Rosa26::rtTA; *Col1a1::tetO-Cre* ESCs; Beard et al., 2006; Hochedlinger et al., 2005). This new ESC line enabled the conditional deletion of the floxed *Rest* genes in the presence of doxycycline. After 3 days of doxycycline exposure, the recombination in both alleles of the *Rest* was confirmed in 70%–80% of these ESCs by FACS (Figure 1F). However, the conditional deletion did not suppress the formation of AP-positive colonies regardless of the presence or absence of feeder cells in comparison to the parental ESCs without doxycycline (Figure 1G). In addition, the expression levels of *Oct3/4* and *Nanog* did not change, whereas the expression level of *Syt4* was derepressed while demonstrating evidence of *Rest* recombination shortly after doxycycline treatment (Figure S1D). These results therefore clearly rule out both the possibility of the adaptation in the long-term culture as well as the notion that feeder cells reduce the requirement of Rest-mediated ESC maintenance. Taken together, our results indicate that *Rest* is not required for the maintenance of ESC pluripotency in these experimental conditions.

Both *Gata4* and *Gata6* were significantly downregulated in the $Rest^{-/-}$ ESCs under confluent culture conditions (Figure 2A), although the findings were not prominent before the cells reached confluence. *Gata4* and *Gata6* are transcriptional factors that promote primitive endoderm differentiation (Fujikura et al., 2002; Niwa, 2007). These findings suggest that the genetic deletion of *Rest* prevents ESCs from differentiating toward the primitive endoderm. The notion of the suppression of primitive endoderm differentiation is confirmed by the decreased expression of both *Sox7* and *Dab2*, markers for the primitive endoderm (Shimoda et al., 2007; Yang et al., 2002), in $Rest^{-/-}$ ESCs (Figure 2A). Consistent with this notion, embryoid bodies (EBs) generated from $Rest^{-/-}$ ESCs revealed a decreased number of Gata4-expressing cells in the periphery of EBs on the histological sections in comparison to the control EBs (13.1 ± 15.0/EB and 30.4 ± 9.02/EB in RestKO8 EBs and V6.5 EBs, respectively, $p < 0.006$ by Student's t test) (Figure S2A). Rescue experiments were performed with a plasmid containing human *REST* cDNA (Grimes et al., 2000) to further investigate the direct association of the genetic deletion of *Rest* and the altered expression of *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs. Importantly, the decreased expression of both *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs were derepressed by the exogenous expression of *REST* (Figure 2B).

The expression of *Nanog*, *Oct3/4*, and *Sox2* were significantly higher in the $Rest^{-/-}$ EB cells than in the control EB cells (Figure 2C). Accordingly, these observations suggest that the delayed repression of self-renewal genes during the early differentiation of ESCs may thus cause the suppression of the early differentiation of $Rest^{-/-}$ ESCs. To further examine the initial response of gene expression upon the early differentiation of $Rest^{-/-}$ ESCs, the differentiation (-LIF, -MEF) of *Cre*-inducible *Rest*-floxed ESCs was induced with/without doxycycline exposure (Figure 2D). At 3 days after doxycycline treatment, the expression of *Nanog*, but not of *Oct3/4*, was observed to be significantly higher in the doxycycline-treated ESCs than that of the nontreated ESCs (Figure 2D). In contrast, a decreased expression of *Gata4* was not detectable at 3 days after doxycycline treatment when the *Syt4* expression had already been derepressed (Figure 2D). These results suggest that a decreased expression of *Gata4* in $Rest^{-/-}$ cells is preceded by an increased expression of *Nanog* and that *Gata4* repression is therefore a secondary effect of *Rest* ablation.

Finally, a doxycycline-inducible *REST* ESC line was generated (Figure 2E; Figure S2B). The forced expression of *REST* led to the rapid morphological changes of ESC colonies into an epithelium-like shape, which was accompanied by decreased AP activity (Figure 2F). In line with such morphological changes, ESCs with exogenous *REST* expressed significantly lower levels of self-renewal genes. The expression of *Gata6* was higher, whereas the expression of an epiblast marker, *Fgf5,* was significantly lower in such ESCs (Figure 2G). Furthermore, an increased number of Gata4-expressing cells in the periphery of EBs was observed in the exogenous *REST*-induced EBs (79.2 ± 19.6/EB and 50.7 ± 17.6/EB in *REST*-induced EBs and control EBs, respectively, $p < 0.004$ by Student's t test) (Figure 2H), thus suggesting that the forced *REST* expression promotes the ESC differentiation into the primitive endoderm. Importantly, the *REST*-induced ESC differentiation was, at least in part, rescued by the *Nanog* overexpression (Figure 2I; Figure S2D).

Although the critical role of the Oct3/4-Sox2-Nanog core transcription circuitry in the maintenance of ESC pluripotency is widely accepted (Boyer et al., 2005, 2006; Chambers et al., 2003; Loh et al., 2006; Mitsui et al., 2003; Niwa et al., 2000), the mechanisms leading to the breakdown of such core circuitry upon the early ESC differentiation are still not well understood (Kunath et al., 2007). The present study demonstrated that *Rest* ablation causes delayed repression of the pluripotent genes, whereas overexpression of *REST* immediately results in the suppression of the pluripotent gene expression. It is noteworthy that the delayed repression of the pluripotent genes by the conditional ablation of *Rest* was predominantly observed in *Nanog*. Given the fact that Rest is a transcriptional repressor and *Nanog* harbors RE1 in its promoter (Johnson et al., 2008), the current results therefore suggest that Rest is involved in the silencing of *Nanog* expression during the early differentiation of ESCs. This notion is also supported by the observation that ectopic *REST* in $Rest^{-/-}$ ESCs predominantly repressed the *Nanog* expression relative to the expression in original $Rest^{-/-}$ ESCs (Figure S2C). These findings suggest that Rest is an external factor connecting to the Oct3/4-Sox2-Nanog regulatory network core circuitry to influence the initial differentiation of ESCs. It is interesting to note that Rest is abundantly expressed in ESCs and it is a target of the Oct3/4-Sox2-Nanog regulatory network core circuitry (Johnson et al., 2008). It is possible that the negative feedback loop through Rest may play

(I) The *Nanog* overexpression dampens the *REST*-mediated ESC differentiation. *REST* was induced in *Nanog*-overexpressing and *EGFP*-overexpressing ESC colonies by the doxycycline exposure. The 24 hr exposure of doxycyline led to the rapid differentiation in *EGFP*-overexpressing ESCs (arrowheads), whereas *Nanog*-overexpressing ESCs retained an undifferentiated morphology. After the 48 hr exposure of doxycyline, 16 out of 25 *EGFP*-overexpressing colonies (68%) started to differentiate, whereas none of *Nanog*-overexpressing colonies (0/21, 0%) revealed the evidence of differentiation (see also Figure S2D).

a role in the stable transcriptional circuitry and in the rapid response upon the early differentiation of ESCs.

The current findings also suggest that Rest promotes the early ESC differentiation. Epiblast and the primitive endoderm are two distinct cell types in the inner cell mass (ICM) of the blastocyst. Genetic evidence indicates that the *Nanog* and *Gata* family transcription factors play a role in the segregation of epiblast and primitive endoderm within ICM (Chambers et al., 2003; Koutsourakis et al., 1999; Mitsui et al., 2003; Soudais et al., 1995). Indeed, Nanog and Gata6 are expressed in the ICM in a mutually exclusive manner (Chazaud et al., 2006), thus indicating the reciprocal control of the gene expression. The current study found that the conditional ablation of *Rest* results in the delayed repression of *Nanog* during the early differentiation of ESCs, whereas *REST* overexpression causes an increased expression of *Gata6*, which is accompanied by the rapid differentiation. In addition, the expression of *Fgf5*, an epiblast marker, was significantly downregulated by the *REST* overexpression. These results suggest that Rest may be involved in the segregation of epiblast and primitive endoderm through modifying the *Nanog* expression.

In summary, the conditional ablation of the *Rest* gene revealed that *Rest* is not absolutely required for the maintenance of ESC pluripotency. These results also indicate that *Rest* plays a role in the suppression of the pluripotent gene expression upon the early differentiation of ESCs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.stem.2009.12.003.

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The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae

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Aberrant DNA methylation is deeply involved in human cancers, but its inducers and targets are still mostly unclear. *Helicobacter pylori* infection was recently shown to induce aberrant methylation in gastric mucosae, and produce a predisposed field for cancerization. Here, we analyzed the presence of target genes in methylation induction by *H. pylori* and the mechanism for the gene specificity. Noncancerous gastric mucosae were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group), and methylation of promoter CpG islands of 48 genes that can be methylated in gastric cancer cell lines was analyzed by methylation-specific PCR. In total, 26 genes were consistently methylated in individuals with current or past infection by *H. pylori*, whereas 7 genes were not methylated at all. In addition, 14 genes were randomly or intermediately methylated in individuals with gastric cancers and the remaining 1 gene was methylated in all the cases. The methylation-susceptible genes had significantly lower mRNA expression levels than the methylation-resistant genes. *H. pylori* infection did not induce mRNA and protein expression of DNA methyltransferases; *DNMT1*, *DNMT3A* or *DNMT3B*. Gene specificity was present in the induction of aberrant DNA methylation by *H. pylori* infection, and low mRNA expression, which could precede methylation, was one of the mechanisms for the gene specificity. These findings open up the possibility that a methylation fingerprint can be used as a novel marker for past exposure to a specific carcinogenic factor.
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Key words: DNA methylation; epigenetic; fingerprint; *Helicobacter pylori*; molecular epidemiology

Aberrant DNA methylation is deeply involved in human cancer development and progression.[1] In some cancer types, such as gastric cancers, tumor-suppressor genes are more frequently inactivated by aberrant DNA methylation than by mutations.[2] Nevertheless, only limited information is available for inducers of aberrant DNA methylation, which include aging, viral infection and ulcerative colitis.[3,4] Also, almost no information is available for gene specificity in methylation induction by a specific factor. Using cancer tissues, it is very difficult to clarify an association between a specific inducer and methylation of a gene. Aberrant methylation of a gene can be present in cancer tissues because its methylation conferred a growth advantage although it was a rare and random event, or because its methylation was carried over from a precursor tissue to a cancer tissue since it was frequently induced in the precursor tissue. In contrast, using a noncancerous tissue, one can assess an effect of a methylation inducer by the fraction of cells with methylation in the polyclonal tissue.

Gastric mucosa infected by *Helicobacter pylori* is a useful model to examine the possible presence of gene specificity in methylation induction by a specific factor since *H. pylori* infection was recently shown to induce aberrant DNA methylation potently in gastric mucosae.[5] Moreover, the fraction of DNA molecules with aberrant methylation (methylation level) in gastric mucosae of individuals without current *H. pylori* infection was correlated with gastric cancer risk,[5,6] indicating that methylation in noncancerous tissues is related to gastric carcinogenesis. So far, 6 CpG islands in gene promoter regions methylated in gastric cancers[7] were analyzed, and all were methylated in gastric mucosae with current and past infection with *H. pylori*. However, it is unknown whether these 6 genes are preferentially methylated by *H. pylori* infection or *H. pylori* infection induces methylation of random genes.

In this study to analyze the presence of gene specificity for methylation induction, firstly we examined the methylation status of 48 promoter CpG islands in the noncancerous gastric mucosae of 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection). The 48 genes were selected as genes that can be methylation-silenced in gastric cancer cell lines[8] because the vast majority of CpG islands in gene promoter regions are not methylated at all in noncancerous tissues, and we had to newly select genes that have better chances to be methylated in noncancerous tissues. Secondly, we analyzed an association between susceptibility to methylation induction and mRNA expression levels in normal tissue without and with *H. pylori* infection.

Material and methods

Tissue samples and DNA/RNA extraction

For methylation analysis, (noncancerous) gastric mucosa samples were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group, average age = 60.8 ± 13.8 years). For analysis of mRNA expression that determines gene specificity of methylation induction, we need to analyze the mRNA expression level in gastric mucosae free of methylation, which, once induced, will cause decreased gene transcription to avoid confusion between cause and consequence. Therefore, samples were collected from 11 healthy volunteers, who were considered to have less chance for methylation induction by *H. pylori* than elderly individuals (7 males and 4 females; 6 with *H. pylori* infection and 5 without; average age = 34.8 ± 3.1 years). Biopsy specimens were taken from one standard site of the stomach (antral regions in the lesser curvature) using sterilized biopsy forceps (Olympus, Tokyo, Japan). *H. pylori* infection status was analyzed by culture test (Eiken, Tokyo, Japan) and rapid urease test (Otsuka, Tokushima, Japan). All the materials were obtained with written informed consents, and the procedures were approved by the institutional review board. High molecular weight DNA was extracted by the standard phenol/chloroform method and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and an RNeasy Mini kit (Qiagen, Valencia, CA).

Additional Supporting Information may be found in the online version of this article.

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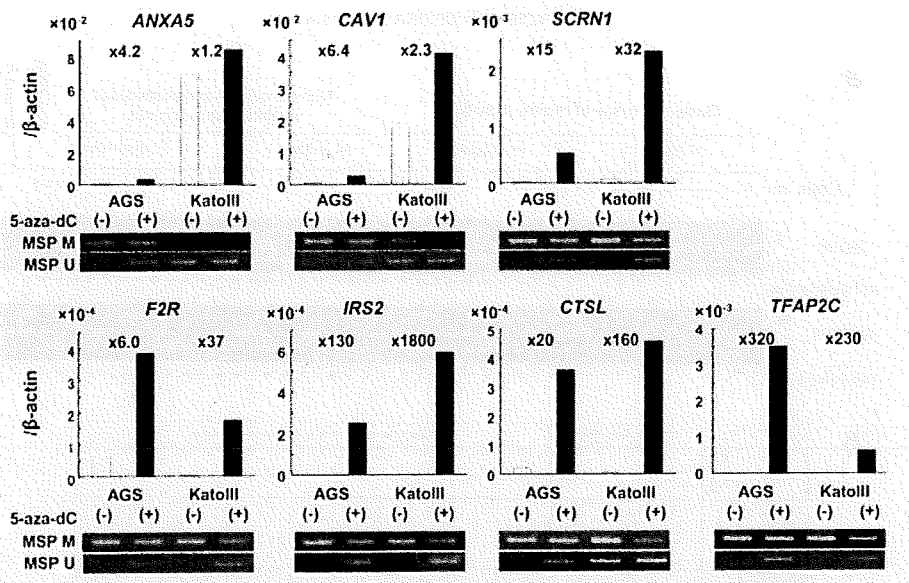

FIGURE 1 – Gene silencing due to methylation of the regions analyzed. mRNA expression and methylation were analyzed by real-time RT-PCR and MSP, respectively, in gastric cancer cell lines (AGS and KATO-III) before and after 5-aza-dC treatment. The fold increases after 5-aza-dC treatment is shown for each cell line. No or little mRNA expression in a cell line(s) without unmethylated DNA molecules and upregulation by the 5-aza-dC treatment was confirmed for the 7 genes randomly selected from the 48 genes.

Cell lines and 5-aza-dC treatment

Gastric cancer cell lines, AGS and KATO-III, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). For treatment with a demethylating agent, 5-aza-2′-deoxycitidine (5-aza-dC, Sigma, St. Louis, MO), cells were seeded on day 0, media containing 0.3 μM 5-aza-dC was freshly added on days 1 and 3, and cells were harvested on day 5. Genomic DNA and total RNA were isolated in the same way as the primary samples.

Bisulfite treatment and methylation-specific PCR

Bisulfite treatment was performed as previously described.[9] 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-sec denaturation at 95°C and 15-min incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), and desulfonated in 0.3 N NaOH. DNA was ethanol precipitated and dissolved in 40 μL of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), respectively,[8] using 2 μL of the sodium bisulfite-treated DNA. A region upstream of a putative transcriptional start site (200 bp or less) was analyzed, and CpG maps of all the genes are shown in the Supporting Information Figure 1. DNA methylated with *Sss*I methylase was used to determine a specific condition of PCR for the M set, and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences) was used for the U set. A number of PCR cycles that would yield a minimal visible band was determined using these fully methylated DNA (for M primers) and fully unmethylated DNA (for U primers), and a further 4 cycles were added for actual analysis of test samples. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that for unmethylated DNA, respectively.

Quantitative reverse transcription PCR

cDNA was synthesized from 1 μg of total RNA using a Superscript II kit (Life Technologies, Rockville, MD) with a random primer. Real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME). The number of molecules of a specific gene in a sample was measured by comparing its amplification with that of standard samples, which contained 10^1–10^7 copies of the gene. The standard samples were produced by PCR amplification and purification using Zymo-Spin I™ Columns (Zymo Research, Orange, CA). The amount of the standard samples was measured by OD 260 nm and also by quantification of band intensities after electrophoresis. The mRNA quantity of each gene was normalized to that of β-actin. The primers and PCR conditions are shown in the Supporting Information Table 1. The difference of mRNA expression levels between 2 groups of genes was analyzed by the Welch *t*-test method (both sided).

Western blot analysis

Each 100 μg whole-cell lysate sample was subjected to SDS-PAGE (10% acrylamide gel) and blotted to PVDF membrane. DNMT1 and DNMT3A were detected using rabbit polyclonal antibody against human DNMT1 (NEB, Beverly, MA), human DNMT3A (Cell Signaling Technology, Danvers, MA), respectively at 1/1,000 dilution. DNMT3B was detected using goat polyclonal antibody against human DNMT3B (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/500 dilution. Horse radish peroxidase-conjugated secondary antibody (antirabbit; Cell Signaling Technology, antigoat; Santa Cruz Biotechnology) was used at 1/5,000 dilution.

Results

Confirmation of gene silencing due to promoter CpG islands

The 48 genes consisted of 32 randomly and 16 arbitrarily selected genes from 421 genes that had been identified as methylation-silenced genes in a gastric cancer cell line using microarray analysis of cells treated with 5-aza-dC, and MSP analysis.[8] First,

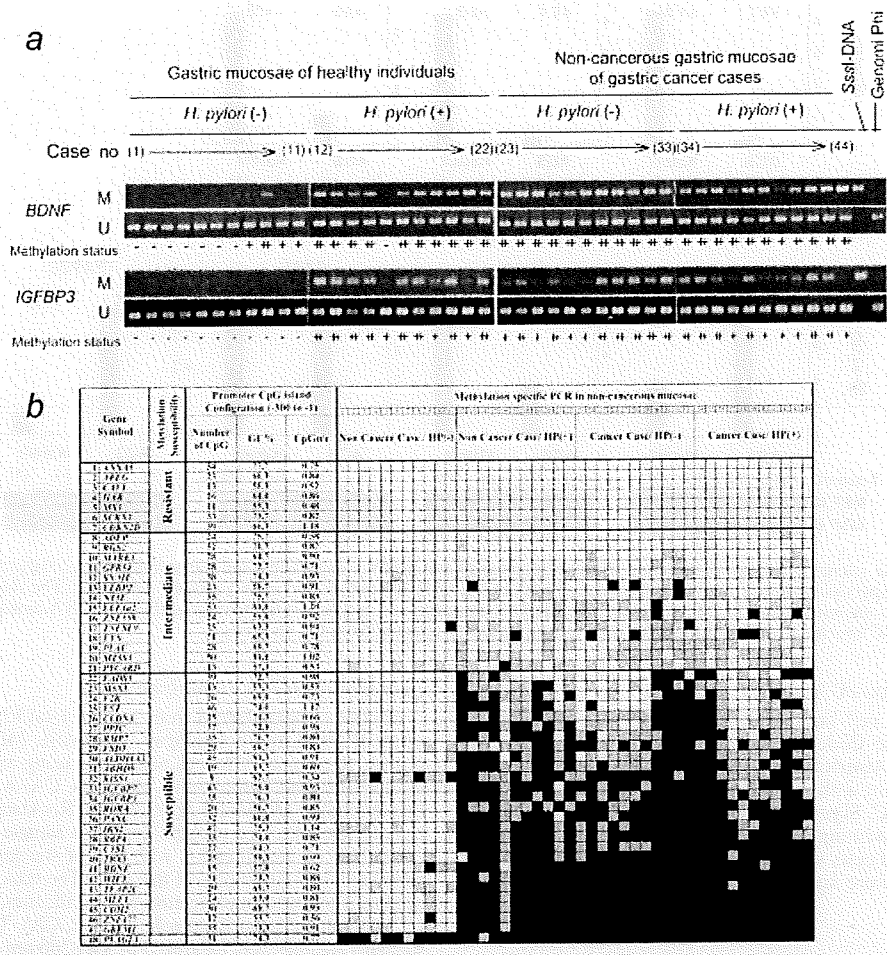

FIGURE 2 – Methylation profile of the 48 genes in noncancerous gastric mucosae. (*a*) Representative results of MSP. Samples 1–11, gastric mucosae of healthy individuals without *H. pylori* infection; 12–22, those with *H. pylori* infection; 23–33, noncancerous gastric mucosae of gastric cancer cases without *H. pylori* infection; and 34–44, those with *H. pylori* infection. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that of fully methylated control DNA. (*b*) Overview of the results of all the 48 genes. The genes were aligned in the order of increasing numbers of individuals with methylation. Closed, hatched, and open boxes represent the methylation levels of high (++), low (+), and none (−), respectively. Rows 1–7, the 7 genes completely resistant to methylation induction in any cases; rows 8–21, genes methylated randomly or more frequently in individuals with cancers; and rows 22–47, genes susceptible to methylation induction by *H. pylori* infection. CpG island configuration (number of CpG sites, G+C content, and CpG score) in 300 bp upstream regions from transcription start sites is also shown. The presence of methylation-resistant and methylation-susceptible genes was clearly revealed. No clear difference in the CpG island configuration was observed between the 2 groups.

we analyzed mRNA expression of 7 of the 48 genes before and after 5-aza-dC treatment using real-time RT-PCR (Fig. 1). It was confirmed that no or little mRNA expression was present in cell lines without unmethylated DNA molecules and that mRNA expression was upregulated by the 5-aza-dC treatment.

Gene specificity in methylation induction by H. pylori infection in gastric mucosae

We then analyzed the methylation status of the promoter CpG islands of the 48 genes in the (noncancerous) gastric mucosae of 4 groups of individuals; those with and without *H. pylori* infection and with and without a gastric cancer. Since MSP can produce inconsistent results if inappropriately performed, we carefully selected a PCR cycle for each primer set so that false positive and negative results were not produced. We scored the methylation status as negative, weakly positive or positive by comparing the band density with that of a fully methylated control (representative results in Fig. 2*a*).

When all the genes were aligned in the order of number of samples with methylation (Fig. 2*b*), the 48 genes were divided into 3 groups: (*i*) 7 genes that were completely unmethylated in any of the 4 groups (genes 1–7 in Fig. 2*b*; methylation-resistant genes), (*ii*) 14 genes that were methylated randomly or more frequently in individuals with cancers (genes 9–21; intermediate genes); and (*iii*) 26 genes that were consistently methylated in the individuals with *H. pylori* infection or with a gastric cancer (genes 22–47; methylation-susceptible genes). The remaining 1 gene, *PLAGL1*, was methylated in all the individuals. This demonstrated that some genes are resistant to methylation induction by *H. pylori* infection while others are susceptible, namely the presence of gene specificity in methylation induction.

Lack of association between CpG island configuration and methylation susceptibility

The 48 genes analyzed here all had CpG islands in their promoter regions. However, based on recent reports,[10] there was a

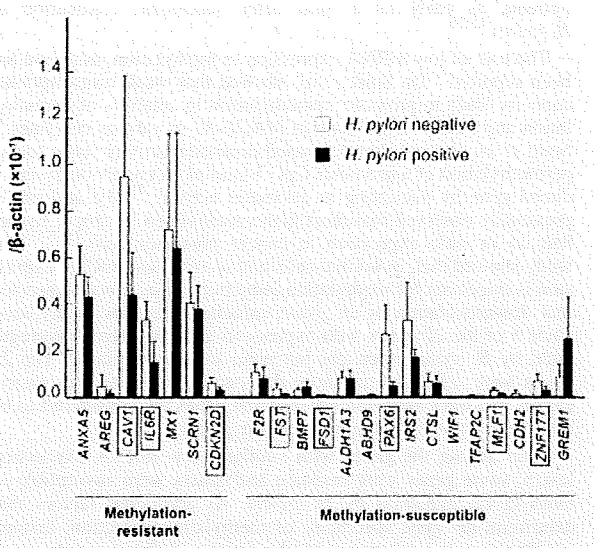

FIGURE 3 – The mRNA expression levels of genes resistant and susceptible to methylation induction. mRNA expression levels of 22 genes (7 resistant and 15 susceptible genes) in the noncancerous gastric mucosae of young healthy individuals with (closed columns) and without (open columns) *H. pylori* infection was analyzed by real-time RT-PCR. Error bar: standard deviation. The average mRNA expression level of methylation-resistant genes was much higher than that of methylation-susceptible genes among individuals without *H. pylori* infection (4.3×10^{-2} *vs.* 7.3×10^{-3}; $p = 0.0008$) and also among individuals with *H. pylori* infection (2.9×10^{-2} *vs.* 5.1×10^{-3}; $p = 0.0012$). The genes whose names are boxed showed a significant decrease in their mRNA expression levels by *H. pylori* infection ($p < 0.05$). Considering that all these 48 genes are those that can be methylated in gastric cancer cell lines, downregulation of mRNA expression could be involved in methylation induction.

possibility that, even among CpG islands, their configurations (number of CpG sites, G+C content, and CpG score) might influence the susceptibility of individual genes to methylation induction by *H. pylori*. Therefore, we examined their configurations in 300 bp upstream regions from transcription start sites (Fig. 2*b*), which corresponded to the nucleosome-free region and whose methylation is critical for gene silencing.[11,12]

The number of CpG sites in the region was 29.2 ± 10.4 (mean ± standard deviation) and 25.4 ± 9.3 for the susceptible and resistant genes, respectively ($p = 0.38$). The G + C content was 68.4 ± 7.4 and 66.4 ± 7.9% for the susceptible and resistant genes, respectively ($p = 0.52$). The CpG score was 0.82 ± 0.18 and 0.75 ± 0.21 for the susceptible and resistant genes, respectively ($p = 0.40$). In short, no significant difference was present between the 2 groups.

Involvement of low mRNA expression levels in gene specificity in methylation induction

To investigate an association between the gene specificity in methylation induction and mRNA expression levels in gastric mucosae, we analyzed mRNA expression levels of all of the 7 methylation-resistant and 15 methylation-susceptible genes, which were randomly selected from the 26 methylation-susceptible genes. To compare mRNA expression levels among different genes, the numbers of cDNA molecules were measured by quantitative RT-PCR after accurate measurement of the weights (converted into the numbers of DNA molecules) of standard DNA samples of all the genes. mRNA expression levels were analyzed in the gastric mucosae of young healthy individuals with and without *H. pylori* infection, who were considered to have no or little methylation of the genes analyzed.

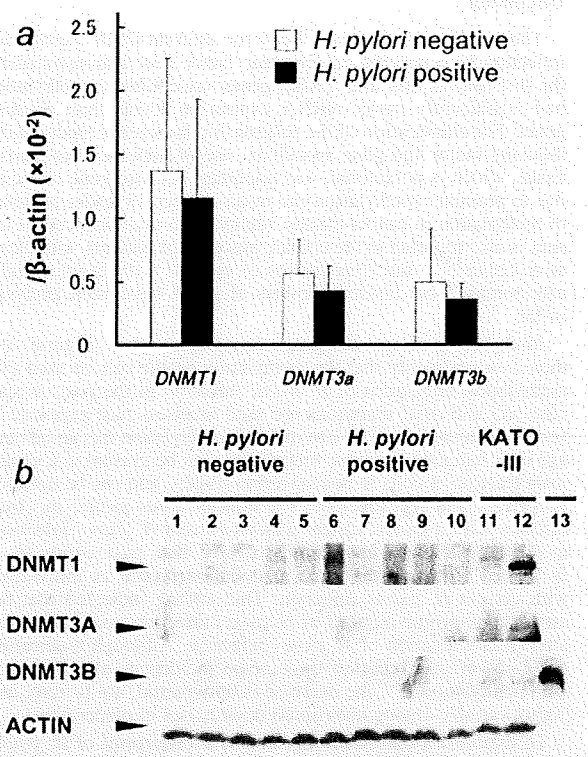

FIGURE 4 – The mRNA and protein expression levels of three DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) in noncancerous gastric mucosae of young healthy individuals with and without *H. pylori* infection. (*a*) mRNA expression levels of *DMNTs*. Closed columns, individuals with *H. pylori* infection; open columns, those without. No significant increase was observed in the mRNA expression levels of these *DNMTs*. (*b*) Western blot analysis of DNMTs. For DNMT1 and DNMT3A, a stomach cancer cell line, KATO-III was used as a positive control (lane 12), and 5-aza-dC (1 μM)-treated KATO-III was used as a negative control (lane 11). ACTIN was used as a loading control. For DNMT3B, a commercially available positive control of DNMT3B (Santa Cruz, lane 13) was used. DNMT protein levels were below the detection limit in the noncancerous gastric mucosae of individuals without (lanes 1–5) and with (lanes 6–10) *H. pylori* infection, and no detectable increase was observed.

The average mRNA expression level of methylation-resistant genes was much higher than that of methylation-susceptible genes among individuals without *H. pylori* infection (4.3×10^{-2} *vs.* 7.3×10^{-3}; $p = 0.0008$) and also among individuals with *H. pylori* infection (2.9×10^{-2} *vs.* 5.1×10^{-3}; $p = 0.0012$) (Fig. 3). Three of the 7 resistant genes and 5 of the 15 susceptible genes showed a significant decrease of mRNA expression levels by *H. pylori* infection, but no genes showed significantly increased mRNA expression.

Expression levels of DNA methyltransferase

To gain an insight into how *H. pylori* infection induces aberrant methylation, we analyzed mRNA expression levels of maintenance DNA methyltransferase, *DNMT1*, and *de novo* methyltransferases, *DNMT3A* and *DNMT3B*, in the gastric mucosae with and without *H. pylori* infection. However, no significant increase in their mRNA expression levels was observed (Fig. 4*a*). Further, at the protein level, expression levels of DNMT1, DNMT3A and DNMT3B were below the detection limit even in the gastric mucosae with *H. pylori* (Fig. 4*b*), indicating no increase was induced by *H. pylori* infection.

Discussion

The presence of gene specificity for aberrant DNA methylation induction by a specific carcinogenic factor was demonstrated for the first time in this study. Also, genes susceptible to methylation had significantly lower mRNA expression levels than resistant genes. For clarification of the relationship between a methylation-inducing factor and gene specificity, use of noncancerous gastric tissue, which is polyclonal, was important because gene silencing due to promoter methylation can result in over- or under-presence of methylation in cancer tissues. Methylation in noncancerous tissues is also reported in the colonic mucosae of patients with ulcerative colitis[13,14] and liver tissues of patients with hepatocellular carcinomas,[15] but limited numbers of genes have been analyzed so far.

Methylation of specific genes can persist for a lifetime, and there is a possibility that the methylation profile can be used as a methylation fingerprint of *H. pylori* infection in the past, as specific *p53* and *APC* mutations are used to assess past exposure to specific carcinogens.[16,17] Use of DNA methylation has an advantage over mutations because methylation can be present in a significant fraction of cells in noncancerous tissues, and can be detected sensitively and reproducibly. The noncancerous gastric mucosae of cases with a gastric cancer without current *H. pylori* infection, most of which are considered to have had past exposure to *H. pylori*,[18] showed the same methylation profile as individuals with current *H. pylori* infection. This finding indicated that the methylation profile induced by *H. pylori* infection can persist even after discontinuation of *H. pylori* infection. Although eradication of *H. pylori* was reported to decrease incidences of individuals with methylation,[19,20] the decrease is only partial, not to zero, and highly variable among individuals (manuscript in preparation).

To establish a methylation profile as a fingerprint of *H. pylori* infection, the profile must be specific. Unfortunately, few gastric cancers can be considered as those induced solely by another carcinogenic factor, such as Epstein-Barr virus infection[21] or high salt intake,[22] and the specificity cannot be examined easily. However, since low mRNA expression levels are involved in gene specificity, there is a possibility that different carcinogenic factors induce different methylation profiles through induction of reduced mRNA expression of different genes. Once the specificity of a methylation profile is established, a methylation fingerprint will be very useful for clinicopathological analysis and epidemiology. Among the clinically used tests for *H. pylori* infection, the culture and rapid urease tests can detect only current *H. pylori* infection.[23,24] The serum antibody test remains positive in only half the patients as early as 1 year after successful eradication of *H. pylori*.[25,26]

The role of low mRNA expression in methylation induction has been reported.[4] De Smet *et al.* showed that weak transcriptional capacity leads to promoter remethylation by analysis of demethylation and mRNA expression of *MAGE-A1* in various cell lines.[27] Song *et al.* showed that decreased promoter activity leads to hypermethylation of a promoter CpG island of an exogenously introduced gene by disrupting its promoter activity.[28] We and others previously observed that most genes methylated in cancer tissues had no or little expression in cancer precursor cells.[29–32] This study showed that, in normal cells and *in vivo*, low mRNA expression is important for methylation induction. Also, it was suggested that downregulation by *H. pylori* infection precedes methylation since 8 of the 22 genes with expression analyses were downregulated by *H. pylori* infection, but none were upregulated. The 22 genes were selected from those that can be methylated in gastric cancer cell lines and even the resistant genes are considered to be relatively susceptible among the entire genes.

Even among the genes with similarly low mRNA expression levels, some genes were resistant and others were susceptible to methylation induction by *H. pylori*. As additional factors that determine the gene specificity of methylation induction, histone modification deregulation could be important. For example, a repressive histone modification, methylation at Lys27 of histone H3 (H3K27) induced by Polycomb group proteins, is associated with genes methylated in cancers.[33,34] Active chromatin marks, associated with active mRNA expression, could be important to protect DNA from methylation. At the same time, without *H. pylori* infection, even the susceptible genes were not methylated, indicating that abnormality in epigenetic regulation was induced by *H. pylori* infection. The final step of aberrant methylation must be mediated by DNA methyltransferases, and actually overexpression of *de novo* methyltransferases enhance methylation of specific genes in a mouse model.[35] Also, some inflammatory cytokines, such as IL-6, are reported to induce DNA methyltransferases.[36] However, contrary to initial expectations, *H. pylori* infection did not induce either mRNA or protein expression of DNMT1, DNMT3A and DNMT3B in gastric mucosae. Abnormalities in epigenetic regulation induced by *H. pylori* infection also need to be investigated.

In summary, methylation of specific genes was induced by *H. pylori* infection in noncancerous gastric membranes, and preceding low mRNA expression was suggested to be involved in the specificity. Use of the specific profile as a methylation fingerprint of past exposure to a specific carcinogenic factor was suggested.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication

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Abstract
Background *Helicobacter pylori* (*HP*) infection potently induces aberrant DNA methylation in gastric mucosae, and its accumulation is associated with gastric cancer risk. Cross-sectional analysis of methylation levels (fraction of methylated DNA molecules) and temporal analysis of methylation incidence suggested that methylation levels decrease after *HP* infection discontinues. We aimed to demonstrate the decrease in methylation levels.
Methods Thirty-five patients with *HP* infection who had undergone curative endoscopic resection and 11 healthy volunteers were recruited. Methylation levels were quantified by real-time methylation-specific PCR. Histology was evaluated according to the updated Sydney System.
Results In the 20 patients with successful eradication, the *FLNc* methylation level, along with infiltration of inflammatory cells, decreased from 0.6 to 0.4% at 6 weeks ($P = 0.049$) and remained low at 1 year. The *THBD* methylation level (30.1%) remained high at 6 weeks, but decreased to 19.0% at 1 year ($P = 0.0032$). Nine healthy volunteers with successful eradication tended to show a decrease of both *FLNc* and *THBD* at 6 weeks. However, the methylation levels after the decrease were still higher than those of healthy individuals without *HP* infection. In the 15 patients with persistent infection, the methylation levels remained the same. Before eradication, the *THBD* methylation level correlated with the degree of inflammatory cell infiltration ($P < 0.05$).
Conclusions Methylation levels in gastric mucosae decreased to certain levels after *HP* eradication in profiles unique to individual markers. Involvement of chronic inflammation in methylation induction was suggested.

Keywords DNA methylation · Epigenetics · *Helicobacter pylori* · Eradication · Molecular epidemiology

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Introduction

Gastric cancer is one of the most common cancers in the world, especially in eastern Asia [1]. To reduce its morbidity and mortality by early detection and early treatment, identification of individuals with high risk is important. Also, endoscopic resection (ER) is becoming a standard treatment for early gastric cancer [2, 3], and this

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

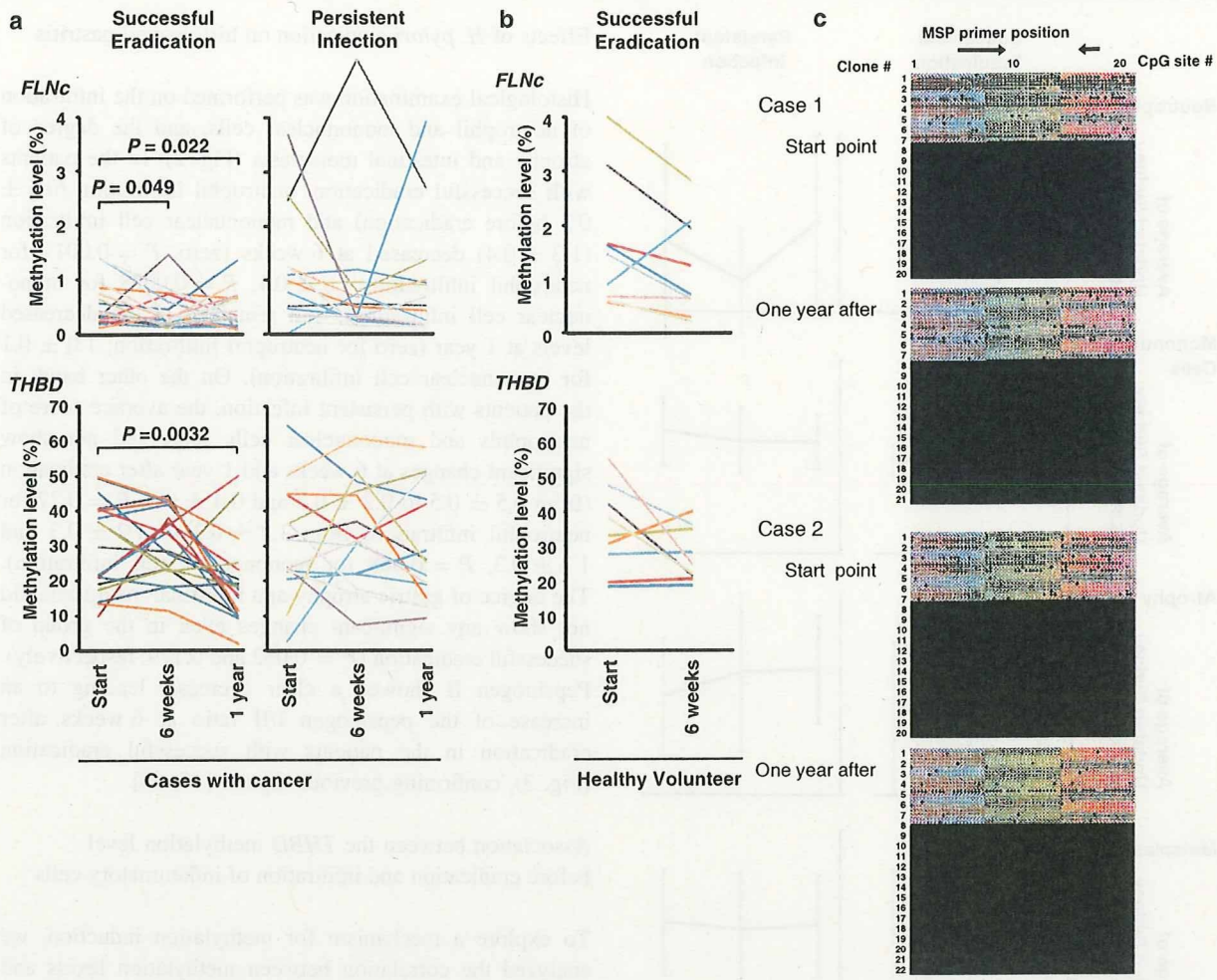

Fig. 1 **a** Effects of *H. pylori* eradication on *FLNc* and *THBD* methylation levels. A methylation level of a sample was measured in triplicate, and an average of three biopsy sites in the stomach was used as a methylation level of an individual. Methylation levels were measured in the group of successful 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

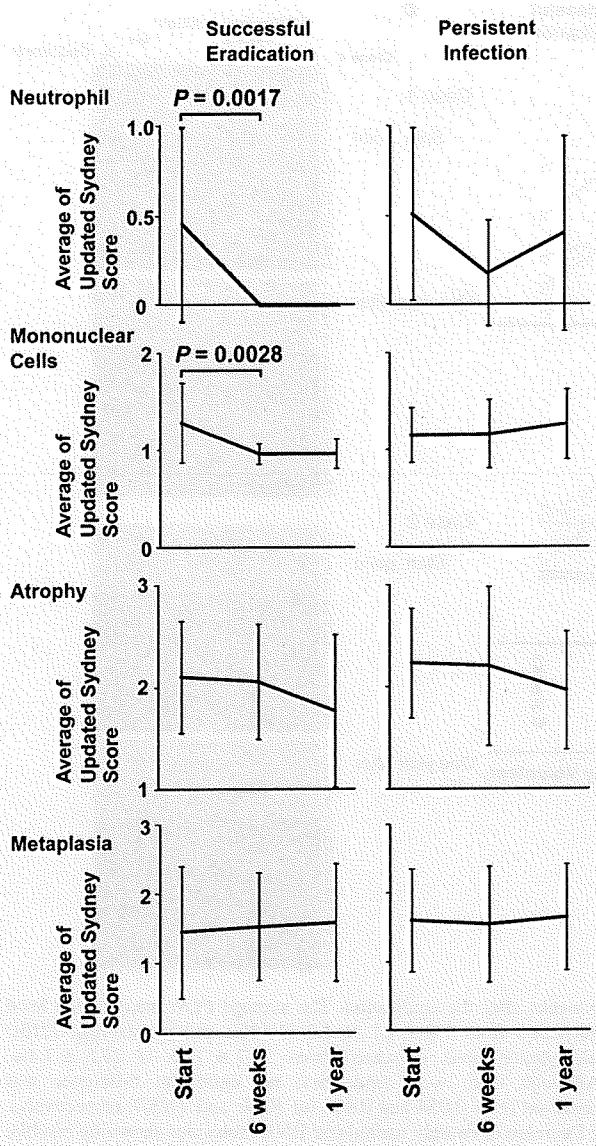

Fig. 2 Histological changes after *H. pylori* eradication. Average scores of histological gastritis of the three biopsy sites in the stomach were measured at three time points. Intensities of acute infiltrates (neutrophil), chronic infiltrates (mononuclear cells), gastric atrophy and intestinal metaplasia 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

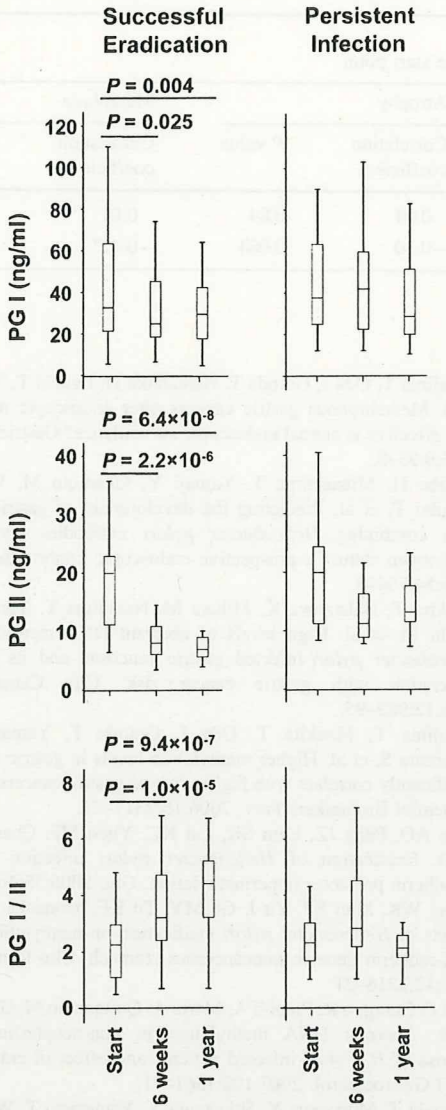

Fig. 3 Changes of serum pepsinogen I and II after *H. pylori* eradication. In the patients with successful eradication, a decrease of pepsinogen I, that of II, and increase of the I/II ratio were observed at 6 weeks after eradication ($P = 0.025$, 2.2×10^{-6}, and 1.0×10^{-5}, respectively) and 1 year ($P = 0.004$, 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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Letter

The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands

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Instructive mechanisms are present for induction of DNA methylation, as shown by methylation of specific CpG islands (CGIs) by specific inducers and in specific cancers. However, instructive factors involved are poorly understood, except for involvement of low transcription and trimethylation of histone H3 lysine 27 (H3K27me3). Here, we used methylated DNA immunoprecipitation (MeDIP) combined with a CGI oligonucleotide microarray analysis, and identified 5510 and 521 genes with promoter CGIs resistant and susceptible, respectively, to DNA methylation in prostate cancer cell lines. Expression analysis revealed that the susceptible genes had low transcription in a normal prostatic epithelial cell line. Chromatin immunoprecipitation with microarray hybridization (CHiP-chip) analysis of RNA polymerase II (Pol II) and histone modifications showed that, even among the genes with low transcription, the presence of Pol II was associated with marked resistance to DNA methylation (OR = 0.22; 95% CI = 0.12–0.38), and H3K27me3 was associated with increased susceptibility (OR = 11.20; 95% CI = 7.14–17.55). The same was true in normal human mammary epithelial cells for 5430 and 733 genes resistant and susceptible, respectively, to DNA methylation in breast cancer cell lines. These results showed that the presence of Pol II, active or stalled, and H3K27me3 can predict the epigenetic fate of promoter CGIs independently of transcription levels.

[Supplemental material is available online at http://www.genome.org. The microarray data from this study have been submitted to Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE15154.]

Epigenetic alterations, along with genetic alterations, are known to play critical roles in human carcinogenesis and other acquired diseases (Laird and Jaenisch 1996; Robertson 2005; Jones and Baylin 2007). Especially, DNA methylation of promoter CpG islands (CGIs) has been known to be involved in silencing of tumor-suppressor and other genes (Ushijima 2005; Eckhardt et al. 2006; Jones and Baylin 2007). In addition, a critical role of methylation of the nucleosome-free region (NFR) just upstream of a transcription start site (TSS) was recently demonstrated in nucleosome occupation and thus in gene silencing (Li et al. 2007; Lin et al. 2007).

Epigenetic alterations, different from genetic alterations, have unique natures, such as gene specificity (Costello et al. 2000; Esteller et al. 2001; Keshet et al. 2006; Nakajima et al. 2009; Oka et al. 2009), high levels of accumulation in normal-appearing tissues (Kondo et al. 2000; Maekita et al. 2006; Ushijima 2007), and deep involvement of inflammation in their induction (Issa et al. 2001; Ushijima and Okochi-Takada 2005; Maekita et al. 2006). Especially, the presence of gene specificity, originally suggested by the presence of tumor type-specific DNA methylation patterns (Costello et al. 2000; Esteller et al. 2001), is now confirmed by methylation of specific genes in non-cancerous tissues exposed to specific carcinogenic factors (Nakajima et al. 2009; Oka et al. 2009). Selection biases for genes with growth advantage can be avoided by analysis of non-cancerous, therefore polyclonal, tissues (Mihara et al. 2006). The gene specificity of DNA methylation induction depending on cell types and carcinogenic factors shows that there are instructive mechanisms for DNA methylation induction, in contrast to the random nature of mutation induction.

As mechanisms for instructive induction, limited information is available so far, including low transcription levels and some histone modifications. Exogenous and endogenous genes are likely to become methylated only when they have low transcription levels (Song et al. 2002; De Smet et al. 2004). Most genes methylated in cancer tissues had no or low transcription in their normal counterpart cells (Ushijima 2005; Keshet et al. 2006). Transcription factors, such as SP1/SP3 and MLL, protected CpG sites from becoming methylated, independent of and dependent on transcription levels, respectively (Boumber et al. 2008; Erfurth et al. 2008). In addition, trimethylation of histone H3 lysine 27 (H3K27me3), a target of Polycomb repressive complex (PRC) 2 (Hansen et al. 2008), was enriched in normal cells and embryonic stem (ES) cells at genes that can be methylated in cancers (Ohm et al. 2007; Schlesinger et al. 2007; Widschwendter et al. 2007; Hahn et al. 2008; Rodriguez et al. 2008). Nevertheless, at a genome level, many genes have low transcription levels and H3K27me3 but are still resistant to DNA methylation induction, indicating that some critical factors are likely to be still missing.

In this study, we hypothesized that RNA polymerase II (Pol II) binding around TSSs can function as a protective factor for DNA methylation induction. Accumulation of Pol II at genes with low transcription levels (stalled Pol II) was recently found in as high as 12% of protein-coding genes in *Drosophila melanogaster* (Muse et al. 2007; Zeitlinger et al. 2007) and in humans (Guenther et al. 2007). We demonstrate in a genome-wide manner that Pol II binding, active or stalled, and histone modifications in normal cells predict genes resistant and susceptible to DNA methylation in cancers.

Results

Identification of genes with promoter CGIs resistant and susceptible to DNA methylation

To identify genes with promoter CGIs resistant and susceptible to induction of DNA methylation in human prostate cancers, four

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prostate cancer cell lines (PC3, LNCaP, 22Rv1, and Du145), along with a normal prostatic epithelial cell line (RWPE1), were analyzed using methylated DNA immunoprecipitation (MeDIP) combined with a human CGI oligonucleotide microarray that covered 27,800 CGIs (MeDIP-CGI microarray analysis).

First, appropriate cutoff values of our original output values "DNA methylation values" (Me values) were determined using 145 samples (29 CGIs in five cell lines) (Supplemental Table S1). As cutoff values with high specificity and little compromise of sensitivity, cutoff values of 0.6 and 0.4 were selected for methylated and unmethylated CGIs, respectively (Supplemental Fig. S1). The specificity and sensitivity for methylated (unmethylated) CGIs with these values were 0.95 (0.96) and 0.85 (0.82), respectively. DNA methylation status of a CGI or putative NFR was judged as unmethylated (UM), moderately methylated (MM), and highly methylated (HM) when the average of Me values of the probes within the region was 0–0.4, 0.4–0.6, and 0.6–1.0, respectively. The validity of our methods was also supported by the fact that promoter CGIs were more likely to be unmethylated (68%–82%) than those in gene bodies (54%–63%), which conformed with previous observations (Supplemental Table S2; Ushijima et al. 2003; Eckhardt et al. 2006; Rakyan et al. 2008).

The susceptibility of genes was determined by methylation analysis of 8930 NFRs (Li et al. 2007). Genes with NFRs unmethylated (Me value, 0–0.4) in the normal cell line and all the four cancer cell lines were defined as DNA methylation-resistant genes. On the other hand, those unmethylated in the normal cell line but highly methylated (Me value, 0.6–1.0) in at least one of the four cancer cell lines were defined as DNA methylation-susceptible genes (Fig. 1A). Susceptible genes were further divided into S1, S2, S3, and S4 subclasses according to the DNA methylation frequency in cancer cell lines (highly methylated in one, two, three, and four, respectively, of the four cancer cell lines). In addition, genes unmethylated in the normal cell line but moderately methylated (Me value, 0.4–0.6) in at least one of the four cancer cell lines were defined as genes with intermediate susceptibility (intermediate genes). In prostate cancers, 5510, 1330, and 521 genes with promoter CGIs were classified as resistant, intermediate, and susceptible genes, respectively (Fig. 1B). DNA methylation levels of NFRs were largely consistent with those of further upstream regions up to −800 bp, and downstream regions up to +800 bp (Fig. 1C).

To avoid any tissue bias and statistical errors, we also analyzed three human breast cancer cell lines (MCF7, ZR-75-1, and MDA-MB-468), along with normal human mammary epithelial cells (HMEC). As in the prostate, the promoter CGIs were more likely to be unmethylated (68%–90%) than the CGIs located in gene bodies (52%–70%) (Supplemental Table S2). Using the same definition as in the prostate cancers, 5430, 1913, and 733 genes with promoter CGIs were classified as resistant, intermediate, and susceptible genes, respectively (Fig. 1B). As in prostate cancers, DNA methylation levels were also largely consistent among the NFRs, further upstream regions, and downstream regions in human breast cancers (Supplemental Fig. S2). Between breast and prostate cancers, only 261 genes, 36% of the susceptible genes in breast cancers and 50% of those in prostate cancers, were commonly susceptible, showing the presence of tissue specificity.

To explore possible selection bias for the resistant and susceptible genes due to gene functions, functional annotation analysis of resistant and susceptible genes was performed. In the prostate, 203 and 154 processes out of 16,621 biological processes were enriched among the resistant and susceptible genes, respectively. Among the resistant genes, processes involved in basic cellular processes such as metabolic process, RNA processing, and RNA splicing were enriched. In contrast, among the susceptible genes, biological processes involved in the developmental processes of

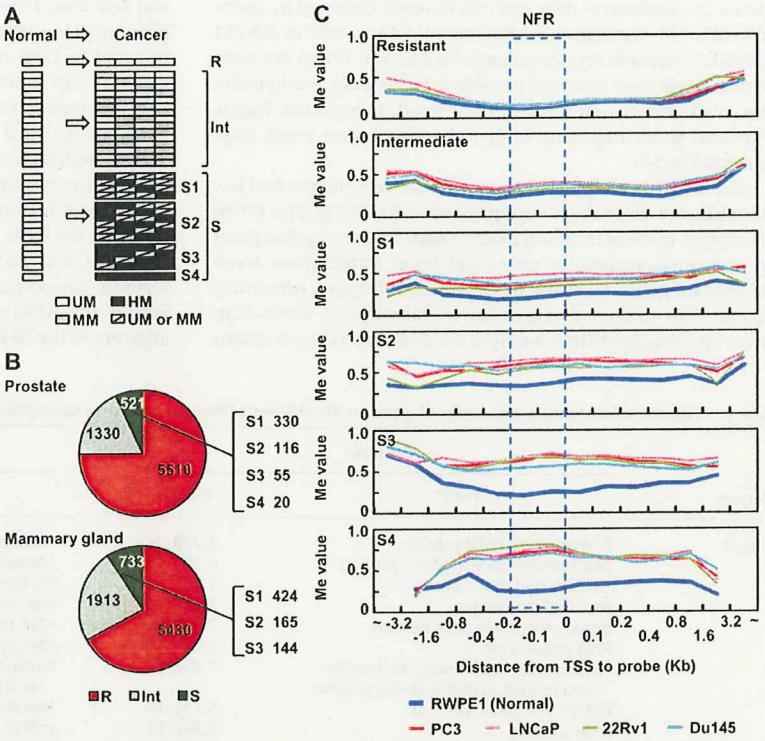

Figure 1. Identification of methylation-resistant and methylation-susceptible genes and their methylation profiles in various genomic regions against TSSs. (*A*) Definition of genes resistant and susceptible to induction of DNA methylation. Genes unmethylated (UM) (white) in the normal cell line (cells) and all cancer cell lines were defined as resistant genes (R). Genes unmethylated in the normal cell line (cells) but highly methylated (HM) (black) in at least one of the four cancer cell lines were defined as susceptible genes (S). Susceptible genes were further divided into four subclasses according to DNA methylation frequency in the cancer cell lines (S1–S4). Genes unmethylated in the normal cell line (cells) but moderately methylated (MM) (gray) in the cancer cell lines were defined as genes with intermediate susceptibility (intermediate genes: Int). (*B*) The fractions of resistant (red), intermediate (light green), and susceptible (green) genes in the prostate and the mammary gland. (*Right* side of the pie graph) Numbers of susceptible genes in each subclass (S1–S4). (C) DNA methylation levels at various positions against the TSSs in the normal prostatic cell line and four cancer cell lines. Average Me values of CGIs continuous from their NFRs are shown. (Blue dotted rectangle) The NFRs. Methylation levels of the NFRs were similar to those of upstream regions up to −800 bp and downstream regions up to +800 bp.

specific cells or tissues, such as nervous system development, and embryonic development, were enriched (Table 1). Similar enrichment of genes involved in specific biological processes was also observed in the mammary glands.

Low transcription levels of DNA methylation-susceptible genes in normal cell lines

For a limited number of genes, the susceptibility of genes with low transcription to DNA methylation has been reported in cell lines (Song et al. 2002; De Smet et al. 2004) and in human tissue (Ushijima and Okochi-Takada 2005; Nakajima et al. 2009). To analyze this susceptibility in a genome-wide manner, we performed expression analysis in the normal prostatic cell line using a GeneChip oligonucleotide microarray. Owing to the difference of array platforms between the CGI oligonucleotide microarray and the GeneChip oligonucleotide expression microarray, we were able to measure transcription levels of the 7574 genes out of 8930 genes with promoter CGIs in the normal prostatic cell line. The accuracy of the transcription levels obtained by the GeneChip oligonucleotide microarray was validated by observing a strong correlation between the microarray data and mRNA levels obtained by quantitative RT-PCR (correlation coefficient = 0.95 and 0.97 in RWPE1 and HMEC, respectively) (Supplemental Fig. S3). When the transcription levels were analyzed according to the DNA methylation status in the normal prostatic cell line itself, as expected, highly methylated genes had remarkably low transcription levels (Supplemental Fig. S4).

Genes highly methylated in prostate cancer cell lines had low transcription levels in the normal prostatic cell line (Fig. 2A). When transcription levels of resistant, intermediate, and susceptible genes were compared, susceptible genes had lower transcription levels than resistant genes. Even among the susceptible genes, genes with frequent DNA methylation had lower transcription levels than those with infrequent DNA methylation (Fig. 2B). When fractions of genes with high, moderate, and low transcription levels were analyzed in the 7574 total, 4567 resistant, and 479 susceptible genes, the susceptible genes had a significantly larger fraction of genes with low transcription (63%) than the total genes (38%; $P < 0.001$, χ^2 test) (Fig. 2C). Even among the susceptible genes, genes with more frequent DNA methylation had the larger fraction of genes with low transcription (Supplemental Fig. S5). These results showed that aberrant DNA methylation is preferentially induced in genes with low transcription, as previously reported (Song et al. 2002; De Smet et al. 2004; Ushijima 2005; Keshet et al. 2006; Nakajima et al. 2009), in a genome-wide manner.

In the mammary glands, the susceptible genes also had a significantly larger fraction of genes with low transcription (74%) than the total genes (37%; $P < 0.001$, χ^2 test) (Supplemental Figs. S5, S6).

Levels of histone modifications and Pol II binding were associated with DNA methylation susceptibility

Although most genes susceptible to DNA methylation in cancers had low transcription in the normal cell line (cells), the converse was not true: 1237 of 2852 (prostate) and 1048 of 2750 (breast) genes with low transcription in the normal cell line (cells) were still resistant to DNA methylation in cancers (Fig. 2C; Supplemental Fig. S6). This indicated that factors besides low transcription are also involved in DNA methylation susceptibility. To address this issue, we analyzed both active (acetylation of histone H3 [H3Ac] and trimethylation of histone H3 lysine 4 [H3K4me3]) and inactive (trimethylation of histone H3 lysine 9 [H3K9me3] and H3K27me3) histone modifications and Pol II binding at and adjacent to the NFRs in a genome-wide manner. Since the length of sheared DNA used for chromatin immunoprecipitation (ChIP) analysis ranged mainly from 200 to 1000 bp, analysis of probes within the NFRs automatically reflected histone modifications adjacent to the NFRs even if nucleosomes were absent in the NFRs.

Table 1. Functional annotation analysis of genes with different DNA methylation susceptibility

	Prostate		Mammary gland	
Category	Term	*P*-value	Term	*P*-value
Resistant	Primary metabolic process	3.72E-22	Cellular metabolic process	3.16E-20
	Macromolecule metabolic process	6.27E-22	Metabolic process	1.74E-19
	Cellular metabolic process	1.08E-21	Primary metabolic process	4.50E-19
	Metabolic process	5.98E-21	Macromolecule metabolic process	2.33E-17
	Biopolymer metabolic process	2.92E-15	RNA processing	9.75E-17
	RNA processing	1.34E-14	RNA splicing	6.54E-14
	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	1.49E-14	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	1.30E-13
	mRNA metabolic process	9.65E-14	Macromolecule localization	1.51E-13
	RNA splicing	3.94E-13	mRNA metabolic process	1.29E-12
	Protein transport	7.19E-13	Biopolymer metabolic process	1.29E-12
Susceptible	Multicellular organismal process	2.41E-16	Multicellular organismal process	8.20E-30
	Multicellular organismal development	3.33E-12	Multicellular organismal development	4.77E-23
	System development	4.77E-11	System development	2.57E-18
	Anatomical structure development	1.10E-09	Anatomical structure development	6.61E-17
	System process	3.09E-08	Developmental process	5.18E-16
	Nervous system development	9.59E-08	Nervous system development	3.96E-13
	Developmental process	4.48E-07	Cell-cell signaling	2.27E-12
	Organ development	7.93E-07	Organ development	2.85E-12
	Cell-cell signaling	3.03E-06	Embryonic development	6.96E-11
	Biological adhesion	9.69E-06	System process	1.01E-10

Enrichment of specific biological processes in Gene Ontology criteria among resistant and susceptible genes was analyzed by DAVID bioinformatics resources. The top 10 significantly enriched biological processes in each gene category are listed. The significance (*P*-value) of enrichment is shown.