Virchows Arch (2008) 452:515–524
DOI 10.1007/s00428-008-0596-7

ORIGINAL ARTICLE

Helicobacter pylori-infection-associated CpG island hypermethylation in the stomach and its possible association with Polycomb repressive marks

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Received: 6 November 2007 / Revised: 2 February 2008 / Accepted: 10 February 2008 / Published online: 12 March 2008

Abstract *Helicobacter pylori* infection can induce CpG island (CGI) hypermethylation in gastric mucosa. Recently, genes occupied by Polycomb proteins in embryonic stem cells were shown to be vulnerable to aberrant DNA hypermethylation in cancers. To explore the relationship between *H. pylori* infection and DNA methylation changes in neoplastic and non-neoplastic stomach, we analyzed 25 CGIs and repetitive DNA elements from 82 chronic gastritis and 69 gastric carcinomas. Twenty-three CGIs showed significantly higher methylation levels in *H. pylori*-negative gastric carcinoma (n=28) than in *H. pylori*-negative chronic gastritis (n=39; P<0.05), indicating cancer-associated methylation. Eight CGIs exhibited significantly higher methylation levels in *H. pylori*-positive chronic gastritis (n=43) than in *H. pylori*-negative chronic gastritis (n=39; P<0.05). Six CGIs showed both cancer-associated and *H. pylori*-associated hypermethylation. Six (75%) of the eight *H. pylori*-associated hypermethylated genes contained at least one of three repressive marks (Suz12 occupancy, Eed occupancy, histone H3 K27 trimethylation), whereas 31% of the remaining cancer-associated hypermethylated genes had at least one mark. The findings suggest that *H. pylori* infection strongly induces CGI hypermethylation in gastric epithelial cells and that susceptibility to *H. pylori*-induced DNA hypermethylation may be determined by Polycomb repressive marks in stem or progenitor cells.

Keywords Chronic gastritis · CpG islands · DNA methylation · Gastric cancer · *Helicobacter pylori*

Electronic supplementary material The online version of this article (doi:10.1007/s00428-008-0596-7) contains supplementary material, which is available to authorized users.

Supported by the Korea Research Foundation Grant (MOEHRD; KRF-2005-041-E00081; G.H.K.) and by the second stage Brain Korea 21 Project.

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Introduction

Helicobacter pylori (*H. pylori*) has been designated as a human class I carcinogen of gastric malignancy by the International Agency for Research on Cancer [16]. Chronic infection with *H. pylori* generally increases the risk for gastric carcinoma by five- to six-fold [28]. The risk has been reported to be even greater when exposure to *H. pylori* infection is estimated based on the presence of serum antibodies to cytotoxin-associated gene product A [11]. Animal models have been developed to demonstrate a close association between *H. pylori* infection and gastric carcinoma [31, 35]. Although the exact mechanism of *H. pylori*-associated gastric carcinogenesis is unknown, long-standing bacterial infection, perpetuated chronic inflammation, and sustained mucosal epithelial cell proliferation are thought to produce a carcinogenic environment. Recently, *H. pylori*

infection was reported to be associated with enhanced hypermethylation of multiple CpG islands (CGIs) in chronic gastritis, and its eradication brings about reversal of the methylation status of multiple CGI loci [5, 6, 24, 25, 37]. This fact raises the possibility that aberrant methylation changes induced by *H. pylori* infection may contribute to *H. pylori* infection-associated gastric carcinogenesis. To date, however, only a limited number of CGI loci have been studied for their hypermethylation in association with *H. pylori* infection.

Aberrant DNA methylation in cancer cells is characterized by focal CGI hypermethylation and generalized genomic hypomethylation. Global hypomethylation contributes to chromosomal instability, as evidenced by recent cell line studies that induced genomic hypomethylation by knocking down DNA methyltransferases and demonstrated aberrant chromosomal structure or number in the cells [20]. It is known that the methylated DNA content of stomach-lining cells in chronic gastritis decreases with progression of the lesion along a multi-step progression to gastric carcinoma [8]. However, little is known about whether *H. pylori* infection leads to genomic hypomethylation.

Polycomb repressive complexes (PRCs) are involved in the initiation of gene repression through epigenetic modification of chromatin structure and three components, namely, enhancer of zeste homolog 2 (Ezh2), suppressor of zeste 12 homolog (Suz12) and embryonic ectodermal development (Eed), forms the core of PRC2 [34]. Ezh2 of PRC2 catalyzes histone H3 lysine 27 (H3K27) trimethylation, which is thought to provide a binding surface for PRC1 [3], which in turn facilitates a closed chromatin structure to repress gene expression. Recent studies have demonstrated that de novo hypermethylation of some promoter CGIs in human cancers may occur in a so-called "instructive manner", led by the chromatin "marks" of H3K27 trimethylation or binding of Polycomb group proteins Suz12 and Eed [29, 32, 43]. CGI loci carrying these chromatin marks in embryonic stem (ES) cells or normal tissue cells where these same loci are unmethylated may be preferentially targeted by aberrant DNA hypermethylation that occurs in association with cancer development [29, 32, 43]. In light of these facts, it can be speculated that in gastric epithelial stem or progenitor cells, CGI loci with these repressive chromatin marks may be vulnerable to hypermethylation induced by *H. pylori* infection. To date, nothing is known about whether CGI loci of *H. pylori*-related hypermethylation have some characteristic chromatin marks for preferred hypermethylation.

In this study, we analyzed the relationship of *H. pylori* infection to aberrant methylation in chronic gastritis and gastric carcinoma with respect to focal CGI hypermethylation and generalized genomic hypomethylation using quantitative methylation analysis. To determine the change of methylation content caused by *H. pylori* infection, we measured the methylation levels of LINE-1 and Alu repetitive elements as surrogates for global genomic methylation because these repetitive elements comprise about 30% of genomic DNA [22] and a strong correlation between 5-methylcytosine content in the human genome and measures of Alu and LINE-1 methylation using polymerase chain reaction has been demonstrated in a previous study [41]. Twenty-five CGI loci were analyzed for their methylation levels by quantitative methylation-specific real-time PCR (MethyLight technology). Using previously published information from stem cells [23], we were able to determine the occupancy by Suz12 and Eed and the trimethylation status of H3K27 for the 25 genes we examined.

Materials and methods

Patients

Four hundred sixty-seven patients who visited Seoul National University Bundang Hospital for gastroscopy from September 2003 to March 2005 were enrolled in the study. Patients underwent endoscopic mucosal biopsy and blood sampling for *H. pylori* detection. Patients with a history of gastric surgery and systemic disease requiring chronic medication were excluded from the enrollment. Neither chemical gastritis nor autoimmune gastritis was included. Written informed consent was obtained from all participating patients, and the study was approved by the Seoul National University Hospital's Institutional Review Board.

Gastroscopic biopsy and serologic evaluation

Three biopsy samples were taken from the greater curvature of the antrum and the body of the stomach. Two samples from the antrum and two samples from the stomach body were fixed in neutral buffered formalin solution and processed for hematoxylin–eosin (H&E) staining and modified Giemsa staining for histologic evaluation and assessment of the presence of *H. pylori*, respectively. The remaining two specimens were subjected to a rapid urease test (CLO test, Delta West, Bentley, Australia), which was monitored for up to 24 h. Blood samples were obtained from the patients and evaluated for the level of anti-*H. pylori* immunoglobulin (Ig) G using an enzyme-linked immunosorbent assay (Genedia *H. pylori* ELISA; Green Cross Sang A Co., Seoul, South Korea), and duplicate determinations were performed according to the manufacturer's protocol. Cases negative for three tests (CLO test, *H. pylori* IgG test, and histological *H. pylori* evaluation) were regarded as *H. pylori*-negative.

DNA extraction and bisulfite modification

Because previous studies [18, 26] revealed that intestinal metaplasia is accompanied by enhanced promoter CGI hypermethylation compared with chronic gastritis without intestinal metaplasia and our preliminary study revealed enhanced CGI hypermethylation in intestinal metaplasia regardless of *H. pylori* infection, cases that showed intestinal metaplasia or epithelial neoplasia in any of the four tissue samples were excluded from the study in order to focus on the effect of *H. pylori* infection on DNA methylation changes in chronic gastritis. Gastric carcinoma cases or chronic gastritis cases without intestinal metaplasia were included in the study. Twenty archival tissue sections of 4-μm thickness were used for DNA extraction. In gastric carcinoma cases, manual microdissection was performed in order not to include non-neoplastic mucosal epithelial cells. The dissected tissues were pooled together in a microtube containing lysis buffer and proteinase K. Sodium bisulfite conversion of genomic DNA was performed as described [41].

MethyLight assay

Twenty-five CGI loci were analyzed by the MethyLight technology using primers and probes targeting promoter sequences or the first exon [9, 41]. The oligonucleotide sequences of the primers and probes used have been described [42]. Briefly, two sets of primers and probes designed specifically to bind to bisulfite-converted DNA were used in each reaction; one set of primers and probe were used for every methylated target to be analyzed (methylated reaction), and another pair of primers and probe were for the reference locus, ALU (normalization control reaction). Normalization control reactions were methylation-independent measurements to control for DNA amplification and normalize for input DNA. *M.Sss*I-treated genomic DNA was used as a reference sample for complete methylation to determine the percentage of methylated reference (PMR) at a particular locus. PMR was defined as $100\times(\text{methylated reaction/normalization control reaction})_{\text{sample}}/(\text{methylated reaction/normalization control reaction})_{\text{M.SssI-Reference}}$.

Combined bisulfite restriction analysis (COBRA) assay

Bisulfite-modified genomic DNA was analyzed by COBRA to determine the methylation level of LINE-1 and Alu, as described [7]. In brief, LINE-1 and Alu were amplified with their respective primers that anneal to short sequences without CpG sites to amplify both methylated and unmethylated alleles equally. For the Alu reaction, *Taq*I was used to digest the methylated alleles, and the digested products were electrophoresed on a 12% polyacrylamide gel. The intensities of the digested and undigested bands were determined using Image J software (http://rsb.info.nih.gov/ij), and the percentage of methylated alleles was calculated by dividing the intensity of the digested band by the sum of the intensities of the digested band and the undigested band and multiplying by 100. For the LINE-1 reaction, *Taq*I and *Tas*I were used to digest the methylated alleles and unmethylated alleles, respectively. The percentage of methylated alleles was determined by dividing the intensity of *Taq*I-digested bands by the sum of *Taq*I-digested bands and *Tas*I-digested bands and multiplying by 100.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS, Ver.11.0, Chicago, IL). The relationship of CGI hypermethylation to *H. pylori* infection or cancer development was assessed by comparing the PMR values of examined CGIs using the Student's *t* test. The methylation levels of Alu or LINE-1 were compared between groups using the Student's *t* test. Comparison of the number of methylated CGI loci between groups was also performed using the Student's *t* test. A P value<0.05 was considered to be statistically significant.

Results

A total of 151 mucosal samples were analyzed for their methylation status in 25 CGI loci by MethyLight and in two repetitive elements (LINE-1 and Alu) by COBRA. The selected CGI loci except for *CDH1* were found to show higher methylation frequency or level in gastric carcinoma than in non-neoplastic gastric mucosa in our previous studies [17, 18] in which 170 CGI loci were analyzed for their methylation status in 16 gastric carcinoma and paired non-neoplastic gastric mucosa samples. *CDH1* was selected because of its known association of promoter CGI hypermethylation with *H. pylori* infection in the stomach [5, 6, 24]. The information regarding PRC2 occupancy was available for 153 of the 170 genes, and 60 genes contain occupancy of at least one component of PRC2. All of the PRC2-occupied 60 CGI loci showed promoter DNA hypermethylation in gastric carcinoma or non-neoplastic gastric mucosa samples, whereas 55 (59%) of 93 PRC2-nonoccupied genes exhibited CGI hypermethylation in gastric carcinoma or non-neoplastic gastric mucosa. Of the selected 24 CGI loci (except for *p14* for which PRC2 occupancy information is not available), 50% harbor occupancy of at least one component of PRC2, whereas 37.2% of the remaining 129 CGI loci contain occupancy of at least one component of PRC2. Forty three *H. pylori*-positive chronic gastritis samples (mean age, 55.1 years; range, 25–78 years), 39 *H. pylori*-negative chronic gastritis (mean age, 55.6 years; range, 24–85 years),

41 *H. pylori*-positive gastric carcinoma (mean age, 63.6 years; range, 40–82 years), and 28 *H. pylori*-negative gastric carcinoma (mean age, 64.7 years; range, 41–83 years) were included in the study. *H. pylori*-positive chronic gastritis cases were selected to match an age distribution and gender distribution comparable to those of the *H. pylori*-negative chronic gastritis cases. The *H. pylori*-positive chronic gastritis was composed of two subgroups, a current *H. pylori* infection group (n=33) and a prior *H. pylori* infection group (n=10). The current infection group was defined by the identification of *H. pylori* on H&E-stained biopsy slides or by a positive CLO test, and the prior infection group was defined by a positive *H. pylori* IgG test but negative CLO test and absence of *H. pylori* infection on histological examination.

CGI hypermethylation of chronic gastritis versus gastric carcinoma

To determine CGI loci showing cancer-related hypermethylation, the methylation levels of 25 CGI loci were compared between *H. pylori*-negative chronic gastritis (n=39) and *H. pylori*-negative gastric carcinoma (n=28). Twenty-three CGI loci (all except for *CDH1* and *DAPK1*) showed significantly higher methylation levels in *H. pylori*-negative gastric carcinoma than in *H. pylori*-negative chronic gastritis (P<0.05), indicating a cancer-related methylation (Fig. 1a). The remaining two loci showed apparent higher methylation levels in gastric carcinoma than in chronic gastritis, but the difference was statistically insignificant. When the methylation levels of examined CGI loci were compared between *H. pylori*-positive chronic gastritis (the current infection group, n=33) and *H. pylori*-positive gastric carcinoma, the cancer-related methylation loci except for seven loci (*p14*, *HOXA1*, *NEUROG1*, *SOCS1*, *CRABP1*, *MLH1*, and *SMAD9*) also displayed significantly higher methylation levels in gastric carcinoma than in chronic gastritis (P<0.05) (Fig. 1b). The level of global genomic methylation, represented by methylation levels of LINE-1 and Alu, was significantly lower in gastric carcinoma than in chronic gastritis (P<0.05), regardless of the status of *H. pylori* infection (Fig. 2a).

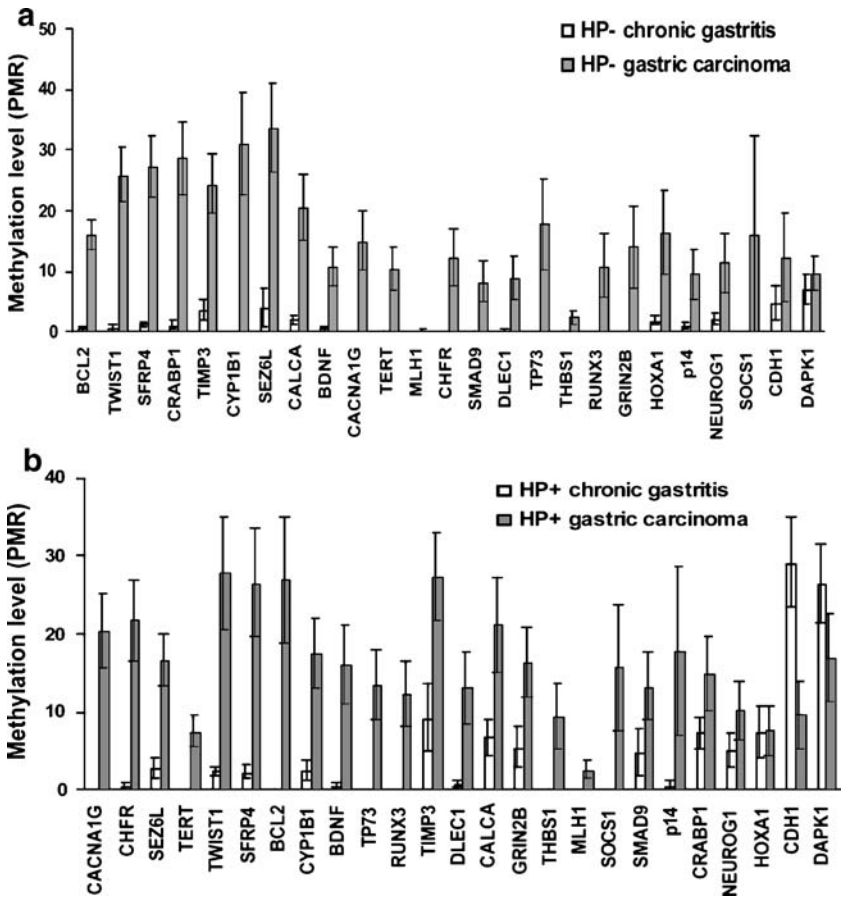

Fig. 1 **a** Comparison of the methylation levels of 25 CGI loci between *H. pylori*-negative (HP−) chronic gastritis specimens from non-cancer patients and HP− gastric carcinoma specimens. **b** Comparison between chronic gastritis cases with current *H. pylori* infection (from non-cancer patients) and *H. pylori*-positive gastric carcinoma cases. The examined CGI loci were sorted ordered from highest to lowest *P* values from left to right along the *x* axis. *Error bars* indicate S.E.M.

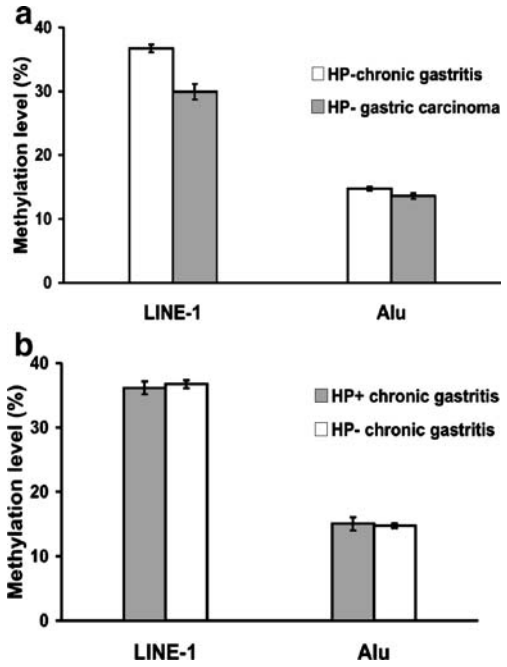

Fig. 2 **a** COBRA assay of LINE-1 and Alu repetitive elements reveal significantly lower methylation levels in *H. pylori*-negative gastric carcinoma than in *H. pylori*-negative chronic gastritis. *Error bars* indicate S.E.M. **b** COBRA assay of LINE-1 or Alu methylation levels demonstrated no difference between *H. pylori*-positive and *H. pylori*-negative chronic gastritis samples

CGI hypermethylation of *H. pylori*-positive chronic gastritis versus *H. pylori*-negative chronic gastritis

Of 25 CGI loci hypermethylated in gastric carcinoma, four loci (*RUNX3*, *SOCS1*, *TERT*, and *TP73*) were never methylated in chronic gastritis samples, regardless of *H. pylori* infection. Figure 3 depicts the summary of the PMR values generated by the MethyLight analysis. When the methylation levels of 21 CGI loci were compared between *H. pylori*-positive chronic gastritis (the current *H. pylori* infection group, n=33) and *H. pylori*-negative chronic gastritis, close association of hypermethylation with *H. pylori* infection was noted in eight CGI loci (*CACNA1G*, *CALCA*, *CDH1*, *CRABP1*, *CYP1B1*, *DAPK1*, *GRIN2B*, and *TWIST1*; Fig. 4). Of these, six loci (*CALCA*, *CACNA1G*, *CRABP1*, *CYP1B1*, *GRIN2B*, and *TWIST1*) showed both cancer-related and *H. pylori*-related hypermethylation. Of the 23 cancer-related methylation loci, 13 loci showed more than a two-fold increase in methylation levels in *H. pylori*-positive chronic gastritis samples than in *H. pylori*-negative chronic gastritis samples. No difference was noted in LINE-1 and Alu methylation levels between *H. pylori*-positive and *H. pylori*-negative chronic gastritis specimens (Fig. 2b).

When a CGI locus was arbitrarily regarded methylated when its PMR value was ≥4, the number of CGI loci methylated varied from 0 to 11 in the *H. pylori*-positive chronic gastritis samples (the current *H. pylori* infection group, n=33) and from 0 to 5 in the *H. pylori*-negative chronic gastritis samples (mean, 4.3 vs. 1.4; P<0.001) (Fig. 5a).

CGI hypermethylation in current *H. pylori* infection versus past *H. pylori* infection

The *H. pylori*-positive chronic gastritis cases were divided into two groups, namely current infection (n=33) and prior infection (n=10). Comparison of methylation levels of the examined CGI loci revealed that the methylation levels of the eight CGI loci showing *H. pylori*-related hypermethylation were apparently lower in the prior infection group than in the current infection group, but the differences were not significant (data not shown). When the number of methylated CGI loci (PMR≥4) was compared between the two groups, the current infection group showed a significantly higher number of methylated loci than the prior infection group (mean number, 4.3 vs. 2.6; P=0.014; Fig. 5b). Moreover, the number of CGI loci methylated was significantly higher in the prior infection group than in the *H. pylori*-negative chronic gastritis group (mean, 2.6 vs. 1.4; P=0.022).

CGI hypermethylation of *H. pylori*-positive gastric carcinoma versus *H. pylori*-negative gastric carcinoma

Although no significant difference was noted in the comparison of the methylation levels of the 24 CGI loci between *H. pylori*-positive and *H. pylori*-negative gastric carcinomas, *THBS1* showed a statistically insignificant 2-fold increase in methylation levels in *H. pylori*-infected gastric carcinoma samples. Alu and LINE-1 methylation levels did not differ between the *H. pylori*-positive and *H. pylori*-negative gastric carcinoma samples.

Discussion

In this study, *H. pylori*-positive and -negative chronic gastritis cases were analyzed for their methylation status of 25 promoter CGI loci using the MethyLight assay. The methylation levels of 15 CGI loci in *H. pylori*-positive chronic gastritis cases were elevated more than two-fold compared with those in *H. pylori*-negative chronic gastritis, and of these CGI loci the differences in the methylation levels in eight loci reached statistical significance. Furthermore, the number of CGI loci hypermethylated (PMR≥4) was significantly higher in the current infection group than in the prior *H. pylori* infection group. These results indicate that *H. pylori* infection is closely associated with aberrant CGI hypermethylation in chronic gastritis, consistent with

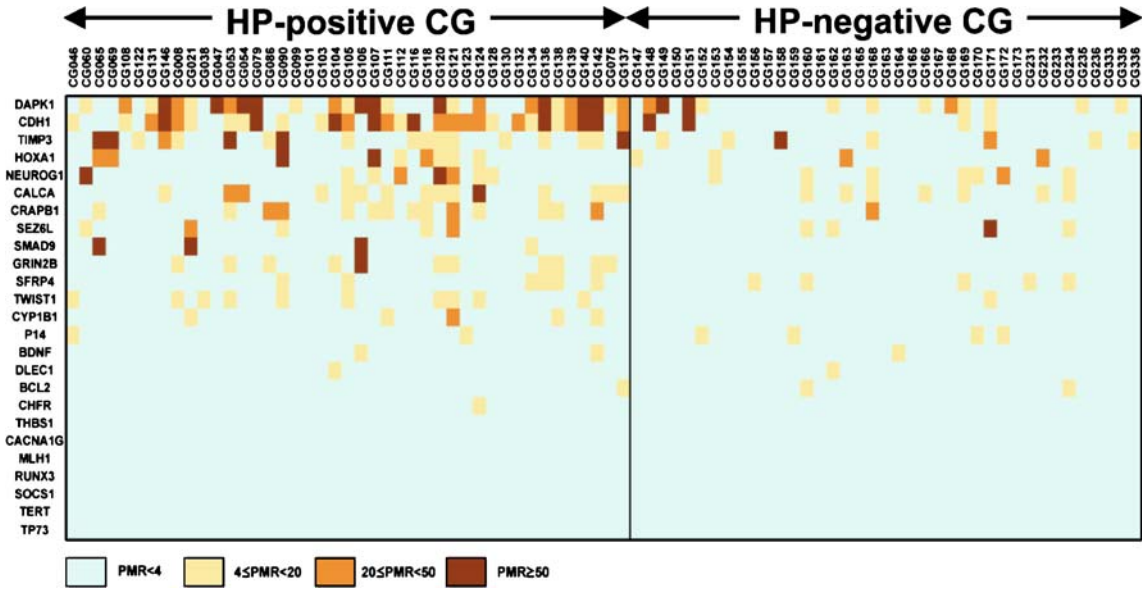

Fig. 3 Methylation map of the percent of methylated reference (*PMR*) values obtained for each CpG island (CGI) locus in *H. pylori*-positive (*HP-positive*) or *H. pylori*-negative chronic gastritis (*HP-negative CG*) cases. In total, 82 CG samples (horizontal axis) were examined for their methylation status at 25 CGI loci (*vertical axis*). The data shows that HP-positive CG samples tend to be more hypermethylated than HP-negative CG samples with regard to methylation levels or methylation frequencies. *Colored boxes* represent four classes of methylation levels [0<PMR<4 (*light blue*), 4≤PMR<20 (*light yellow*), 20≤PMR<50 (*light orange*), PMR≥50 (*brown*)], as indicated in the figure

the findings of other researchers [5, 24, 25]. However, it should be noted that variability existed in the susceptibility to hypermethylation induced by *H. pylori* infection depending on the type of CGI loci: 15 CGI loci showed more than two-fold increases in methylation levels in *H. pylori*-positive chronic gastritis compared to *H. pylori*-negative chronic gastritis cases, whereas the remaining ten CGI loci did not. Even within the current *H. pylori*-infection chronic gastritis subjects, the number of CGI loci hypermethylated per case displayed a wide distribution, indicating the existence of other factors, including host factors, in the vulnerability of CGIs to hypermethylation provoked by *H. pylori* infection. Host factors may include the duration of *H. pylori* infection and inflammatory response, and genetic variables. For example, genetic polymorphisms of the inflammatory cytokines involved in *H. pylori*

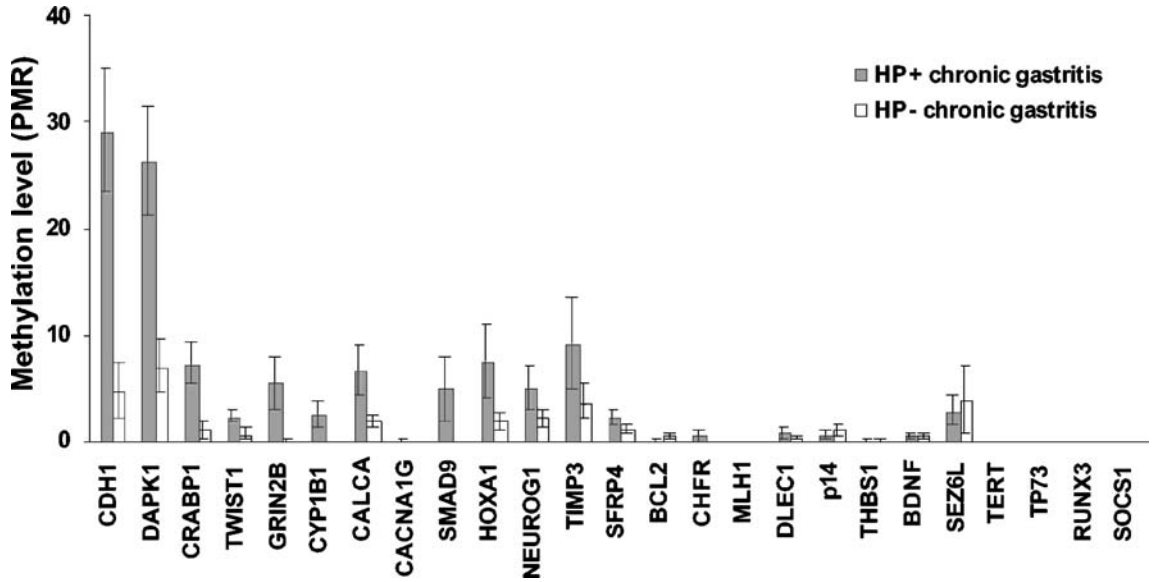

Fig. 4 Comparison between chronic gastritis cases with current *H. pylori* infection (from non-cancer patients) and chronic gastritis cases with no current *H. pylori* infection and no past history of *H. pylori* infection (from non-cancer patients). The examined CGI loci were sorted ordered from highest to lowest *P* values from *left to right* along the *x* axis. The *left eight CGI loci* showed a statistically significant difference in methylation levels ($P<0.05$)

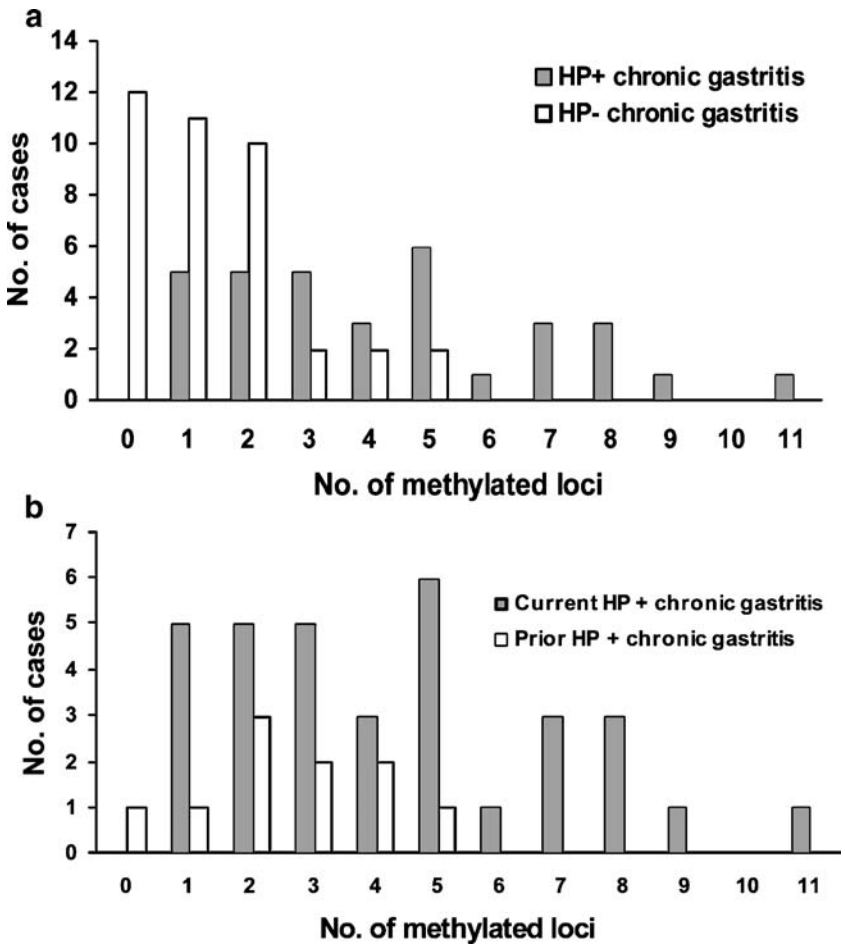

Fig. 5 **a** Distribution of the number of methylated CGI loci in *H. pylori*-positive chronic gastritis (current *H. pylori* infection) and *H. pylori*-negative chronic gastritis (no current *H. pylori* infection and no history of *H. pylori* infection). A CGI locus was considered methylated if the PMR was more than 4. **b** Comparison of the number of methylated CGI loci between chronic gastritis with current *H. pylori* infection and chronic gastritis with past *H. pylori* infection. Note the marked difference in the number of methylated CGI loci between the two groups

infection may affect susceptibility to *H. pylori*-induced hypermethylation [4].

Histologically, *H. pylori*-positive chronic gastritis tends to show more mononuclear and polymorphonuclear cell infiltration in the lamina propria than *H. pylori*-negative chronic gastritis, which was also the case in our study. Because we did not perform laser capture microdissection, inflammatory cells may give rise to a bias in the comparison of the methylation levels between *H. pylori*-positive and *H. pylori*-negative chronic gastritis. To identify whether the increased methylation levels of CGI loci in *H. pylori*-positive chronic gastritis were related to increased infiltration of inflammatory cells in the gastric mucosa, we examined peripheral blood leukocytes (from 50 healthy volunteers) for the methylation status of 25 CGI loci using the MethyLight technology. None of the CGI loci gave a PMR≥4 in peripheral blood leukocyte DNA (Supplementary data).

The present study provides supportive evidence that *H. pylori* infection is closely associated with aberrant CGI hypermethylation in the stomach. However, the mechanism of methylation induction in *H. pylori*-infected gastric mucosa remains unclear. A plausible explanation for the mechanism is as follows: an intense inflammatory response and an increased production of pro-inflammatory cytokines in the gastric mucosa provoked by *H. pylori* infection might participate in the induction of aberrant hypermethylation. (1) Interleukin-6, one of the cytokines produced in the *H. pylori*-infected gastric mucosa [1], has been shown to regulate the *DNMT1* promoter and the resulting enzyme activity [14, 15] and to increase methylcytosine content and modulate CGI hypermethylation [40]. (2) DNA methyltransferase expression has been reported to increase in the presence of *H. pylori* infection [36]. Overexpression of DNA methyltransferase is itself capable of bringing about de novo methylation of selective CGI loci [12]. (3)

5-Halogenated cytosine damage products, including 5-chlorocytosine and 5-bromocytosine, are formed in vivo by inflammation-mediated reactive molecules [2, 13] and it has been suggested that these modified pyrimidines mimic 5-methylcytosine and induce de novo methylation of previously unmethylated CpG sites [38].

CGI loci that are methylated in human cancers may be marked for preferred methylation. In recent studies, genes hypermethylated in human cancers were found to be often those genes which are marked by H3K27 trimethylation or Polycomb component occupancy in ES cells, where they are unmethylated [29, 32, 43]. In other words, PRC2 target genes of ES cells are predisposed to DNA hypermethylation occurring in association with cancer development. Because PRCs are not only essential regulators of embryonic development but also key players in the maintenance of adult stem cells [27, 39], gastric epithelial stem cells are expected to carry many genes under the control of PRCs and these Polycomb target genes may preferentially succumb to DNA hypermethylation induced by *H. pylori* infection. Although we could not explore the PRC occupancy of the examined 25 genes in gastric epithelial stem cells, published information about the occupancy by Polycomb group components (Suz12 and Eed) or H3K27 trimethylation at the genes in ES cells enabled us to analyze the association [23]. Based on the results of our methylation analysis, the examined 25 genes could be classified into three groups: (1) *H. pylori*-associated methylation only (*CDH1*, and *DAPK*), (2) cancer-associated methylation only (*BCL2*, *BDNF*, *CHFR*, *DLEC1*, *HOXA1*, *MLH1*, *NEUROG1*, *p14*, *RUNX3*, *SEZ6L*, *SFRP4*, *SMAD9*, *SOCS1*, *TERT*, *THBS1*, *TIMP3*, and *TP73*), and (3) both *H. pylori*- and cancer-associated methylation (*CACNA1G*, *CALCA*, *CRABP1*, *CYP1B1*, *GRIN2B*, and *TWIST1*). Seventy five percent of the genes showing *H. pylori*-associated methylation (the first and third groups) contain at least one of the three repressive marks, namely Suz12 occupancy, Eed occupancy, or H3K27 methylation, whereas 69% of the genes with cancer-associated methylation (the second group) do not carry any of the three repressive marks (Fig. 6). Furthermore, all of the genes with methylation associated with both *H. pylori* and cancer (the third group) contain one or more of the repressive marks. This fact suggests that repressive chromatin marks may leave genes vulnerable to later aberrant DNA hypermethylation induced by *H. pylori* infection, on the assumption that repressive chromatin marks established on genes early in development are maintained in gastric epithelial stem cells and progenitor cells. However, it should be noted that *CDH1* and *DAPK*, showing association of their hypermethylation with *H. pylori* infection, are not occupied by PRC2 in ES cells, and also that a large portion of the genes showing cancer-related methylation do not contain the repressive marks. Thus, other potential factors capable of directing targeted methylation should exist and may include the local DNA sequence motifs. Genome-wide DNA methylation studies have demonstrated the presence of such motifs which are significantly enriched in methylated promoter CGIs relative to the bulk of unmethylated CGIs [21, 33].

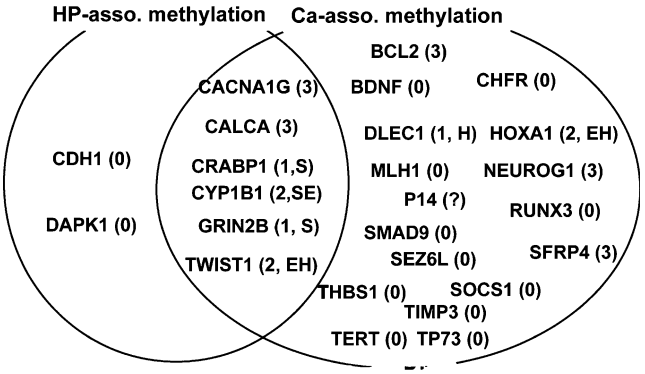

Fig. 6 Venn diagram showing CGI loci of *H. pylori*-associated methylation and those of gastric cancer-associated methylation. CGI loci having significantly higher methylation levels in *H. pylori*-negative gastric cancer specimens compared to *H. pylori*-negative chronic gastritis specimens are referred to as cancer-associated loci. The loci for which DNA hypermethylation was significantly associated with *H. pylori* infection are referred to as *H. pylori*-associated loci. The occupancy of the three components of PRC2 (Suz12 (*S*), Eed (*E*), and Ezh2 (*H*)) at the examined 25 genes in ES cells could be established from the published information and is given in parentheses and the numbers in parenthesis indicates the total number of occupying components. The status of PRC2 occupancy or H3K27 methylation for *p14* in ES cells is not known

Generalized genomic hypomethylation as well as focal CGI hypermethylation constitute aberrant methylation changes characteristic of human cancer cells and aged cells [10, 30]. Both methylation changes are frequently found in gastric carcinoma cells and even in multi-step lesions prior to gastric carcinoma [8, 19]. Given these facts, *H. pylori* infection was expected to affect the global genomic methylation status of the gastric epithelial cells. However, Alu and LINE-1 methylation levels, surrogates for genomic methylation levels, did not decrease in *H. pylori*-positive chronic gastritis samples compared with those of *H. pylori*-negative chronic gastritis samples, but they were significantly lower in gastric carcinoma compared with chronic gastritis cases, regardless of *H. pylori* infection. Although the number of analyzed cases was low, the findings suggest that *H. pylori* infection affects methylation changes of CGI loci but does not change the methylation content of the repetitive elements.

We have analyzed the relationship of aberrant DNA methylation changes in the stomach relative to *H. pylori* infection using the MethyLight assay on 25 genes and the COBRA assay on Alu and LINE-1 repetitive elements. The results suggest that *H. pylori* infection is strongly associated

with focal CGI hypermethylation but does not affect the genomic methylation contents. Identification of Polycomb group protein occupancy in stem cells of the 25 genes examined revealed that genes occupied by Polycomb group proteins during embryonic development seem to be vulnerable to *H. pylori*-related DNA hypermethylation.

Conflict of interest statement We declare that we have no conflict of interest.

References

1. Ando T, Kusugami K, Ohsuga M, Ina K, Shinoda M, Konagaya T, Sakai T, Imada A, Kasuga N, Nada T, Ichiyama S, Blaser MJ (1998) Differential normalization of mucosal interleukin-8 and interleukin-6 activity after *Helicobacter pylori* eradication. Infect Immun 66:4742–47
2. Badouard C, Masuda M, Nishino H, Cadet J, Favier A, Ravanat JL (2005) Detection of chlorinated DNA and RNA nucleosides by *H. pylori*LC coupled to tandem mass spectrometry as potential biomarkers of inflammation. J Chromatogr B Analyt Technol Biomed Life Sci 827:26–31
3. Cao R, Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 14:155–164
4. Chan AO, Chu KM, Huang C, Lam KF, Leung SY, Sun YW, Ko S, Xia HH, Cho CH, Hui WM, Lam SK, Rashid A (2007) Association between *Helicobacter pylori* infection and interleukin 1 beta polymorphism predispose to CpG island methylation in gastric cancer. Gut 56:595–597
5. Chan AO, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, Hui WM, Rashid A, Kwong YL (2003) Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer. Gut 52:502–506
6. Chan AO, Peng JZ, Lam SK, Lai KC, Yuen MF, Cheung HK, Kwong YL, Rashid A, Chan CK, Wong BC (2006) Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter hypermethylation. Gut 55:463–468
7. Cho NY, Kim BH, Choi M, Yoo EJ, Moon KC, Cho YM, Kim D, Kang GH (2007) Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol 211:269–277
8. Cravo M, Pinto R, Fidalgo P, Chaves P, Gloria L, Nobre-Leitao C, Costa Mira F (1996) Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. Gut 39:434–438
9. Eads CA, Lord RV, Wickramasinghe K, Long TI, Kurumboor SK, Bernstein L, Peters JH, DeMeester SR, DeMeester TR, Skinner KA, Laird PW (2001) Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 61:3410–3418
10. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400–5413
11. Ekstrom AM, Held M, Hansson LE, Engstrand L, Nyren O (2001) *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. Gastroenterology 121:784–791
12. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM (2003) Predicting aberrant CpG island methylation. Proc Natl Acad Sci USA 100:12253–12258
13. Henderson JP, Byun J, Williams MV, Mueller DM, McCormick ML, Heinecke JW (2001) Production of brominating intermediates by myeloperoxidase. A transhalogenation pathway for generating mutagenic nucleobases during inflammation. J Biol Chem 276:7867–7875
14. Hodge DR, Peng B, Cherry JC, Hurt EM, Fox SD, Kelley JA, Munroe DJ, Farrar WL (2005) Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. Cancer Res 65:4673–4682
15. Hodge DR, Xiao W, Clausen PA, Heidecker G, Szyf M, Farrar WL (2001) Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. J Biol Chem 276:39508–39511
16. IARC working group on the evaluation of carcinogenic risks to humans, schistosomes, liver flukes, Helicobacter pylori (1994) IARC monographs on the evaluation of carcinogenic risks to humans, vol 61. International Agency for Research on Cancer, Lyon, pp 1–241
17. Kang GH, Lee S, Cho NY, Gandamihardja T, Long TI, Weisenberger DJ, Campan M, Laird PW (2008) DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. Lab Invest 88:161–170
18. Kang GH, Lee S, Kim JS, Jung HY (2003) Profile of aberrant CpG island methylation along multistep gastric carcinogenesis. Lab Invest 83:519–526
19. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG (2001) CpG island methylation in premalignant stages of gastric carcinoma. Cancer Res 61:2847–2851
20. Karpf AR, Matsui S (2005) Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 65:8635–8639
21. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38:149–153
22. Kochanek S, Renz D, Doerfler W (1993) DNA methylation in the Alu sequences of diploid and haploid primary human cells. EMBO J 12:1141–1151
23. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125:301–313
24. Leung WK, Man EP, Yu J, Go MY, To KF, Yamaoka Y, Cheng VY, Ng EK, Sung JJ (2006) Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res 12:3216–3221
25. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T (2006) High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 12:989–995
26. Mihara M, Yoshida Y, Tsukamoto T, Inada K, Nakanishi Y, Yagi Y, Imai K, Sugimura T, Tatematsu M, Ushijima T (2006) Methylation of multiple genes in gastric glands with intestinal metaplasia: a disorder with polyclonal origins. Am J Pathol 169:1643–1651
27. Molofsky AV, Pardal R, Morrison SJ (2004) Diverse mechanisms regulate stem cell self-renewal. Curr Opin Cell Biol 16:700–707
28. Normark S, Nilsson C, Normark BH, Hornef MW (2003) Persistent infection with *Helicobacter pylori* and the development of gastric cancer. Adv Cancer Res 90:63–89
29. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, Mohammad HP, Chen W, Daniel VC, Yu W, Berman DM, Jenuwein T, Pruitt K, Sharkis SJ, Watkins DN, Herman JG, Baylin SB (2007) A

stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39:237–242
30. Richardson B (2003) Impact of aging on DNA methylation. Aging Res Rev 2:245–261
31. Rieder G, Merchant JL, Haas R (2005) *Helicobacter pylori* cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. Gastroenterology 128:1229–1242
32. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, Eden E, Yakhini Z, Ben-Shushan E, Reubinoff BE, Bergman Y, Simon I, Cedar H (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39:232–236
33. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3:2023–2036
34. Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856
35. Sugiyama A, Maruta F, Ikeno T, Ishida K, Kawasaki S, Katsuyama T, Shimizu N, Tatematsu M (1998) *Helicobacter pylori* infection enhances *N*-methyl-*N*-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. Cancer Res 58:2067–2069
36. Tamura G (2004) Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia. Histol Histopathol 19:221–228
37. Ushijima T, Nakajima T, Maekita T (2006) DNA methylation as a marker for the past and future. J Gastroenterol 41:401–407
38. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
39. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M (2004) Stem cells and cancer; the polycomb connection. Cell 118: 409–418
40. Wehbe H, Henson R, Meng F, Mize-Berge J, Patel T (2006) Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res 66:10517–10524
41. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33:6823–6836
42. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38:787–793
43. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW (2007) Epigenetic stem cell signature in cancer. Nat Genet 39:157–158