCCs was much broader than the levels in background mucosae. Also, 11 of 12 marker genes (*CLDN6*, *GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PPP1R14A*, *RSPO4*, *TSPYL5*, *UCHL1*, *ZIK1*, and *ZSCAN18*) and 2 tumor-suppressor genes (*CDH1* and *RASSF1A*) had large numbers of methylation-negative samples at the same time.

Correlations Between Methylation Levels in the Background Mucosae and Exposure Levels to ESCC Risk Factors

Next, we examined correlations between methylation levels in the background mucosae and risk factors for ESCCs; age, smoking duration, and mean daily alcohol intake (Table 2). From the initial 14 genes, *CLDN6*, which did