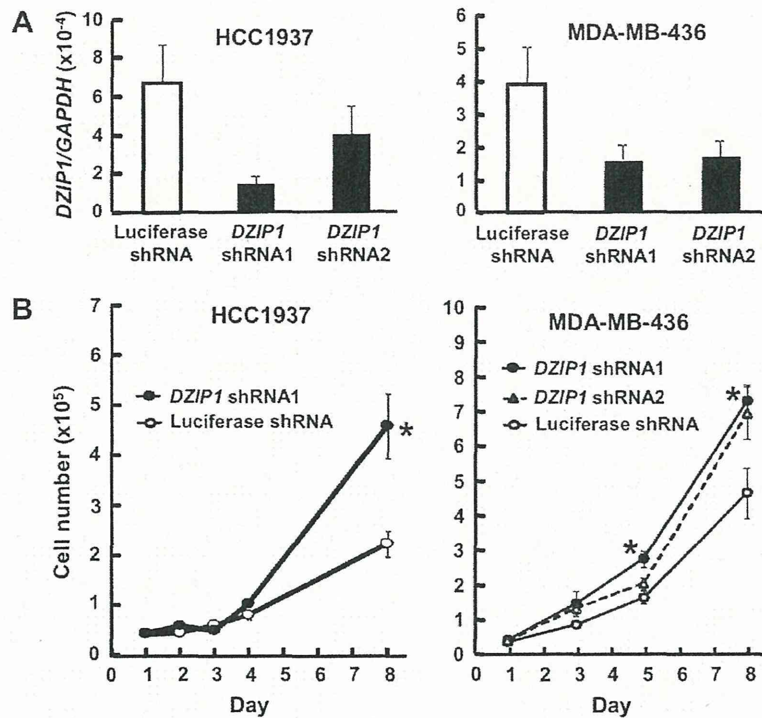

Fig. 5. Suppression of cell growth by *DZIP1*. (A) Knockdown of *DZIP1* in HCC1937 and MDA-MB-436 cells. The expression level of *DZIP1* mRNA in HCC1937 cells was reduced to about 20% and 60% of that of HCC1937 cells with control shRNA by shRNA1 and shRNA2, respectively. The expression level in MDA-MB-436 cells was reduced to about 40% of that in MDA-MB-436 cells with control shRNA by both shRNA1 and shRNA2. The mean ± SE values of three independent experiments of *DZIP1* expression levels are shown. (B) Increased growth of the HCC1937 and MDA-MB-436 cells by *DZIP1* knockdown. Cell numbers were counted at time points designated in the panels, and the mean ± SE values of three independent experiments of cell number are shown. Differences of cell growth were tested by the Student's t test. $^*p < 0.05$.

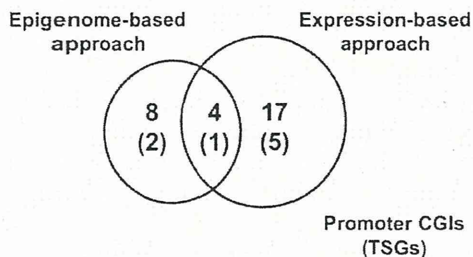

Fig. 6. The overlap of promoter CGIs identified by the epigenome-based outlier approach and those by the expression-based outlier approach. Among the 12 promoter CGIs with unique downstream genes identified by the epigenome-based approach, four promoter CGIs overlapped with those identified by the expression-based approach. *DZIP1* could be identified only by the epigenome-based approach.

pathway [42,52]. Therefore, there is a possibility that methylation-silencing of *DZIP1* induces abnormal expression of the downstream genes of the Hh signaling pathway during human carcinogenesis. Further investigations into *DZIP1* functions are necessary. *DZIP1* was also repressed in breast cancer cell lines such as BT-474 and SK-BR-3 that had unmethylated promoter CGIs of *DZIP1*. As possible causes of this repression, involvement of repressive histone modifications and defects in signaling pathways that regulate *DZIP1* expression were considered.

Among the seven known TSGs initially analyzed, *BRCA1*, *HOXA5*, and *MASPIN* were outliers in breast cancer, and *MLH1* and *RASSF1A* were in colon cancer. In contrast, *CDKN2A*, *RASSF1A*, *RBP1* in breast cancer and *CDKN2A* in colon cancer were not outliers. Especially, *RASSF1A* was an outlier in colon cancer, but not in breast cancer. This difference might explain the different incidence of aberrant DNA methylation of *RASSF1A* between breast cancers that show 50–60% incidence [44,53] and colon cancers that show 20–45% incidence [54,55].

Among the TSGs confirmed as outliers in the initial analysis, *HOXA5* was identified by the following genome-wide screening, but *BRCA1* and *MASPIN* were not. This was because the microarray used in this study did not have probes in the NFRs of *BRCA1* and *MASPIN*, and these genes cannot be identified as frequently methylated genes using the microarray used here.

Cancer cell lines were used to obtain DNA methylation-susceptible genes in this study. However, some TSGs, such as *BRCA1*, are reported to be frequently methylated in primary breast cancer tissues [56–58], but infrequently in cancer cell lines [59]. By use of primary cancer samples, identification of such types of TSGs will be facilitated.

In summary, we showed that a significant fraction of TSGs are outliers to the general rule of genes methylated in cancer cells, and that a different set of TSGs could be identified by the epigenome-based outlier approach compared to the expression-based outlier approach.

Acknowledgment

M.K. is a recipient of Research Resident Fellowships from the Foundation for Promotion of Cancer Research. This work was supported by Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan; for Young Scientists (B) from Japan Society for the Promotion of Science (JSPS); and a grant from the Foundation for Promotion of Cancer Research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.03.016.

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Gastric Cancer (2012) 15:382–388
DOI 10.1007/s10120-011-0126-1

ORIGINAL ARTICLE

Identification of gastric cancer risk markers that are informative in individuals with past *H. pylori* infection

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Received: 25 August 2011 / Accepted: 26 November 2011 / Published online: 12 January 2012
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Abstract

Background Epigenomic damage induced by *Helicobacter pylori* infection is accumulated in gastric mucosae before the development of malignancy. In individuals without current *H. pylori* infection, DNA methylation levels of specific CpG islands (CGIs) are associated with gastric cancer risk. Because risk estimation in individuals with past infection is clinically important, we here aimed to identify the risk markers that reflect epigenomic damage induced by *H. pylori* infection, and that are informative in these individuals.

Methods Gastric mucosae were obtained from 55 gastric cancer patients (GC-Pt) (21 with current infection and 34 with past infection) and 55 healthy volunteers (HV) (7 never-infected, 21 with current infection, and 27 with past infection). Hypermethylated CGIs were searched for by methylated DNA immunoprecipitation-CGI microarray, and methylation levels were analyzed by quantitative methylation-specific polymerase chain reaction (PCR).

Results By microarray analysis of a pool of three samples from GC-Pt with past infection and another pool of samples from HV with past infection, 15 hypermethylated CGIs in the former pool were isolated. Seven of them had significantly higher methylation levels in GC-Pt with past infection ($n = 10$) than in HV with past infection ($n = 10$) ($P < 0.001$). In a validation cohort (21 GC-Pt with past infection and 14 HV with past infection), the seven new markers had large areas under the receiver-operating characteristic curves (0.78–0.84) and high odds ratios (12.7–36.0) compared with two currently available markers (0.60–0.65, 5.0–5.7).

Conclusions We identified seven novel gastric cancer risk markers that are highly informative in individuals with past infection.

Keywords Carcinogenesis · DNA methylation · Gastric cancer · *Helicobacter pylori*

Electronic supplementary material The online version of this article (doi:10.1007/s10120-011-0126-1) contains supplementary material, which is available to authorized users.

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Introduction

Early detection of cancer is critically important to reduce its morbidity and mortality, and early detection can be achieved by identifying individuals at high risk of developing cancers. In the risk estimation of gastric cancers, a history of *Helicobacter pylori* infection, which increases gastric cancer risk 2.2- to 21-fold [1–4], plays the major role, but the vast majority of individuals with a history of *H. pylori* infection do not develop gastric cancers. Also, gene polymorphisms associated with gastric cancers have been identified, and they have been shown to confer odds ratios (ORs) mostly between 1.0 and 2.0 [5, 6]. To obtain

clinically useful risk markers, we have to develop markers that are informative even in individuals with a history of *H. pylori* infection and that confer higher ORs.

Recently, we showed that *H. pylori* infection induces epigenomic damage, especially aberrant DNA methylation, in gastric mucosae [7]. DNA methylation levels of specific CpG islands (CGIs) were very high in the gastric mucosae of individuals with active *H. pylori* infection irrespective of gastric cancer risk, and decreased to certain levels after *H. pylori* was eradicated [8]. Importantly, these methylation levels in individuals without active *H. pylori* infection were correlated with gastric cancer risk [7, 9]. It is considered that aberrant DNA methylation is induced both in gastric stem cells and in non-stem cells, that methylation induced in stem cells will remain even after *H. pylori* eradication, and that methylation levels in individuals without current *H. pylori* infection reflect gastric cancer risk (degree of the epigenetic field defect) [10].

The correlation between methylation levels and gastric cancer risk has been analyzed in individuals without current *H. pylori* infection [7, 9]. Based on the data in our previous study [7], currently available methylation risk markers, *FLNc* and *THBD*, have ORs of 4.2–7.0 to detect gastric cancer patients (GC-Pt) among such individuals. However, individuals without current *H. pylori* infection indeed consist of never-infected individuals and those with past infection, and risk estimation is important in individuals with past infection.

In this study, we aimed to identify gastric cancer risk markers that reflect epigenomic damage induced by *H. pylori* infection, and that are informative in individuals with past infection.

Materials and methods

Tissue samples and determination of *H. pylori* infection status

Fifty-five healthy volunteers (HV) with endoscopic findings of no malignancy were recruited, with written informed consents, on the occasion of a gastric cancer screening program, with the approval of the institutional review board. Fifty-five GC-Pt who had undergone curative endoscopic submucosal dissection (ESD) of a well-differentiated adenocarcinoma in the non-cardia according to the Japanese classification of gastric carcinoma [11] were also recruited, with written informed consents, with the approval of the Institutional Review Board. Gastric mucosae were collected by endoscopic biopsy of the antrum. The biopsy specimens were frozen in liquid nitrogen immediately after biopsy, and stored at −80°C until DNA extraction. High molecular weight DNA was extracted by the phenol/chloroform method.

Current *H. pylori* infection was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan) in HV and by urea breath test (Otsuka Pharmaceutical, Tokushima, Japan) in GC-Pt. Also, the presence of current or past *H. pylori* infection was detected by the endoscopic presence of atrophic gastritis in the antrum, because atrophic change induced by *H. pylori* infection arises in the antrum in 83% of individuals with *H. pylori* infection [12] and remains in all individuals who have had *H. pylori* eradication therapy [13]. "Never-infected individuals" were defined as those who were negative for *H. pylori* analysis and did not have atrophic gastritis in the antrum. "Individuals with current infection" were defined as those who were positive for *H. pylori* analysis. "Individuals with past infection" were defined as those who were negative for *H. pylori* analysis and had atrophic gastritis in the antrum.

Methylated DNA immunoprecipitation-CGI microarray analysis

Methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis was performed as previously described [14, 15]. Briefly, 5 μg of genomic DNA was immunoprecipitated with an anti-5-methylcytidine antibody (Diagnode, Liége, Belgium), and the precipitated DNA and the input DNA were labeled with cyanin (Cy) 5 and Cy3, respectively. A human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA, USA) was hybridized with the labeled probes and scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). Scanned data were processed with Feature Extraction Software Version 9.1 (Agilent Technology) and Agilent G4477AA ChIP Analytics 1.3 software. The signal of a probe was converted into a "Me value", which represented the methylation level as a value from 0 (unmethylated) to 1 (methylated). Differentially methylated regions were detected by comparison between the Me values of two samples, and data were visualized in the UCSC Genome Browser (http://genome.ucsc.edu/) on NCBI36/hg18 assembly (National Center for Biotechnology Information, Bethesda, MD, USA).

Sodium bisulfite modification and quantitative methylation-specific polymerase chain reaction

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *SssI* methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA, and the modified

DNA was suspended in 40 µl of Tris–ethylenediamine tetraacetic acid (EDTA) buffer [16]. An aliquot of 2 µl of sodium bisulfite-treated DNA was used in one reaction of quantitative methylation-specific polymerase chain reaction (PCR; qMSP).

qMSP was performed using primer sets specific to methylated and unmethylated sequences (Supplementary Table 1), SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME, USA), and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA that contained known numbers of molecules (10^1–10^9 molecules). Standard DNA was prepared by purifying the PCR products using the Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA). The methylation level was calculated as the fraction of methylated (M) molecules in the total number of DNA molecules (number of M molecules + number of unmethylated molecules). The percentage of methylated reference (PMR) was calculated as the fraction of the methylated reference {(number of M molecules in a sample)/(number of *Alu* repeat sequences in a sample)}/{(number of M molecules in *SssI*-treated DNA)/(number of *Alu* repeat sequences in *SssI*-treated DNA)} [17].

Statistical analysis

Differences in mean methylation levels or PMR were analyzed by the Student's *t*-test. The receiver-operating characteristic (ROC) curve was drawn, and the area under the curve (AUC) and OR were analyzed by binomial distribution and binomial logistic regression analysis, respectively. All the analysis was performed using PASW statistics (SPSS, Chicago, IL, USA), and the results were considered significant when *P* values of less than 0.05 were obtained by two-sided tests.

Results

Isolation of hypermethylated CGIs in GC-Pt compared with HV in individuals with past *H. pylori* infection

A pool of three samples from HV with past infection and another pool of three samples from GC-Pt with past infection were analyzed by MeDIP-CGI microarray analysis. CGIs that were hypermethylated in the latter group compared with the former group were selected as follows: (1) Me value in the latter pool was higher than that in the former pool by 0.2 or more, (2) Me value in the former pool was lower than 0.4, and (3) criteria (1) and (2) were satisfied in three consecutive probes. A total of 15 CGIs were isolated by these criteria (Table 1), and representative data around CGI #5 are shown in Fig. 1.

From the 15 CGIs, those differentially methylated in a screening set, which consisted of 10 HV with past infection and 10 GC-Pt with past infection, were searched for by evaluating PMRs by qMSP (Supplementary Table 2). Seven CGIs (#1 to #7; Table 1), distributed on various chromosomes, were methylated at significantly higher

Table 1 CGIs identified by MeDIP-CGI microarray

CGI no.	Gene symbol	Name	Chromosomal position	Location around a gene
#1	*EMX1*	Empty spiracles, homeobox 1	2p13.2	Intron 1
#2	*miR663*	MicroRNA 663	20p11.1	Overlap
#3	*NKX6-1*	NK6, homeobox 1	4q21.23	Intron 1
#4	*OTP*	Orthopedia homeobox	5q13.3	Downstream
#5	*OPLAH*	5-Oxoprolinase (ATP-hydrolysing)	8q24.3	Downstream
#6	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1	2p22.2	Exon 1
#7	*NEFM*	Neurofilament, medium polypeptide	8p21	Exon 1
#8	*PMF1*	Polyamine-modulated factor 1	1q22	Intron 1
#9	*BDNF*	Brain-derived neurotrophic factor	11p14.1	Intron 1
#10	*SSTR5*	Somatostatin receptor 5	16p13.3	Promoter
#11	*MYO1D*	Myosin ID	17q11.2	Intron 1
#12	*CAMK2N2*	Calcium/calmodulin-dependent protein kinase II inhibitor 2	3q27.1	Promoter
#13	*GATA4*	GATA binding protein 4	8p23.1	Promoter
#14	*NFATC1*	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	18q23	Promoter
#15	*ANKRD9*	Ankyrin repeat domain 9	14q32.31	Exon 1

CGI CpG island, *MeDIP* methylated DNA immunoprecipitation

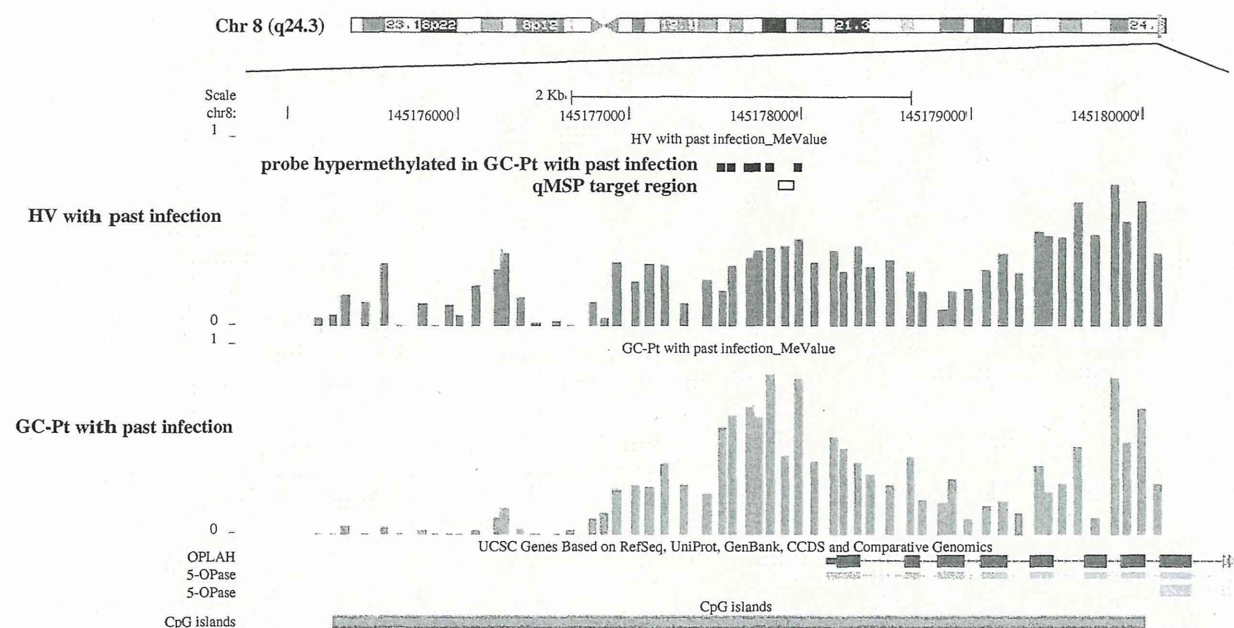

Fig. 1 Data of methylated DNA immunoprecipitation-CpG island (MeDIP-CGI) microarray analysis in the genomic region around CGI #5. Methylation levels were assessed by Me values, and the Me values of the two pools were visualized by the UCSC Genome Browser (http://genome.ucsc.edu/) for a genomic region (from nt. 145,174,733 to nt. 145,180,586 on chromosome 8 in NCBI36/hg18 assembly). *Vertical bars* show Me values of individual probes. *Closed boxes* above the Me values indicate the differentially methylated probes. Quantitative methylation-specific polymerase chain reaction (*qMSP*) primers were designed in the area shown by the *open box*. *HV* healthy volunteers, *GC-Pt* gastric cancer patients

levels in GC-Pt than in HV ($P < 0.05$). Relative positions against a gene also varied—two CGIs being located in exon 1, two in intron 1, two 300 bp downstream of the annotated end, and one overlapping with *pre-microRNA 663*.

Validation of the usefulness of the seven markers

The usefulness of the seven CGIs was validated by qMSP analysis of an independent set of samples (Fig. 2). The validation set consisted of seven never-infected HV (Group [G] 1), 21 HV with current infection (G2), 14 HV with past infection (G3), 21 GC-Pt with current infection (G4), and 21 GC-Pt with past infection (G5) (Supplementary Table 3). For comparison, two currently available markers (*FLNc* and *THBD*) were also analyzed. In the individuals with past infection (G3 and G5), the seven CGIs had levels that were 2.8-, 1.5-, 3.8-, 2.3-, 2.5-, 1.8-, and 3.8-fold, respectively, higher in G5 than in G3 ($P < 0.01$). *FLNc* tended to have a higher level in G5 than in G3 ($P = 0.087$), but *THBD* did not show any significant difference ($P = 0.341$). These data showed that the methylation levels of all the seven CGIs had the power of cancer risk estimation even in individuals with past infection.

In the HV, methylation levels in G2 were much higher than those in G1 ($P < 0.05$), but those in G3 were lower than those in G2. This observation supported the model that active infection by *H. pylori* induces methylation potently in non-stem cells, in addition to stem cells, and that methylation levels will eventually decrease after *H. pylori* infection has been eradicated. Also, methylation levels in G3 were significantly higher (four of the seven CGIs, $P < 0.05$) or tended to be higher than those in G1. This observation again supported the model that methylation induced in stem cells will remain even after *H. pylori* infection is eradicated.

Power of the seven CGIs as gastric cancer risk markers

AUCs to detect individuals in G5 were calculated using individuals in G3 and G5 (Table 2; Fig. 3). AUCs for the seven CGIs ranged between 0.78 and 0.84 and were significantly larger than 0.5 ($P < 0.01$). In contrast, the AUCs for the two currently available markers were 0.69 (95% CI 0.51–0.87) and 0.65 (95% CI 0.45–0.84), respectively, and were not significantly different from 0.5. Using optimal cut-off values obtained by the ROCs, ORs for the seven CGIs were calculated to be 12.7–36.0 (Table 2). ORs for

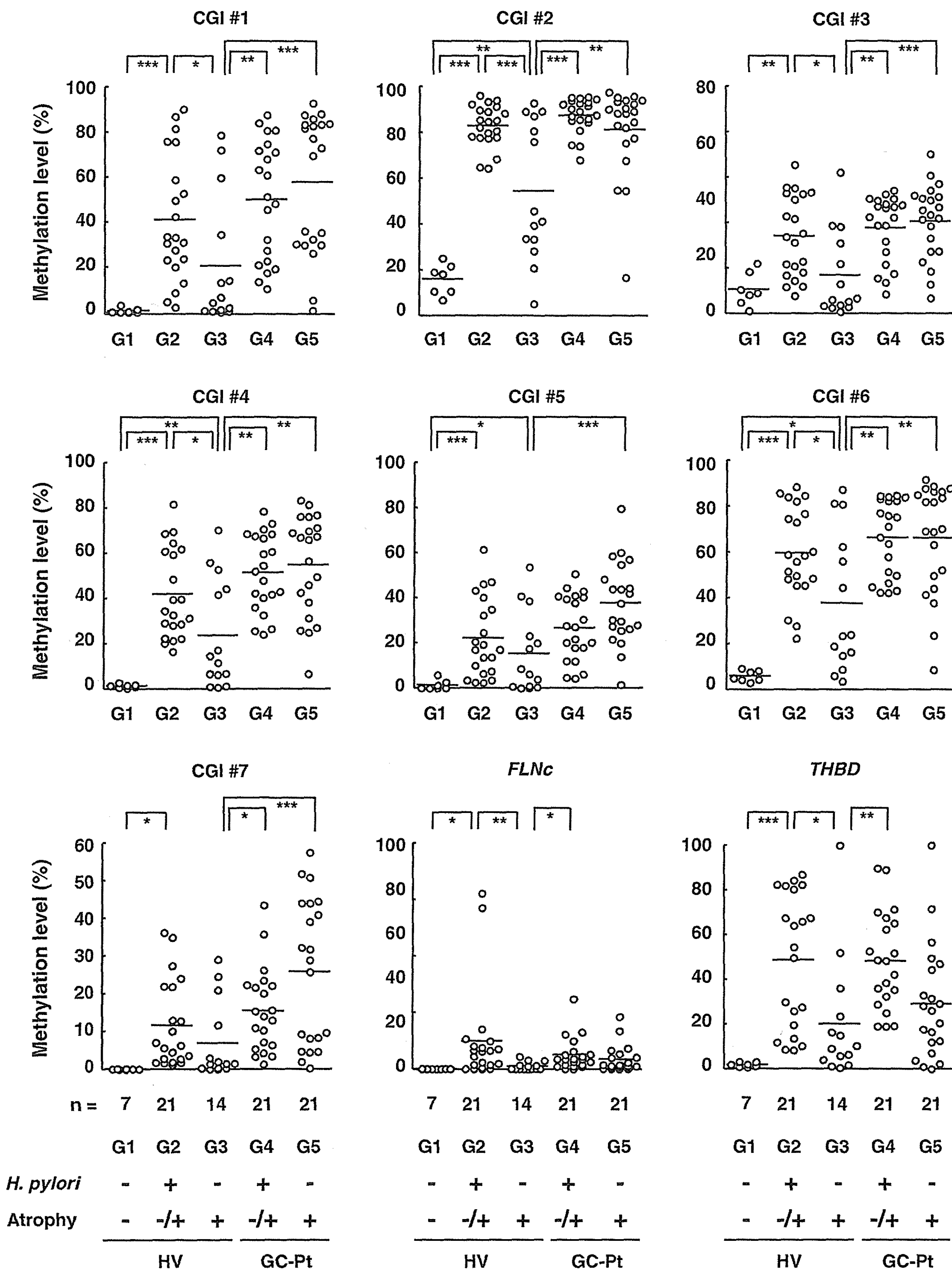

Fig. 2 Methylation levels of the seven CGIs and two currently available markers, *FLNc* and *THBD*, in the validation set. The *horizontal line* represents the mean methylation level in each group. Methylation levels of the seven CGIs in Group 5 (G5) were significantly higher than those in G3 ($P < 0.01$), but there were no significant differences for the two currently available markers. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Table 2 AUC and OR for new and currently available markers

CGI no.	Gene symbol	AUC	95% CI	*P* value	OR	95% CI	*P* value
#1	*EMX1*	0.84	0.70–0.97	<0.001	23.8	3.7–153	<0.001
#2	*miR663*	0.78	0.62–0.94	0.006	26.7	2.8–258	0.005
#3	*NKX6-1*	0.84	0.69–0.99	<0.001	15.0	2.8–80.1	0.002
#4	*OTP*	0.83	0.69–0.97	0.001	36.0	3.7–354	0.002
#5	*OPLAH*	0.83	0.69–0.98	0.001	15.6	2.9–83.5	0.001
#6	*CYP1B1*	0.78	0.62–0.94	0.006	12.7	2.1–76.7	0.006
#7	*NEFM*	0.84	0.71–0.98	<0.001	23.8	3.7–153	<0.001
–	*FLNc*	0.69	0.51–0.87	0.055	5.7	1.2–25.9	0.025
–	*THBD*	0.65	0.45–0.84	0.152	5.0	1.1–21.8	0.032

CGI CpG island, *AUC* area under the curve, *CI* confidence interval, *OR* odds ratio

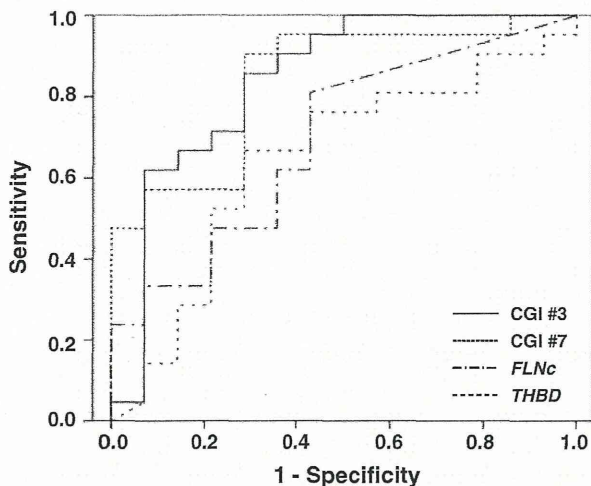

Fig. 3 Receiver-operating characteristic (ROC) curves of CGI #3 and #7, whose AUC values were the largest in the seven CGIs, are shown with those of two currently available markers, *FLNc* and *THBD*. *Black line*, *dotted line*, *dot-and-dash line*, and *dashed line* show ROC curves of CGI #3, #7, *FLNc*, and *THBD*, respectively. The AUC values of CGI #3 and #7 were larger than those of *FLNc* and *THBD*

the two currently available markers, *FLNc* and *THBD*, were 5.7 (95% CI 1.2–25.9) and 5.0 (95% CI 1.1–21.8), respectively. These results clearly showed that the methylation levels of the seven CGIs had greater power than the two currently available markers to estimate gastric cancer risk in individuals with past infection.

Discussion

In the present study, by carrying out genome-wide methylation analysis of gastric cancer patients (GC-Pt) and healthy volunteers (HV), both with past infection, we screened seven gastric cancer risk markers that are highly informative in individuals with past infection. Their usefulness was validated in 35 individuals (21 GC-Pt and 14 age-matched HV). To our knowledge, this is the first study that has evaluated epigenetic gastric cancer risk markers in individuals with past infection, and these markers are expected to be especially useful. This is because the number of individuals with past infection is increasing as more and more people receive *H. pylori* eradication therapy [18], but the usefulness of the current methods for gastric cancer risk estimation, i.e., a combination of the detection of *H. pylori* infection and the serum pepsinogen test, in this population has not been established [18–20].

None of the seven CGIs were located in promoter regions. We analyzed the association between the methylation levels of the seven CGIs and the expression levels of genes close to them, but no association was observed for any of the seven CGIs (data not shown). This was in line with the current knowledge that DNA methylation of only promoter CGIs consistently causes gene silencing, but that methylation of gene bodies may or may not be associated with increased expression [14, 21, 22]. The lack of association between methylation and gene expression supported the hypothesis that the methylation of these seven CGIs reflects the degree of overall epigenomic damage in gastric stem cells, and that the degree of epigenomic damage, and not the change of expression of individual genes, is associated with gastric cancer risk.

Epigenomic damage induced by *H. pylori* infection is one of the major causes of gastric cancer [23–26], but it is not known whether the epigenomic damage is independent of other risk factors. For example, salt intake is a risk factor for gastric cancer [27, 28], and although it does not induce methylation in gastric mucosae by itself in a Mongolian gerbil model [29, 30], it shows synergistic effects with *H. pylori* on cancer development [31]. It is not known yet whether epigenomic damage in the gastric mucosa provides independent information from past salt exposure or whether the exposure is already reflected in methylation levels. Multivariate analysis in a large cohort with a reliable record of history of salt intake will clarify this issue, and might provide a risk marker that complements the epigenetic gastric cancer risk markers.

In conclusion, we identified seven CGIs whose methylation levels are increased after *H. pylori* infection, and

are associated with gastric cancer risk even in individuals with past infection. These seven CGIs are promising candidate markers to estimate gastric cancer risk.

Acknowledgments This study was supported by Grants-in-Aid for Pioneering Basic Research and for the Third-term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan.

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