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DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis

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Transcriptional silencing by CpG island hypermethylation is a potential mechanism for the inactivation of tumor-related genes. Virtually, all types of human cancers show CpG island hypermethylation, and gastric carcinoma (GC) is one of the tumors with a high frequency of aberrant CpG island hypermethylation. In this study, we prescreened DNA methylation of 170 CpG island loci in a training set of 8 paired GC and GC-associated non-neoplastic mucosae (GCN) using MethyLight technology and selected 27 DNA methylation markers showing higher methylation frequency or level in GC than in GCN. These markers were then analyzed in a tester set of 25 paired GC and GCN and 27 chronic gastritis (CG) from non-cancer patients to generate their DNA methylation profiles. We identified 17 novel methylation markers in GC, including *SFRP4*, *SEZ6L*, *TWIST1*, *BCL2*, *KL*, *TERT*, *SCGB3A1*, *IGF2*, *GRIN2B*, *SFRP5*, *DLEC1*, *HOXA1*, *CYP1B1*, *SMAD9*, *MT1G*, *NR3C1*, and *HOXA10*. Of the 27 selected CpG island loci, 23 were methylated in GC, GCN, and CG and the remainder four loci (*DLEC1*, *CHFR*, *CYP1B1*, and *NR3C1*) were only methylated in GC. We found that the number of methylated loci was significantly higher in GC than in GCN or CG and that *Helicobacter pylori* infection was strongly associated with aberrant CpG island hypermethylation in CG. Hypermethylation was more prevalent in Epstein–Barr virus (EBV)-positive GC than in EBV-negative GC and in diffuse-type GC than in intestinal-type GC. Through our large-scale screening of 170 CpG island loci, we found 17 new DNA methylation markers of GC, which may serve as useful markers that may identify a distinct subset of GC.

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CpG islands are DNA segments, at least 0.5 kb in size, rich in G:C and CpG content, and often located in the promoter or 5′-exon sequences of genes.[1] Promoter CpG islands have traditionally been thought to be unmethylated in normal cells, with the exception of those on the inactive X chromosome and those associated with imprinted genes. Although the cause is unclear, promoter CpG island hypermethylation can occur in association with cancer development or aging. Promoter CpG island hypermethylation is closely associated with gene inactivation and thought to act as an alternative to genetic change for inactivation of tumor suppressor genes in human cancers. Virtually, all types of human cancer display promoter CpG island hypermethylation, although there are variations in the prevalence of CpG island hypermethylation among tumor types.[2,3] In addition to its potential role in gene inactivation in human cancers, CpG island hypermethylation is now gaining attention as a molecular marker for tumor detection and prediction of development or progression of cancer. DNA methylation markers are actively being investigated for their utility as biomarkers to detect human cancers in blood, secretions, or exfoliated cytology specimens and to predict the risk of the progression or development of cancer.[4,5]

Gastric carcinoma (GC) is the most common cancer in eastern Asia and the third most frequent cancer across the world.[6] The stomach is one of the organs where aberrant CpG island hypermethylation occurs frequently during cancer development or aging.[7] To date, about 87 genes have been characterized to be inactivated by hypermethylation of their promoter CpG islands in GC.[8] Considering the fact that

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more than 40% of human genes contain promoters within CpG islands[9–11] and the number of the genes characterized as hypermethylated in GCs is very limited, the vast majority of genes with CpG islands remain to be tested as to their DNA methylation status in GC.

Methylation-specific polymerase chain reaction (MSP) is a highly sensitive method and can detect one methylated allele in 10 000 unmethylated alleles.[12] However, MSP does not provide quantitative information on the proportion of the methylated alleles among total alleles. Combined bisulfite restriction analysis (COBRA) is a quantitative method detecting methylated alleles but less sensitive than MSP. Furthermore, it cannot be applied to all CpG island loci because of its dependence upon the limited number of restriction enzymes available that recognize CG sites in bisulfite-converted sequences. Most of the studies investigating CpG island hypermethylation in GCs used MSP, and the methylation frequencies of the specific CpG islands loci in GCs varied among the studies. Although the discrepancies might be related to ethnic differences of the study populations (materials), it is also likely that some of the variability in observed methylation frequency of specific CpG island loci could be caused by the differences in the number of the cycles of polymerase chain reaction (PCR), the amount of template DNA, the PCR mixture conditions, or the specific target CpG sites of the CpG island locus. The quantitative, real-time PCR-based MethyLight assay can overcome issues related to PCR cycling and can sensitively detect DNA methylation in a small amount of template DNA. MethyLight assay has been evaluated and validated for its precision and performance characteristics.[13,14]

In the present study, we identified DNA methylation markers that can differentiate GC from non-neoplastic gastric mucosae in terms of the methylation frequency or methylation level (the proportion of the methylated alleles among the total alleles, including methylated and unmethylated alleles). After prescreening of 170 CpG island loci against a training set (8 paired GC and the associated non-neoplastic stomach tissues), we selected and analyzed 27 CpG island loci for their methylation status in a tester set of GCs ($n = 25$), paired GC-associated non-neoplastic mucosae (GCN), and chronic gastritis (CG) samples from patients without GC ($n = 27$) using MethyLight. The results of DNA methylation were analyzed in relation to clinicopathologic factors, including histologic type, microsatellite instability (MSI) status, and the presence of *Helicobacter pylori* (HP) or Epstein–Barr virus (EBV).

MATERIALS AND METHODS

Patients

Formalin-fixed, paraffin-embedded archival tissues from 33 GC patients and 27 CG patients were retrieved from the file of the Department of Pathology, Seoul National University Hospital (Seoul, Korea). The age of cancer patients ranged 36–81 years (mean: 62.4 years; median: 63 years) and male to female ratio was 4:1. The age of CG patients ranged 41–68 years (mean: 53.3 years; median: 53 years) and male to female ratio was 19:10. This study was approved by the Institutional Review Board.

DNA Extraction and Bisulfite Modification

Ten sections of 10 μm thickness of paraffin-embedded tissues were used for DNA extraction. The paraffin was removed from the tissue by rinsing in xylene and genomic DNA was isolated using a QIAamp tissue kit (Qiagen, Valencia, CA, USA). Sodium bisulfite conversion of genomic DNA was performed as follows. Briefly, 2 μg of genomic DNA was denatured at 100°C for 10 min, followed by incubation in 0.3 M NaOH at 42°C for 20 min. Bisulfite conversion was performed with 2.5 M sodium metabisulfite solution at 50°C for 16 h in the dark. DNA was purified from the bisulfite solution using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's recommendations, except that each sample was loaded two times onto each spin column. After the entire sample was loaded and the spin column washed, the sample was eluted twice in 40 μl elution buffer. The 80 μl sample was then desulfonated by incubation in 0.077 M NaOH solution at room temperature for 15 min. After neutralization with 1 M HCl, DNA was purified a second time using the QIAamp Viral RNA Mini Kit (Qiagen). The eluted sample (80 μl) was then diluted accordingly for use in MethyLight analysis.

DNA Methylation Analysis

DNA methylation analyses were performed using MethyLight. We screened 170 DNA MethyLight reactions to identify those that are cancer-specifically methylated in GCs. Most of these MethyLight reactions had been developed for other purposes, including studies of esophageal cancer, lung cancer, pancreatic cancer, ovarian cancer, brain cancer, and neurodegenerative disorders. From this prescreen, we selected 27 MethyLight reactions for subsequent DNA methylation analyses. These MethyLight reaction primers, probes, and information are shown in Supplementary Table 1. MethyLight PCR was performed in a 30 μl reaction volume with 200 mM dNTPs, 0.3 μM forward and reverse PCR primers, 0.1 μM probe, 3.5 mM $MgCl_2$, 0.01% Tween-20, 0.05% gelatin and 0.1 U of *Taq* polymerase using the following PCR program: 95°C for 10 min, then 50 cycles of 95°C for 15 s followed by 60°C for 1 min. The samples in 96-well plates were analyzed on an Opticon DNA Engine Continuous Fluorescence Detector (MJ Research/Bio-Rad, Hercules, CA, USA). Each MethyLight reaction signal was compared to two control reactions, *ALU* and *COL2A1*, to normalize for input bisulfite-converted DNA levels. The *COL2A1* reaction and the *ALU* reaction were described previously.[15,16] Both *COL2A1* and *ALU* reactions are specific for bisulfite-converted DNA but are not DNA methylation specific. The specificity of the reactions for methylated DNA was confirmed separately using *M.Sss*I (New England Biolabs

Inc., Beverly, MA, USA)-treated human peripheral blood lymphocyte DNA (Promega Co., Madison, WI, USA). The percentage of fully methylated alleles (percentage of methylated reference (PMR)[4]) at each locus was calculated by dividing the *GENE*:*ALU* ratio of a sample by the *GENE*:*ALU* ratio of the *M.Sss*I-treated human genomic DNA sample and multiplying by 100. The same calculation was performed for each sample using the *COL2A1* control reaction, and the final PMR values were obtained by averaging the PMR values based on the individual *ALU* and *COL2A1* control reactions. Since the PMR value is calculated as a ratio of the signal between the sample and *in vitro M.Sss*I-treated reference sample, there may be instances in which the PMR value is greater than 100 if there was incomplete methylation of the reference sample. PMR calculations have also been described extensively.[4,13,16] There is no standard cutoff value to classify methylated and unmethylated loci using DNA methylation analyses, so we considered a CpG island locus methylated if the PMR value was >4. Our rationale is that samples with PMR values less than 4 are not substantially methylated, and therefore may not serve well as DNA methylation markers in GC, and the PMR cutoff value of 4 has been validated by previous studies based on the distributions of PMR values in the tested CpG island loci and correlation of protein expression loss with methylation positivity in *CDKN2A*, *MLH1*, and *MGMT* determined by the PMR cutoff of 4.[14,17]

Microsatellite Instability Analysis

The MSI status of each tumor and paired normal mucosa sample was determined based on an examination of five microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26). Either forward or reverse primer for each marker was labeled with fluorescence, and PCR products were electrophoresed and analyzed by ABI 3730 (Applied Biosystems, Foster City, CA, USA). We classified tumors with instability at two or more microsatellite loci as MSI-positive.

Epstein–Barr Virus-Encoded RNA *In Situ* Hybridization

The EBV RNA *in situ* hybridization was performed using a fluorescein-conjugated peptide nucleic acid probe complimentary to a portion of the small EBV-encoded RNAs (EBERs) 1 and 2. Sections (5 μm thick) on slides coated with poly-L-lysine were routinely deparaffinized, dehydrated, and predigested with 3 μg/ml proteinase K, and then hybridized for 2 h at 37°C. Anti-fluorescein antibody conjugated to alkaline phosphatase was used with an NBT/BCIP kit (Dako, Copenhagen, Denmark) to detect the EBER signals. Counterstaining was performed with Mayer's hematoxylin.

Assays For *H. pylori* Infection

The presence of HP infection was determined by light microscopic examination of Giemsa-stained histologic slides, PCR for HP 16S rDNA, or rapid urease test (CLO test; Delta West, Bentley, WA, Australia). Cases that showed positivity in any of the three assays were regarded as HP-positive. PCR for HP 16S rDNA was performed as previously described.[18]

5-Aza-2′-deoxycytidine and Trichostatin A treatments

Cells were seeded at a density of 3×10^5 cells/10 cm dish on day 0 and treated daily with 1 μM 5-aza-2′-deoxycytidine (5-Aza-CdR) (Sigma Chemical Co., St Louis, MO, USA) for 72 h. We also treated cells with 150 nM Trichostatin A (Sigma Chemical Co.) alone for 24 h or in combination of 1 μM 5-Aza-CdR (72 h) plus 150 nM Trichostatin A (24 h). After the treatments, the cells were harvested and RNA was extracted for reverse transcription-PCR (RT-PCR).

RT-PCR

cDNA was synthesized from 1 μg of total RNA treated with DNase I (Ambion, Austin, TX, USA) with a Superscript II kit (Invitrogen, Carlsbad, CA, USA). The quantity of mRNA was normalized to that of GAPDH. The primers and PCR conditions are shown in Supplementary Table 2. Real-time RT-PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems) in an iCycler Optical Module (Bio-Rad).

Statistical Analysis

The Student's *t*-test was used to compare the number of CpG island loci methylated or methylation index (MI) in GC, GC-paired normal mucosa, and normal mucosa from non-cancer patients. To compare means between paired samples, paired *t*-test was used. SPSS software was used throughout (SPSS for Windows Release, Ver.11.0; SPSS Inc., Chicago, IL, USA). *P*-values of <0.05 were accepted as being statistically significant.

RESULTS

Selection of 27 CpG Islands Loci from the MethyLight Assay of 170 CpG Islands Loci in a Training Set

DNA methylation markers ($n = 170$) were prescreened for a training set of eight normal tumor pairs of GCN and GC samples to determine those markers that differentiate between GC and GCN on the combined aspects of methylation frequencies and DNA methylation levels. We first removed 32 markers that exhibited a maximum PMR value <0.01 in GCN and GC samples, and 38 markers displaying a minimal PMR value >4 only in GCN and GC samples. From the remaining 100 methylation markers, 27 informative markers were selected by the following criteria: first, we determined the mean PMR values of each marker for both normal (GCN) and tumor (GC) samples. We next determined the ratio of mean tumor and normal PMR values using the following formula: [Mean PMR_{tumor}/(Mean $PMR_{normal} + 1$)]. Second, we determined the number of cases in which the PMR value of each methylation marker was greater than 0 for the GC samples and paired GCN samples. GC/GCN frequencies were determined using the formula [$Number_{tumor}$/($Number_{normal} + 1$)]. Finally, we

determined the product of the PMR values and frequencies $[(\text{mean PMR}_{tumor}/(\text{Mean PMR}_{normal}+1))\times(\text{Number}_{tumor}/(\text{Number}_{normal}+1))]$ among the GC and GCN samples and ranked these in descending order. The best 27 markers were selected and examined on a tester set of 25 paired GCN and GC samples and 27 CG samples from non-cancer patients. The oligonucleotide sequences of the primers and probes of the 27 methylation markers are listed in Supplementary Table 1.

MethyLight Analysis of 27 CpG Islands Loci in a Tester Set of Gastric Carcinomas, Paired Gastric Carcinoma-Associated Non-Neoplastic Mucosae, and Chronic Gastritis from Non-Cancer Patients

We performed MethyLight analysis of 27 CpG island loci on GC and paired GCN, as well as CG from non-cancer patients, and we calculated PMR values for each sample and MethyLight reaction (Figure 1). We also calculated DNA methylation frequencies (PMR > 4) for each sample. These values are summarized in Table 1. The methylation frequencies of 27 CpG island loci ranged from 24 to 96% in a tester set of GC samples. High methylation frequencies (> 50% of GC samples) were evident for *ITGA4*, *SFRP4*, *TWIST1*, *SEZ6L*, *BCL2*, *TERT*, *KL*, *RBP1*, *SCGB3A1*, *RUNX3*, *GRIN2B*, *SFRP5*, *IGF2*, and *DLEC1* (in descending order of methylation frequency). *CACNA1G*, *CHFR*, *HOXA1*, *CYP1B1*, *BDNF*, *CDKN2A*, *SMAD9*, *RARRES1*, *CDKN1C*, and *MT1G* (in order of decreasing methylation frequency) showed an intermediate frequency of methylation (30–50%), while *NR3C1*, *TP73*, and *HOXA10* were methylated between 20 and 30% of GC samples.

When the PMR values of each CpG island locus were compared between GC and paired GCN samples, all loci except *RARRES1*, *CDKN1C*, *MT1G*, *NR3C1*, and *HOXA10* were methylated in GC at levels significantly higher than those of the respective ones in GCN samples ($P<0.05$, paired *t*-test). Although the differences of methylation levels were not significant, *RARRES1*, *CDKN1C*, *NR3C1*, and *HOXA10* were methylated in GC at levels three times higher than those of GCN.

RNA Expression Levels of 17 CpG Island Loci in Gastric Cancer Cell Lines

To evaluate whether the DNA hypermethylation events are inversely correlative with gene expression, we treated eight gastric cancer cell lines with 5-Aza-CdR and or Trichostatin A. After the drug treatment(s), we harvested total RNA and evaluated the expression of 17 gene mRNAs by RT-PCR. The primers are described in Supplementary Table 2. We also obtained MethyLight-based DNA methylation measurements of the CpG islands associated with each gene (Figure 2a). In general, there was an inverse correlation between MethyLight PMR value and the gene expression levels and many of the genes had upregulated expression after 5-Aza-CdR treatment (Figure 2a–c). These findings suggest that the DNA hypermethylation seen in the clinical GC cases may have a functional and biological relevance. However, there were some loci that did not show an increase in gene expression after drug treatments.

Comparison of Methylation Levels or Frequencies between *H. pylori*-Positive and -Negative Non-Neoplastic Gastric Mucosae

We determined the status of HP infection using microscopic examination of Giemsa-stained histologic slides, PCR assays to amplify HP 16S rDNA (Supplementary Figure 1), and CLO test. The cases were regarded as HP-positive when they showed positive findings in any of the above three tests. Of 27 CG samples from non-cancer subjects, 13 samples were HP-negative and 14 samples were HP-positive. The methylation levels of 10 CpG island loci, including *SEZ6L*, *CDKN1C*, *TWIST1*, *CDKN2A*, *RBP1*, *SFRP4*, *MT1G*, *SFRP5*, *RARRES1*, and *SMAD9*, were significantly higher in HP-positive CG than those in HP-negative CG (Figure 3a). When the number of CpG island loci methylated was compared between HP-positive and HP-negative CGs, HP-positive CG exhibited significantly higher number of CpG island loci methylated than HP-negative CG (12.1 *vs* 5.7, $P=0.001$, Student's *t*-test) (Figure 3b).

In addition, we found that 22 of 25 (88%) GCN samples from GC patients were found to be HP-positive, and the remaining three cases were HP-negative. The differences in the methylation levels of the examined CpG island loci between HP-positive and HP-negative GCN from GC patients displayed similar trend seen in the above comparison, although the limitation of the case number of HP-negative GCN cases did not give credit upon this comparison.

The Distribution of CpG Island Hypermethylation in Normal Gastric Mucosae from Non-Cancer Patients, Normal Gastric Mucosae Associated with Gastric Carcinomas, and Gastric Carcinomas

The number of CpG island loci methylated varied from 0 to 17 in CG samples from non-cancer patients (median: 9) and from 5 to 17 in GCN samples (median: 12) and from 2 to 27 loci in GC samples (median: 14). When the MI was defined as the number of methylated loci/the number of tested loci, the average MI was 0.33, 0.43, and 0.54 for CG, GCN, and GC, respectively. The differences in MI values between CG and GCN and between GCN and GC were near statistical significance ($P=0.049$, two-tailed *t*-test and $P=0.077$, two-tailed paired sample test, respectively). However, after taking into consideration the fact that HP prevalence was higher in GCN than in CG, we performed comparison of MI between HP-positive CG and HP-positive GCN, which revealed no difference of MI between them (MI, 0.45 *vs* 0.44).

The Relationship between CpG Island Hypermethylation and Histologic Type of Gastric Carcinoma, Epstein–Barr Virus, or Microsatellite Instability

According to the Lauren's classification,[19] there are two major histologic types of GC, intestinal and diffuse types. Of the GC

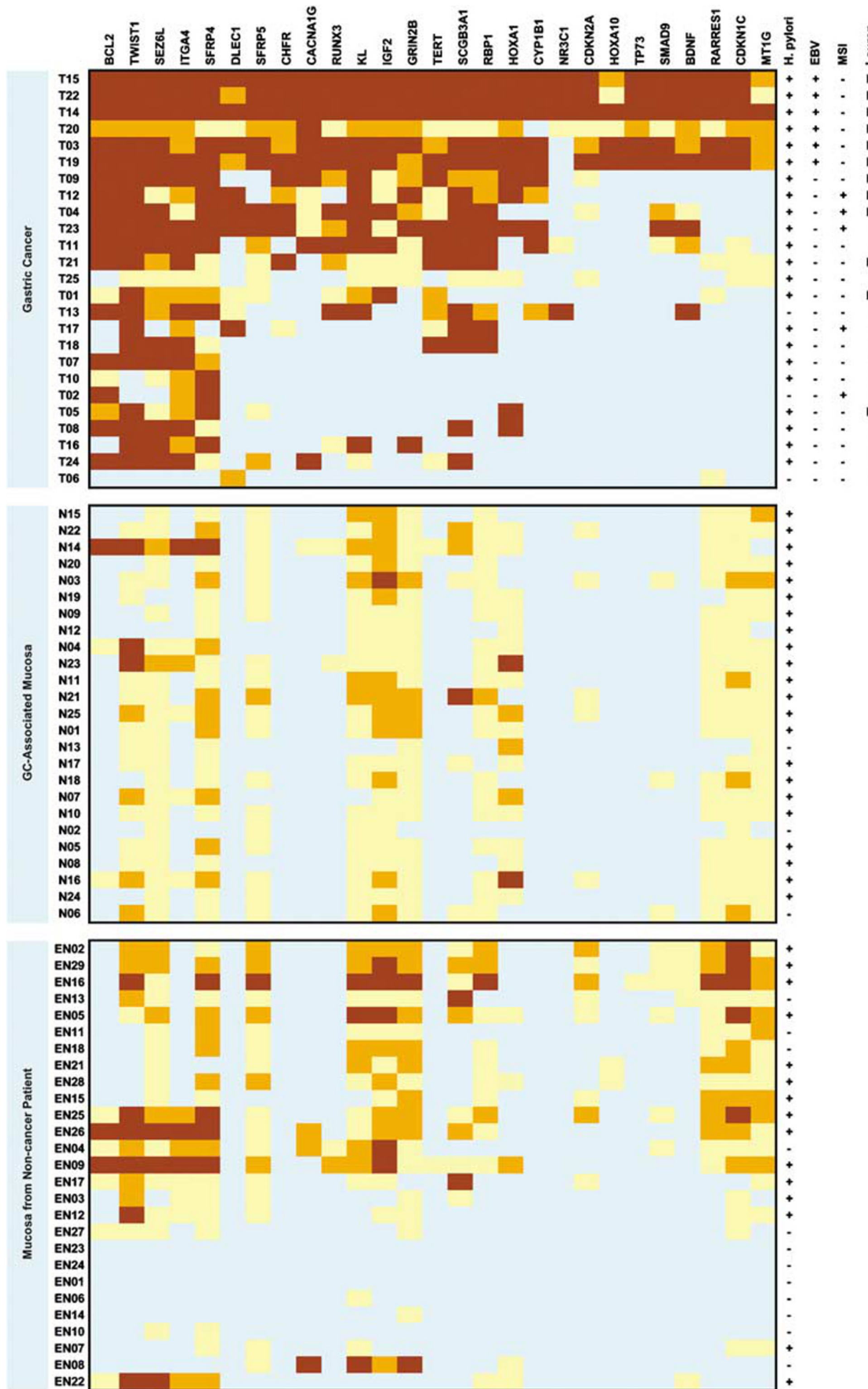

Figure 1 Methylation map of the PMR values obtained for each CpG island locus in gastric carcinoma (GC), GC-associated normal gastric mucosa, and normal gastric mucosa from non-cancer patients. Colored boxes represent four classes of methylation levels (0 < PMR < 4 (light blue), 4 ≤ PMR < 20 (light yellow), 20 ≤ PMR < 50 (light orange), PMR ≥ 50 (brown)). EBV: +, EBV-positive GC; MSI: +, MSI-positive GC. Lauren's classification of histologic type: D, diffuse type; I, intestinal type.

tester samples, 13 were of intestinal type and 12 were classified as diffuse type. Diffuse-type GCs showed higher MI than that of intestinal type (0.33 and 0.76 for intestinal type and diffuse type, respectively; $P < 0.001$, two-tailed Student's t-test).

A close association has been known between CpG island hypermethylation and EBV positivity or MSI positivity in GCs.[20–23] To confirm the association, EBER *in situ* hybridization was performed for tissue array of 25 cases of

Table 1 Methylation frequencies and PMR values of 27 analyzed genes in GC, GC-associated normal gastric mucosa, and normal gastric mucosa from non-cancer patients

	GC		GC-associated gastric mucosa		Normal gastric mucosa from non-cancer patients	
	Frequency (%)	PMR	Frequency (%)	PMR	Frequency (%)	PMR
ITGA4	96	80.2	24	6.3	29.6	10.7
SFRP4	92	78.6	96	19.3	74.1	26.0
SEZ6L	88	78.2	92	12.0	70.4	17.1
TWIST1	88	214.7	72	16.4	51.9	39.1
BCL2	80	97.3	12	4.5	25.9	10.7
KL	68	105.3	92	13.2	63.0	22.2
TERT	68	44.4	4	0.3	3.7	0.6
RBP1	64	58.1	80	8.7	44.4	8.8
SCGB3A1	64	152.6	24	9.0	37.0	11.9
IGF2	56	56.1	96	21.0	63.0	20.7
GRIN2B	56	52.0	96	13.5	70.4	28.0
SFRP5	56	29.8	76	9.1	66.7	13.2
RUNX3	56	43.9	8	0.9	7.4	1.3
DLEC1	52	28.4	0	0.2	0.0	0.2
HOXA1	48	76.3	72	14.7	18.5	2.2
CHFR	48	59.7	0	0.1	0.0	0.2
CACNA1G	48	76.7	4	0.5	11.1	6.2
CYP1B1	40	68.1	0	0.1	0.0	0.0
BDNF	40	31.5	0	0.5	18.5	1.8
RARRES1	36	25.2	84	7.6	51.9	14.3
CDKN1C	36	43.7	96	14.5	66.7	24.3
CDKN2A	36	18.1	20	1.9	29.6	6.0
SMAD9	36	33.3	12	1.4	22.2	2.4
MT1G	32	13.1	88	9.4	63.0	13.0
NR3C1	24	22.2	0	0.0	0.0	0.0
TP73	24	33.8	0	0.1	3.7	0.3
HOXA10	24	10.4	0	1.5	7.4	1.2

GC samples (Supplementary Figure 2) and MSI of BAT25, BAT26, D2S123, D5S346, and D17S250 was assessed for 25 cases of GC. GCs with MSI in two or more loci were regarded as MSI-positive. Six cases were positive for EBV and five cases were positive for MSI. However, there was no case that was positive for both EBV and MSI. The average MI values were 0.98, 0.48, and 0.37 for EBV-positive GCs, MSI-positive GCs, and GCs negative for EBV and MSI, respectively. The MI difference between EBV-positive GCs and EBV-negative GCs was statistically significant ($P<0.001$, two-tailed Student's *t*-test); however, we did not find a statistically significant difference between MSI-positive GCs and GCs negative for EBV and MSI ($P>0.05$, two-tailed Student's *t*-test).

DISCUSSION

Methylation in GC was revealed for the first time in this study for 17 of the 27 markers, including *SFRP4*, *SEZ6L*, *TWIST1*, *BCL2*, *KL*, *TERT*, *SCGB3A1*, *IGF2*, *GRIN2B*, *SFRP5*, *DLEC1*, *HOXA1*, *CYP1B1*, *SMAD9*, *MT1G*, *NR3C1*, and *HOXA10*. RNA expression levels of the 17 genes were analyzed in eight GC cell lines and correlated with the methylation status of the respective genes, suggesting that the DNA hypermethylation events in human GC are biologically important for gene function. A connection between DNA methylation and gene expression has been frequently demonstrated in the literature, however, not all CpG island hypermethylation show this correlation, as seen here as well. Many genes are not expressed in normal or tumor epithelium and their methylation in cancer is simply a marking of cancer, and has nothing to do with a functional or biological role. The co-authors of the present study recently published a report describing that there is a strong agreement between colorectal cancer-specific CpG island DNA methylation and their polycomb (PcG) occupancy in human embryonic stem cells.[24] Gene methylation of several PcG-target genes, including *NEUROG1*, *NEUROD1*, and *MYOD1*, which are not expressed in the normal colonic epithelium, occurs frequently in colorectal cancer. The expression of muscle differentiation (*MYOD1*) and neuronal development genes (*NEUROG1* and *NEUROD1*) is not needed in normal colonic mucosa. Similar genes exist in gastric and other human cancers.

These newly developed methylation markers showed methylation frequencies $\geq$24% in GC. The methylation frequencies of *BCL2*, *TERT*, *DLEC1*, *CYP1B1*, and *NR3C1* in GC were three times higher than those of the respective gene in GCN or CG from non-cancer patients. The methylation frequency of *HOXA10* in GC was two times higher than that in GCN or CG. The methylation frequencies of *SFRP4*, *SEZ6L*, *TWIST1*, *KL*, *IGF2*, *GRIN2B*, *SFRP5*, *HOXA1*, and *SMAD9* were not different between GC and GCN or CG, although the methylation level of each gene was at least twice higher in GC than that of the respective gene in CG or GCN.

The number of methylated CpG island loci was higher in HP-positive CG than in HP-negative CG. This result is consistent with those of previous studies that have demonstrated the close association of HP infection with aberrant CpG island hypermethylation.[25–27] We found that HP infection was a confounding factor in the comparison of methylation between CG and GCN and the analysis restricted to HP-positive cases did not show any difference in the number of methylated genes between CG and GCN. This was in contrast to the previous study of Waki *et al*,[28] who demonstrated higher number of methylated CpG island loci in GCN than in CG using MSP. However, they did not analyze the association between CpG island hypermethylation and HP infection. Recent studies exhibited a marked difference of methylation levels between CG and GCN in HP-negative cases but no difference between them in HP-positive cases.[27,29]

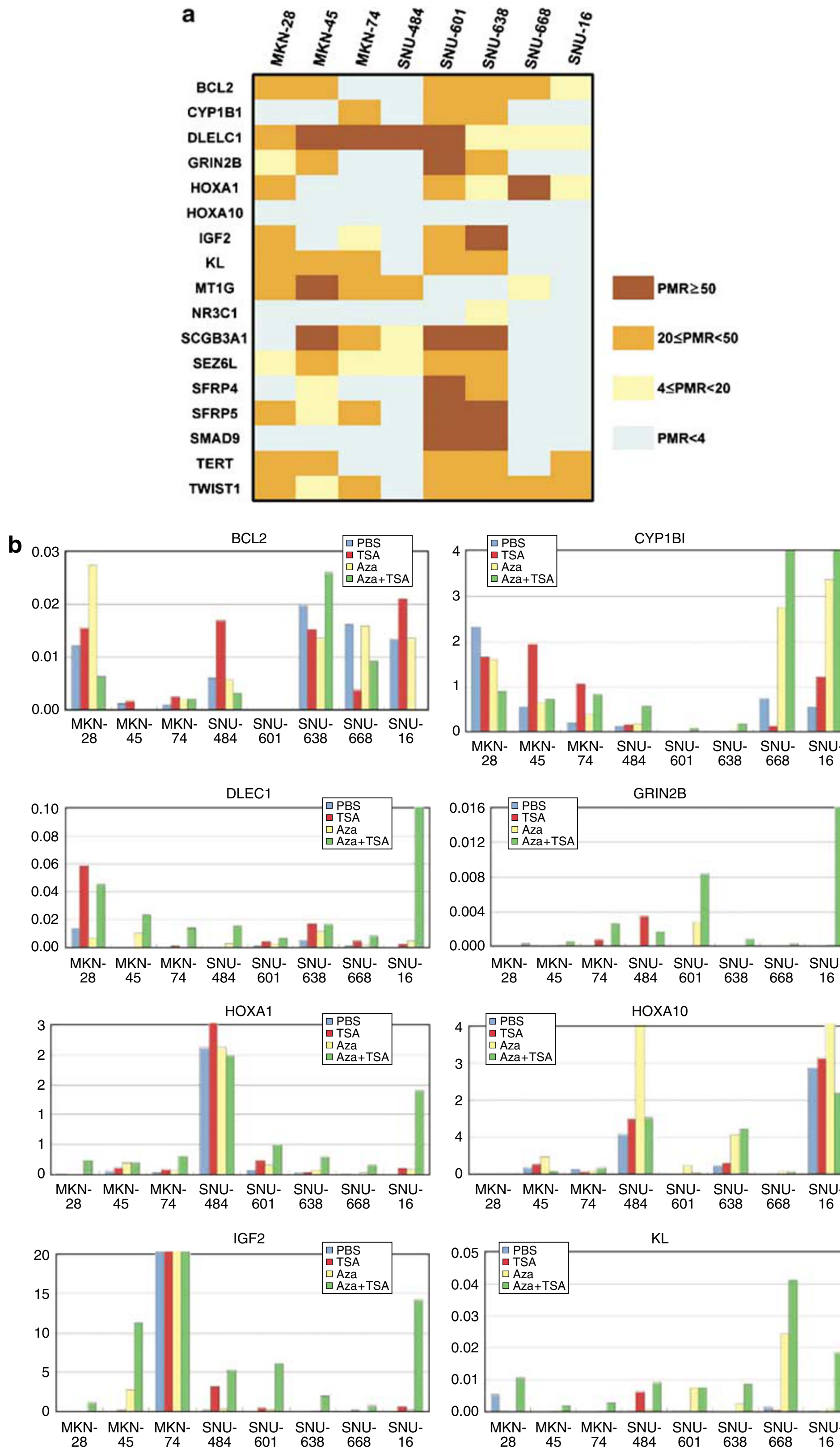

Figure 2 MethyLight analysis results of 17 genes in eight gastric cancer cell lines (**a**). Relative expression levels of 17 genes in eight gastric cancer cell lines (**b** and **c**). Cells were treated with phosphate-buffered saline (PBS), 5-aza-2′-deoxycytidine (Aza), Trichostatin A (TSA), or combination of AZA plus TSA. The expression levels are displayed as the ratios between the individual genes and the endogenous control GAPDH and multiplied by a factor of 100. General inverse correlation between gene expression and CpG island hypermethylation was found in the vast majority of genes except for *BCL2*, *IGF2*, and *TERT*.

The CpG island locus of *CACNA1G* corresponds to MINT31, which was used as one of the reference CpG island loci to determine CpG island methylator phenotype of GCs or colorectal carcinomas.[20,30,31] According to Toyota *et al*'s[30] study using COBRA, MINT31 was never methylated in GC-associated normal stomach samples. However, in our

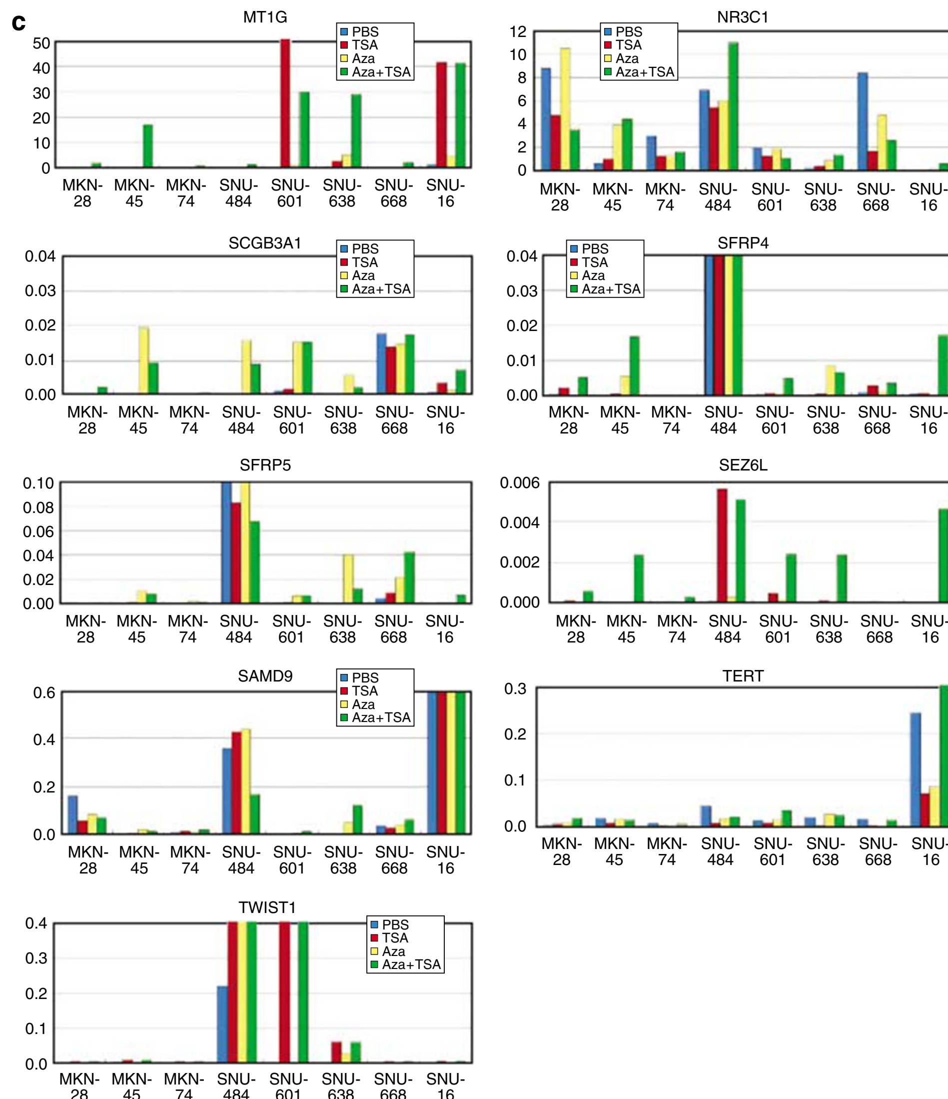

Figure 2 Continued.

study using MethyLight assay, *CACNA1G* was methylated in 3 of 27 CG samples from non-cancer patients and the mean PMR value of these 3 cases was 55. Considering the high PMR values of the three cases, it is likely that the same cases would be positive in the methylation analysis using COBRA for MINT31. Thus, the classification of MINT31 as a type C gene may need to be re-evaluated for gastric samples.

In previous studies, EBV-positive GCs and MSI-positive GCs have been demonstrated to contain a higher frequency of aberrant CpG island hypermethylation than GCs negative for both EBV and MSI.[21–23,30,32,33] However, these previous studies used a small number of CpG island loci by MSP. In our study analyzing 27 CpG island loci by MethyLight, which overcomes some variability problems of MSP, the average MI of EBV-positive GCs and MSI-positive GCs was 0.98 and 0.48, respectively, whereas that of GCs negative for both was 0.37. Although a strong association was confirmed between CpG island hypermethylation and EBV, the association between CpG island hypermethylation and MSI was not found. This might be related to the small number of GCs studied in the present study. *TP73* hypermethylation has been previously reported to be overrepresented in EBV-positive GCs (92.3%, 12 of 13 EBV-positive GCs) but under-represented in EBV-negative GCs (5.3%, 2 of 38 EBV-negative GCs).[34] In the present study, *TP73* hypermethylation was found in all the EBV-positive GCs but not found in EBV-negative GCs. Additionally, *HOXA10* hypermethylation was also found in all the EBV-positive GCs but not found in EBV-negative GCs. These two DNA methylation markers are expected to serve as useful biomarkers to detect a distinct type of GC (EBV-positive GC).

GCs can be classified into two major histologic types: 'intestinal' and 'diffuse'. Intestinal-type GCs are associated

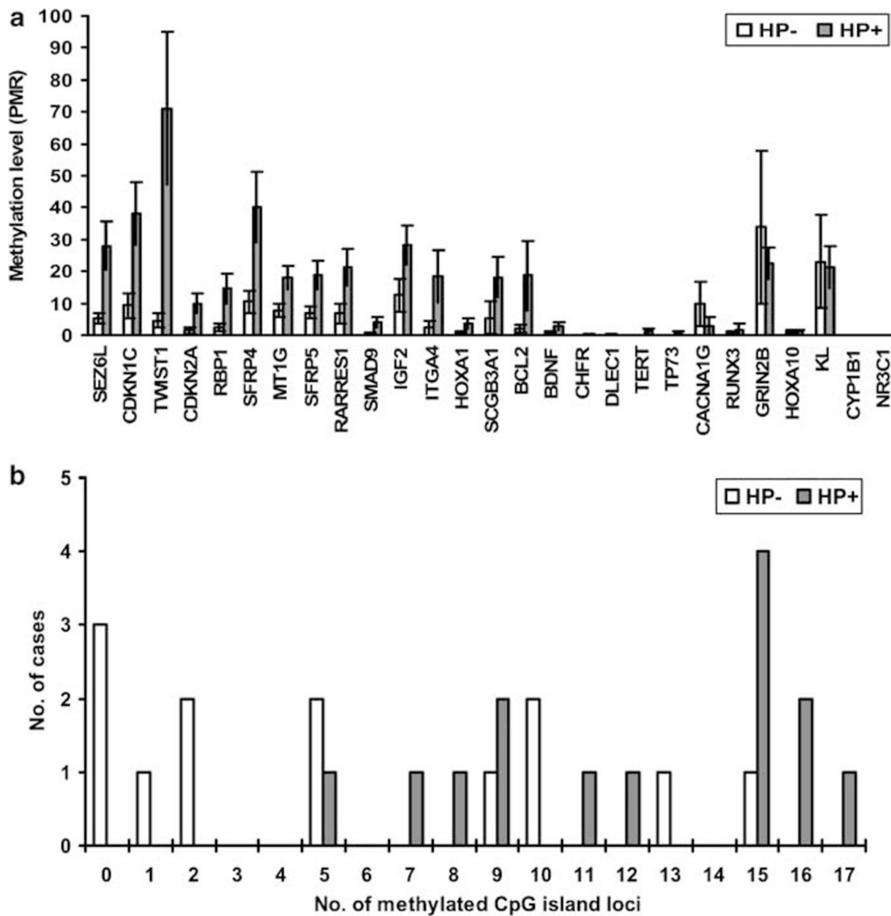

Figure 3 (**a**) The methylation levels of each of 27 CpG island loci were compared between HP-positive and HP-negative CG from non-cancer patients. Nine CpG island loci (from SEZ6L to SMD9) were methylated in HP-positive CG at significantly higher methylation levels compared with those of HP-negative CG. (**b**) The frequency of coincident number of gene hypermethylation was compared between HP-positive and HP-negative CG from non-cancer patients.

with intestinal metaplasia, often preceded by sequential steps of precancerous changes, including atrophic gastritis, intestinal metaplasia, and dysplasia, whereas diffuse-type GCs arise *de novo* and originate from gastric mucosa proper. The pathogenesis and molecular alterations leading to these two types of GC are generally considered to differ.[35–37] The difference in CpG island hypermethylation between intestinal and diffuse types has not been well characterized. Using different DNA methylation markers and different methodology, CpG island methylator phenotype was found to be associated with 'diffuse' histology in some reports[33,38] but not in others.[39,40] These controversial findings are most likely due to the use of differing DNA methylation marker panels as well as methods of analysis. In the present study, we analyzed 27 DNA methylation markers using the MethyLight technology and diffuse-type GC showed a significantly higher frequency of CpG island hypermethylation than intestinal-type GC, although the number of cases was limited. Considering that all the EBV-positive GCs were of diffuse type and comprised 50% of diffuse-type GCs of the present study, the relationship between CpG island hypermethylation and diffuse type might depend on the proportion of EBV-positive GCs among the diffuse-type GCs. When EBV-positive GCs were excluded from the diffuse-type GCs, still diffuse type showed higher MI than that of intestinal type (0.54 *vs* 0.33, $P = 0.054$, two-tailed Student's *t*-test).

In conclusion, we have generated DNA methylation profiles of 27 CGs from non-cancer patients and 25 paired GCs and GCNs and developed 17 new methylation markers for GC. We found that HP infection was strongly associated with aberrant CpG island hypermethylation in CG and the number of methylated CpG island loci was significantly higher in GC than in its associated GCN. CpG island hypermethylation was more prevalent in diffuse-type GC than in intestinal-type GC and EBV-positive GC showed methylation in more than 95% of the loci interrogated.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/DUALITY OF INTEREST

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