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Identification of a DNA methylation marker that detects the presence of lymph node metastases of gastric cancers

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Abstract. The accurate detection of the presence of lymph node metastases (LNM) of gastric cancers (GCs) is useful for the implementation of necessary and sufficient treatment, but current methods of detection are unsatisfactory. In the present study, we focused on DNA methylation markers since they have several advantages, including biological and chemical stability and informativeness even in the presence of contaminating cells. Using three metastatic lymph nodes and three primary GCs without LNM, methylation bead array analyses were performed, which enabled the interrogation of 485,577 CpG sites. A total of 31 CpG sites that were hypermethylated in the metastatic lymph nodes, compared with the GCs without LNM, were isolated. Using primary GCs with and without LNM (28 GCs with LNM and 10 without), their methylation levels were measured using quantitative PCR following treatment with sodium bisulfite or a methylation-sensitive restriction enzyme. Of the genomic regions around the 31 CpG sites, 10 regions demonstrated higher methylation levels in the GCs with LNM compared with the GCs without LNM ($P<0.05$). Finally, the hypermethylation of the 10 regions was validated using another set of samples (129 GCs with LNM and 20 without). Hypermethylation of the region around the cg06436185 CpG site predicted the presence of LNM at a sensitivity of 43% and specificity of 85%. Additionally, the hypermethylation of the region was associated with a poor survival rate among GC patients with LNM. The results of the present study indicated that the methylation status of the region was a promising candidate marker to detect the presence of LNM of GCs and may reflect the malignant potential of GCs.

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Introduction

Gastric cancer (GC) is one of the most prevalent malignancies worldwide and remains a leading cause of cancer-related mortality (1,2). Since the presence of lymph node metastases (LNM) is associated with a significantly poorer prognosis of GC patients (3-5), radical resection with free-margin gastrectomy and extended lymphadenectomy are performed for patients with advanced GC to eradicate LNM (6). Such an aggressive resection of the lymph nodes is associated with higher patient morbidity and/or mortality rates (7-9). Alternatively, the absence of LNM allows for minimally invasive surgery, which provides an improved quality of life following treatment. Therefore, the accurate detection of LNM is useful for the implementation of necessary and sufficient treatment.

To detect the presence of LNM, much effort has been made in the fields of imaging and molecular markers. Imaging modalities, including computed tomography (CT), endoscopic ultrasonography (EUS) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) are used in clinical practice. However, the sensitivities of these modalities are 77.2, 82.8 and 71%, respectively, and the specificities are 78.3, 74.2 and 74%, respectively (10-13). Moreover, these imaging modalities are almost powerless to detect micrometastases (14,15). With regard to molecular markers, analyses that targeted specific RNA and protein expression have been made. Although a number of these markers were associated with the presence of LNM of GCs (16-19), their utility has not been confirmed by independent studies. Therefore, genome-wide or comprehensive analysis of molecular markers for LNM of GCs is required and validation of the utility of the markers is essential for clinical application.

As a molecular marker, DNA methylation is advantageous, as its status is stable even if a cell is placed in different environments (biologically stable) and DNA is chemically stable, even in clinical materials. In addition, DNA methylation profiles are not disturbed by the presence of a small population of contaminating cells. As a strategy, we used metastatic lymph nodes and primary GCs without LNM for genome-wide analysis as cells with the abililty of LNM may constitute only a small population of the cells in primary GCs with LNM. Differences in methylation levels may be extremely small and may not be detected by the analysis between primary GCs with and without LNM. Alternatively, in metastatic lymph nodes, cancer cells are expected to possess the aberrant DNA methylation following clonal selection. Moreover, the methylation levels of appropriate marker CpG sites in the metastatic lymph nodes are expected to be relatively high compared with those in primary GCs with LNM.

In the present study, we aimed to identify CpG sites with a methylation status associated with the presence of LNM of GCs via a genome-wide methylation analysis using metastatic lymph nodes and primary GCs without LNM and to validate the isolated candidate markers.

Materials and methods

Patients, tissue samples and DNA extraction. A total of 187 GC surgical samples were obtained from patients who underwent gastrectomy with extended lymph node dissection (D2) at the National Cancer Center Hospital (Tokyo, Japan) and Aichi Cancer Center Hospital (Aichi, Japan) between 1994 and 2011 with informed consent. A total of three metastatic lymph nodes were obtained from 3 of the 187 patients. No patients had undergone prior chemotherapy or radiotherapy. Prognostic information of 55 GC patients with LNM was available and the mean follow-up period after surgery was 3,024 days. Disease grades were classified according to the 6th edition of the TNM classification by the UICC. Samples were stored at -80˚C and a high molecular weight DNA was extracted using the phenol/chloroform method. The 187 samples were divided into screening (28 GCs with LNM and 10 without) and validation (129 GCs with LNM and 20 without) sets in advance, between which no significant differences in clinicopathlogical data were observed (Table I). This study was conducted with the approval of the Aichi Cancer Center and National Cancer Center.

Genome-wide methylation analysis. Genome-wide screening of differentially methylated CpG sites was performed using an Infinium HumanMethylation450 BeadChip array, which covers 485,577 CpG sites (Illumina, San Diego, CA, USA) (20). Genomic DNA (1 μg) was treated with sodium bisulfite using a Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA) and the bisulfite-modified DNA was amplified prior to hybridization to the array. The array was scanned with an iScan System (Illumina) and the data were analyzed using GenomeStudio Methylation Module Software (Illumina). A CpG site was considered to be informative if the sum of the signals for methylated and unmethylated sequences at the CpG site was significantly higher (at $P<0.05$) than signals of the negative control probes on the same array. Methylation levels were represented by β values, with a β value of 0 corresponding to no methylation and 1 corresponding to full methylation.

Quantitative methylation-specific PCR (qMSP). Sample DNA was treated with sodium bisulfite and purified as described previously (21). qMSP was performed using real-time PCR with bisulfite-modified DNA and specific primers (Table II, Fig. 1A). A methylation level was expressed as a percentage of the value of methylated DNA reference (PMR) calculated as the [(number of fragments methylated at a target locus in sample/number of the *Alu* sequences in sample)/(number of fragments methylated at a target locus in *Sss*I-treated DNA/number of the *Alu* sequences in *Sss*I-treated DNA)]x100 (22).

Quantitative PCR following treatment with a methylation-dependent restriction enzyme (qPTMR). A fully unmethylated control was prepared by amplifying human blood genomic DNA with phi29 DNA polymerase (Illustra GenomiPhi HY kit, GE Healthcare, Buckinghamshire, UK) (23). DNA (1 μg) was treated with *Msp*JI (New England Biolabs, Beverly, MA, USA), which cleaves DNA 9 bp downstream from the ^{m}CNNR sequence (24,25), in a 30 μl reaction [4 U of *Msp*JI, 1X NEB buffer 4 (New England Biolabs) and 0.1 mg/ml BSA] at 37˚C for 20 h. Following purification, the DNA was treated with *Msp*JI again and dissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at a concentration of 5 ng/μl without purification. Using 1 μl of the solution, quantitative PCR (qPCR) was performed by real-time PCR with primers that encompassed a target *Msp*JI site (Fig. 1B). To normalize the quantity of input DNA, the number of copies of a standard sequence, which may be amplified with a primer pair (5'-TTGCTTGAAGTTTTGTTGCTGTAGT-3' and 5'-AATAAACTCAGTTGTGACATGGACA-3') and contains no *Msp*JI site, was measured by qPCR. A percentage of the value of unmethylated reference (PUR) was calculated as the [(number of fragments at target locus in sample/number of the standard sequence in sample)/(number of fragments at target locus in GenomiPhi-amplified DNA/number of the standard sequences in GenomiPhi-amplified DNA)]x100. For convenience, the methylation level was expressed as 100-PUR.

Statistical analysis. Statistical analyses were conducted using PASW statistics version 18.0.0 (SPSS Japan Inc., Tokyo, Japan). The difference between the mean values of the two groups of samples was evaluated using Welch's t-test. The Fisher's exact test was used to evaluate the significant difference in relative frequency of the phenomena between two independent groups. Survival curves were computed according to the Kaplan-Meier method and the log-rank test was employed to evaluate the level of significant difference. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Genome-wide screening using metastatic lymph nodes and GCs without LNM. To isolate the CpG sites that are hypermethylated specifically in GCs with LNM, genome-wide methylation analysis was performed using metastatic lymph nodes (n=3) and GCs without LNM (n=3) using an Infinium HumanMethylation450 BeadChip array. The samples used for this analysis were prepared from 6 patients in the screening

Table I. Clinicopathological data of sample sets.

	N	Age (years)	P-value	Gender	N	P-value	T stage	N	P-value
Genome-wide analysis set[a]									
Meta (-)	3	72±4	0.17	Male	2	1.0	T1	0	0.51
							T2	1	
				Female	1		T3	1	
							T4	1	
Meta (+)	3	59±13		Male	2		T1	0	
							T2	0	
				Female	1		T3	1	
							T4	2	
Screening set									
Meta (-)	10	69±6	0.13	Male	7	0.53	T1	0	0.17
							T2	1	
				Female	3		T3	6	
							T4	3	
Meta (+)	28	63±11		Male	18		T1	0	
							T2	0	
				Female	10		T3	14	
							T4	14	
Validation set									
Meta (-)	20	63±11	0.71	Male	13	0.6	T1	0	0.14
							T2	3	
				Female	7		T3	8	
							T4	9	
Meta (+)	129	62±10		Male	91		T1	0	
							T2	4	
				Female	38		T3	55	
							T4	70	

[a]This set comprised samples from the screening set.

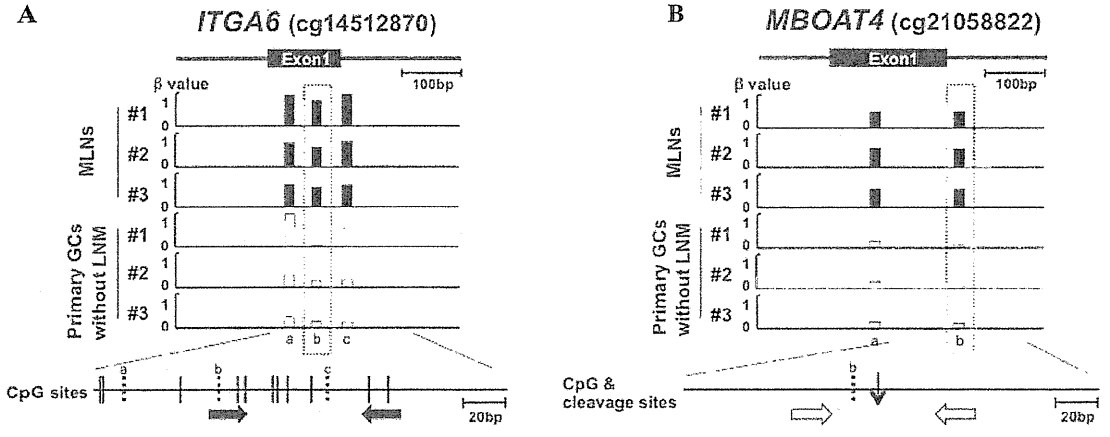

Figure 1. Representative genomic regions around the CpG sites differentially methylated between metastatic lymph nodes and GCs without LNM and primer design in the regions. Below the genomic structure of a region, β values (methylation levels) of the CpG sites carried by Infinium bead array are shown. The differentially methylated CpG site is marked by a rectangle with dotted line. A CpG map is drawn at the bottom, vertical lines (solid and broken lines) indicate CpG sites and broken lines indicate CpG sites whose β values were measured. (A) A region whose methylation level was assessed by qMSP. Primers specific to the methylated sequence (closed arrows) were designed on CpG sites around the differentially methylated sites based on the bisulfite-modified sequence. (B) A region whose methylation level was assessed by qPTMR. Primers (open arrows) were designed to amplify the region encompassing the *Msp*JI-cleaved site (thin vertical arrow) based on the unmodified sequence. GC, gastric cancer; MLN, metastatic lymph nodes; LNM, lymph node metastases; qMSP, quantitative methylation-specific PCR; qPTMR, quantitative PCR following treatment with a methylation-dependent restriction enzyme.

Table II. CpG sites identified by bead-chip array analysis.

No.	Probe name (IlmnID)[a]	Gene symbol	Location (Chr: base)	Relation to CpG island	Position to gene	P-value[b]		Cut-off (YI)	Primer sequences (5'-3')		Annealing temp.	PCR type	Mg^{2+c} (μM)
						Screening	Validation		Forward	Reverse			
1	cg23218354	-	Chr1: 2885244	Island	-	0.05	0.17	10.1 (0.48)	TGGTTTTTATACGGGGGATTTAC	ACTAAACCAAAACGACGATTACG	60	qMSP	1.5
2	cg13239126	*KIAA1026*	Chr1:15256136	-	Body	0.24	-	-	CTCCAGAGAGACAGGCATGGTT	CAAGCCTGACCTTCCCTCTCC	60	qPTMR	1.5
3	cg16112880	*TMEM9*	Chr1:201123745	Island	TSS200	0.41	-	-	CCCGCCCTCTCCTAGCTTCTAT	GGCTGACGTTCCCTTTTCTGGT	63	qPTMR	1.5
4	cg14512870	*ITGA6*	Chr2:173330342	-	Body	0.01	0.07	32.5 (0.44)	TATAGTTGCGATATTATCGTTC	AAACTACCGAAATAAACGCT	51	qMSP	2.5
5	cg09866366	*ABCF3*	Chr3:183903315	Shore	TSS1500	0.34	-	-	TCGTTAGATTACGGGTGTTTC	CAAAACGCATATATAACGATAACG	58	qMSP	2.5
6	cg08812108	-	Chr6:2515318	-	-	0.03	0.24	56.3 (0.44)	AGCGTTGGCGTTAGGTAGGGTAGTTC	CCAAATAACCACCTACGTCTTTACG	63	qMSP	1.5
7	cg06728252	*ABT1*	Chr6:26598149	Island	Body	0.24	-	-	CGCGTAGATCGGTTCGTGAGAC	GCCACGCGCTTAACTATACG	63	qMSP	1.5
8	cg08972588	*TNXB*	Chr6:32014674	-	Body	0.64	-	-	CCTGAGCAAGAATGAGGCCAGA	GGGGACAAGGGGGAGATCACA	65	qPTMR	2.5
9	cg22126965	*COX19*	Chr7:1015501	Shore	TSS1500	0.50	-	-	GGTTTAGAAAGGTTTAGCGAATTGTTC	AACAACCGCAAACAACG	62	qMSP	2.5
10	cg18450582	*DYNC1I1*	Chr7:95546539	-	Body	0.32	-	-	ACCTTGGCCTCTGGATTGTGGA	GCACTGCCTGCCTGAAAGGAGA	64	qPTMR	1.5
11	cg02005782	-	Chr7:105857664	-	-	0.59	-	-	GAAGTCAGCCAGGCATTGGAAG	CCCAGCTGCCTTTCTGATCTCT	65	qPTMR	1.5
12	**cg06436185**	*PRKAG2*	Chr7:151442351	-	Body	0.04	0.03	28.8 (0.24)	ATTTAGTTTTTTGTACGGTTGC	CCCAATAAAACGACGTAACG	55	qMSP	2.5
13	cg21058822	*MBOAT4*	Chr8:30002223	-	TSS200	0.38	-	-	GGCTGTCTCTGGTCTTTTTATC	AGAAAGCCAGTTTTTATTCTGC	61	qPTMR	1.5
14	cg12089032	-	Chr8:72881203	-	-	0.03	0.09	40.6 (0.41)	GCAAGTTAAGGCATCGTAGGAAAGC	GGCAGAGAGGAACAGCTCCTAAG	66	qPTMR	1.5
15	cg23170346	-	Chr8:134863880	-	-	0.95	-	-	CTAGCCACATCCATAGCAGACAGG	CACTCAGCAATGCAAACAGTCTTG	66	qPTMR	1.5
16	cg19878482	*C8orf73*	Chr8:144655026	Shore	TSS200	0.10	-	-	GGAGTTTTTCGGGTTCGGTTTC	CAAAAACCCATTATAAACACGTCCGT	65	qMSP	2.5
17	cg01263942	*DIP2C*	Chr10:695859	-	Body	0.01	0.12	23.2 (0.38)	GTTCGTTATTTGCGTTTTCGTGC	CAACGAAAAAACTCCATAAACCG	59	qMSP	2.5
18	cg03015672	*ARHGAP12*	Chr10:32216066	Shore	5'UTR	0.88	-	-	AGAACAGTGGAGCCGCATGCAA	CCAAAGCAGGCAGTGAAAGCGT	66	qPTMR	1.5
19	cg10326726	*MSMB*	Chr10:51549505	-	TSS200	0.16	-	-	CAACCCTCTGTAAACACTCAAT	TATAGACAGGTACATCCAGGCA	57	qPTMR	2.5
20	cg19864370	-	Chr10:80354592	-	-	0.00	0.29	70.6 (0.69)	GAATAGCTTAGGCCCCTGTCAT	GATAGTGCTAGCCCTTGGGAAT	60	qPTMR	1.5
21	cg03850986	*ABLIM1*	Chr10:116408382	-	Body	0.38	-	-	TGATAAAAATGCTCTGGAATTAG	TGGAGATGTAATGTAGTACACCATA	51	qPTMR	1.5
22	cg25885280	*SHANK2*	Chr11:70760166	-	Body	0.34	-	-	GCGGTGGGGGATTTCTGTAAGGA	GAGCAGGGTGTGCCTTCTCAGGG	68	qPTMR	1.5
23	cg26894278	*CRYL1*	Chr13:21016241	-	Body	0.22	-	-	GTTAAGTTTAAATGGAGCCTTG	TGACAGGATTACAATAAGGCTA	56	qPTMR	1.5
24	cg04339360	*KLF5*	Chr13:73635568	Shore	Body	0.04	0.31	25.4 (0.43)	TAGTCAAGAAAAGAAACCTGTGCAA	TGCCAAACTACCTCAATTCTGTTTA	61	qPTMR	1.5
25	cg16206504	-	Chr13:114917223	Shelf	-	0.02	0.35	35.2 (0.41)	CGAGATTGTAGGCGGTTGTTC	CCTAACTATTACAACAATACCGAACG	63	qMSP	1.5
26	cg14851578	-	Chr14:106187192	Shore	-	0.08	-	-	GGAGTGTGGGTTACGTGTGATTAC	CAATCTCGCCCACTCACG	66	qMSP	1.5
27	cg02990302	*C16orf80*	Chr16:58155189	-	Body	0.04	0.45	56.2 (0.65)	TCCTTTCCTTAGCTCCTTCCAG	AAAAACAGTCGGCTCTTTGTGA	63	qPTMR	1.5
28	cg08292959	*MGAT5B*	Chr17:74878420	Island	Body	0.97	-	-	GGCACCTGCCACTCCATCCG	TGCACTCTGGGCTGTACCACAGTG	63	qPTMR	1.5
29	cg15645685	*PBX4*	Chr19:19730175	Shore	TSS1500	0.26	-	-	CTAATGCTCCCTGCATCCTCAG	TAAACAAGCGAGGTCACTCTTCAGC	64	qPTMR	1.5
30	cg14571622	*NLRP8*	Chr19:56499348	-	3'UTR	0.01	-	-	TGGGGCTTGATTGATCAGTTCC	CCAGGGTTCAAAGCTGAGGTTC	62	qPTMR	1.5
31	cg27050343	*OTC*	ChrX:38211596	-	TSS200	0.15	-	-	AATTTTTGGGTTTAAGTGATTCGTTC	AAAAAAATAATTACTAACCGAACACG	62	qMSP	1.5

[a]IlmnID is a unique CpG site identifier from the Illumina CG database; underlined IlmnID indicates P<0.05 in the screening set; bold IlmnID, P<0.05 in the validation set. [b]Difference was evaluated by Welch's t-test. [c]Final concentration in a PCR analysis. Island, CpG island; Shore, within 2,000 bp of end of CpG island; Shelf, within 2,000 bp of end of Shore; TSS200, within 200 bp upstream of the transcription start site; TSS1500, 200-1,500 bp upstream of the TSS; 5'UTR, in the 5' untranslated region of the gene; Body, within exons or introns of the gene; 3'UTR, in the 3' untranslated region of the gene; YI, maximized Yoden index.

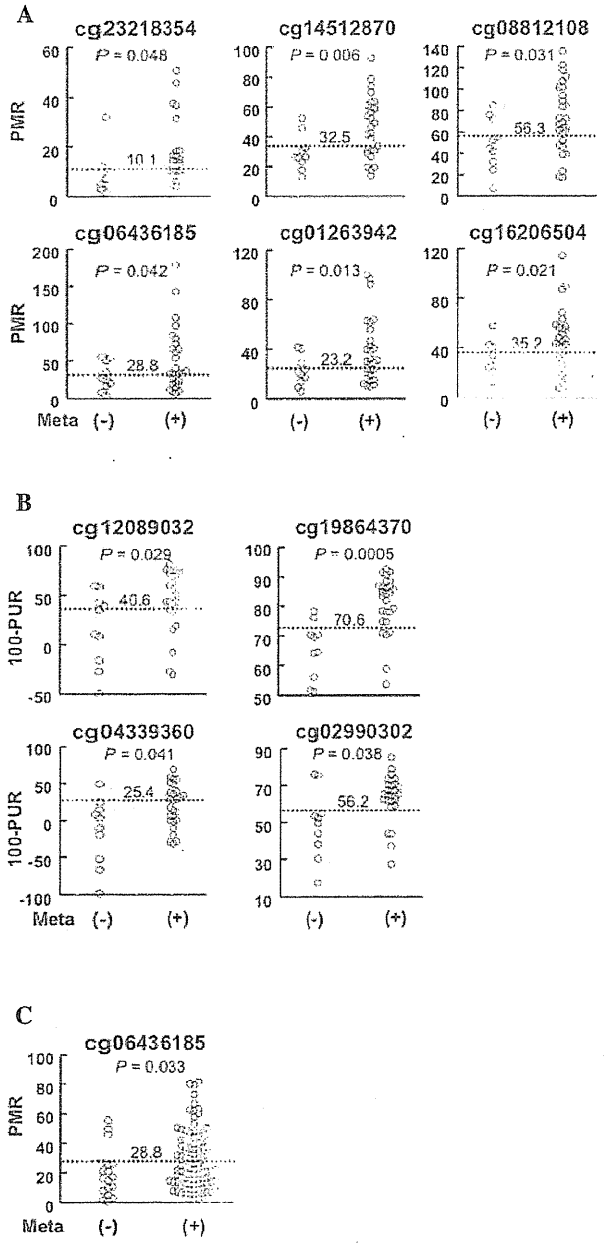

Figure 2. Methylation levels of the candidate genomic regions in primary GCs with and without LNM. Methylation levels were measured by (A) qMSP and (B) qPTMR in the screening sets. The screening set consisted of 10 GCs without LNM and 28 with LNM. (C) Methylation level of the region around cg06436185 in the validation sets was measured by qMSP. The validation set consisted of 20 GCs without LNM and 129 with LNM. Meta (-), GCs without LNM; Meta (+), those with LNM. Horizontal dotted lines are the cut-off methylation levels and the number on the line indicates the value of the level. GC, gastric cancer; LNM, lymph node metastases; qMSP, quantitative methylation-specific PCR; qPTMR, quantitative PCR following treatment with a methylation-dependent restriction enzyme; PUR, percentage of the value of unmethylated reference; PMR, percentage of the value of methylated DNA reference.

set (Table I). The mean number of informative CpG sites was 485,170 (SD 209) in the metastatic lymph nodes and 485,001 (SD 514) in the GCs without LNM (P=0.63). We searched for CpG sites that were highly methylated in the three metastatic lymph nodes [β value > a) 0.6, b) 0.5 and c) 0.4] and hardly methylated in the three primary GCs without LNM (β value <0.2) and the number of hypermethylated CpG sites was a) 1, b) 31 and c) 209, respectively. To obtain a practicable number of candidate CpG sites, we adopted a cut-off β value of 0.5 and the 31 CpG sites were selected for further analysis (Table II).

Table III. Association between methylation levels of the genomic region around cg0643618 and clinical characteristics.

		Methylation level		
Parameters	N	Mean	SD	P-value
Age				
≤60	77	32.2	24.0	0.26
>60	110	28.1	25.2	
Gender				
Female	58	34.9	26.4	0.07
Male	129	27.6	23.6	
T category				
T3	83	26.8	27.1	0.07
T4	96	33.6	22.5	

Selection of informative candidate genomic regions among primary GCs. Using primary GCs with and without LNM (screening set, Table I), the methylation levels of genomic regions around the 31 CpG sites were measured by qMSP or qPTMR, which are accurate and sensitive enough to detect aberrant DNA methylation in a small population of cells. Of the 31 regions, 10 regions exhibited higher methylation levels in GCs with LNM (1.4- to 1.9-fold) than in those without LNM (Table II and Fig. 2A and B). For each of the 10 genomic regions, a cut-off methylation level was established in order that the Youden index (sensitivity + specificity - 1) would be maximized (Table II and Fig. 2).

Validation of the candidate genomic regions in a different set of samples. To validate the hypermethylation of the 10 candidate genomic regions in GCs with LNM, the methylation levels were analyzed in an independent sample set (validation set, Table I). A region around the cg06436185 CpG site revealed significantly higher methylation levels in GCs with LNM (1.5-fold) than those without (P=0.033, Fig. 2C), whereas the other nine regions were not validated (Table II). The region was located in the gene body of the *PRKAG2* gene and did not belong to a CpG island (Table II). Therefore, it was unlikely that the methylation status of the region around cg06436185 affected the transcription of a gene. Using a cut-off level established in the analysis of the screening set (28.8%), the presence of LNM was detected at a sensitivity of 43% and a specificity of 85%. This result indicated that a methylation level of this region is a candidate marker for the detection of the presence of LNM.

Association between the methylation level of the genomic region around the cg06436185 CpG site and clinicopathological characteristics. Associations between the methylation level of the genomic region around cg06436185

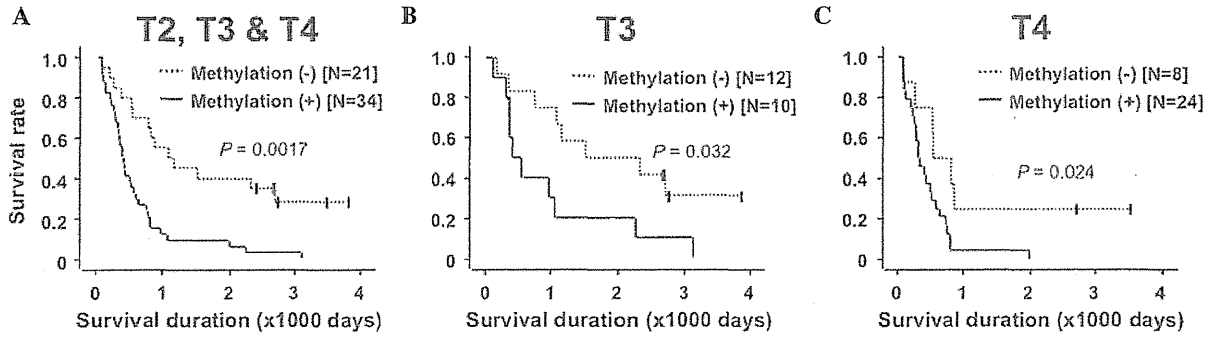

Figure 3. Kaplan-Meier analysis of 55 GC patients with LNM. (A) Overall survival in all the patients with LNM. (B and C) The survival curves of patients categorized into T3 and T4. Methylation (+), GCs with PMR of the region around cg06436185>28.8; Methylation (-), GCs with the PMR<28.8. GC, gastric cancer; LNM, lymph node metastases; PMR, percentage of the value of methylated DNA reference.

and clinicopathological characteristics (age, gender and T category) were analyzed in 157 GC patients with LNM and 30 without LNM. No difference in methylation levels according to age, gender or T category was found (Table III). Using 55 of the 157 GC patients with LNM, whose prognostic information was available (T2, one patient; T3, 22 patients; T4, 32 patients), a correlation between the methylation level and survival rate was analyzed. Patients with high methylation levels (>28.8%; the value used to detect the presence or absence of LNM) had a significantly poorer overall survival rate compared to those with low methylation levels (P=0.0017; Fig. 3A). Since the T category is known to be the major prognostic factor in GC patients (26), patients in the T3 and T4 categories were analyzed separately. In the T3 and T4 subgroups, the patients with high methylation levels demonstrated a significantly poorer overall survival rate than those with low methylation (P=0.032 and 0.024, respectively; Fig. 3B and C). These results revealed that the high methylation level of the genomic region around cg06436185 was associated with an unfavorable prognosis, regardless of the depth of tumor invasion.

Discussion

Using a genome-wide methylation analysis using metastatic lymph nodes and primary GCs without LNM, a genomic region (around cg06436185) whose methylation level in primary GCs was associated with the presence of LNM was successfully identified. Notably, the association was also significant in an independent validation set (P=0.033). Generally, markers isolated by genome-wide analyses need to be validated in a different set of samples due to the overfitting issues caused by multiple testing (27). Even in the present study, 9 of the 10 candidate genomic regions that revealed significant hypermethylation in GCs with LNM in the screening set (P=0.0005-0.048) were not reproduced in the validation set. This observation emphasizes the value of the methylation level of the genomic region around cg06436185. Since it had a sensitivity of 43% and specificity of 85%, the combined use of this novel methylation marker with imaging tools is predicted to improve the diagnostic accuracy of LNM of GCs.

The mean methylation levels of GCs with and without LNM were 18.7 and 27.5%. This small difference is extremely difficult to detect by a genome-wide screening method. Our strategy in the present study was to benefit from the monoclonal growth of cells in metastatic lymph nodes and compare metastatic lymph nodes and GCs without LNM. The methylation levels of the genomic regions around cg06436185 were 13.2 and 54.3%, respectively, in these samples. This relatively significant difference was identified using genome-wide screening, which has a relatively low accuracy in the analysis of methylation levels. Using a more accurate and sensitive method, qMSP, the small difference between GCs with and without LNM (18.7 and 27.5%, respectively) was clearly demonstrated.

A method to measure methylation levels in CpG-poor genomic regions, qPTMR, was developed using a combination of digestion with a methylation-dependent restriction enzyme and qPCR. qPTMR had an error range of 5% in this study. It is difficult to measure methylation levels in CpG-poor genomic regions by qMSP, a well-established method with a high accuracy, due to the difficulty in designing primers. Alternatively, *Msp*JI, a recently developed methylation-sensitive restriction enzyme, recognizes ^{m}CNNR (N=A, T, G or C; R=G or C) sequences and cleaves DNA when the C is methylated (24,25). Since the recognition sequence is applicable to the majority of CpG sites and cytosines in non-CpG sites are not methylated in somatic cells, the positive cleavage by *Msp*JI is used to determine methylation status of most CpG sites. Using qPTMR, the methylation levels of all the 19 candidate regions with few CpG sites were quantified. This new method is predicted to have various applications.

The methylation status of the genomic region around cg06436185 was unlikely to affect transcription of a known nearby gene (*PRKAG2*). However, its high methylation level in GCs, namely large fractions of cancer cells with methylation in cancer tissue, was associated with the presence of LNM and also with a poorer prognosis of the GC patients. One possible reason is that the region is located in a promoter region of unknown genes, including microRNA genes, or in enhancer regions whose methylation is critical for the regulation of gene expression levels. Another possible reason is that the methylation of the region is caused by an abnormality of unknown methylation regulation and that this abnormality is critical for tumor metsastasis or malignancy. In this case, other genomic regions are likely to be methylated in GCs with LNM or a poorer prognosis.

In conclusion, we identified one genomic region with a methylation status in primary GCs that was associated with the presence of LNM and a poorer prognosis of GC patients.

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Clinical Cancer Research

Molecular Pathways

Molecular Pathways: Involvement of *Helicobacter pylori*–Triggered Inflammation in the Formation of an Epigenetic Field Defect, and Its Usefulness as Cancer Risk and Exposure Markers

Toshikazu Ushijima and Naoko Hattori

Abstract

Infection-associated cancers account for a large proportion of human cancers, and gastric cancer, the vast majority of which is associated with *Helicobacter pylori* infection, is a typical example of such cancers. Epigenetic alterations are known to occur frequently in gastric cancers, and *H. pylori* infection has now been shown to induce aberrant DNA methylation in gastric mucosae. Accumulation of aberrant methylation in gastric mucosae produces a field for cancerization, and methylation levels correlate with gastric cancer risk. *H. pylori* infection induces methylation of specific genes, and such specificity is determined by the epigenetic status in normal cells, including the presence of H3K27me3 and RNA polymerase II (active or stalled). Specific types of inflammation, such as that induced by *H. pylori* infection, are important for methylation induction, and infiltration of monocytes appears to be involved. The presence of an epigenetic field defect is not limited to gastric cancers and is observed in various types of cancers. It provides translational opportunities for cancer risk diagnosis incorporating life history, assessment of past exposure to carcinogenic factors, and cancer prevention. *Clin Cancer Res; 18(4); 923–9. ©2011 AACR.*

Background

Infection-associated cancers account for a large proportion of human cancers. These include gastric cancers induced by *Helicobacter pylori* (1), hepatocellular carcinomas induced by hepatitis C virus (HCV) and hepatitis B virus [HBV (2–4)], cervical cancers induced by human papilloma virus [HPV (5, 6)], and lymphomas and nasopharyngeal cancers associated with Epstein-Barr virus [EBV (7, 8)]. The carcinogenic mechanisms of these infection-associated cancers have been extensively investigated, and although multiple contributing mechanisms have been clarified, they are not yet completely understood.

General mechanisms of infection-associated cancers

Virus-associated cancers have complex mechanisms of carcinogenesis. Viral oncogenes, such as E6 and E7 of HPV and X protein of HBV, can be integrated into host cells and produce aberrant growth signals and inactivate tumor-suppressor genes (6). Also, integration of virus genes into the host genome can alter the expression of nearby tumor-related genes and induce a genomic instability that will eventually contribute to cancer development (4). Even if the virus genes are not integrated, they can be persistently expressed and perturb important cellular signaling, such as cell proliferation, apoptosis, and cytokine expression, as in the case of HCV and EBV (7).

Both bacterial and viral infections can be associated with severe tissue damage and resultant chronic inflammation (4, 6, 7). Tissue damage itself activates cell proliferation and increases the chance that mutations will occur. In addition, chronic inflammation is considered to be deeply involved in cancer development and progression by multiple mechanisms, such as increased production of active oxygen species, induction of inflammation-mediated cell proliferation, and increased cytokine production (9, 10). In addition to this, induction of epigenetic alterations is now recognized as one of the mechanisms underlying induction of cancer by chronic inflammation.

H. pylori infection and epigenetic alterations in gastric cancers

Gastric cancer is still the third-leading cause of death from cancer in men and the fifth-leading cause in women worldwide, although its incidence is gradually decreasing (11). The vast majority of gastric cancers are caused by *H. pylori* infection (12), which is a Gram-negative bacterium (13). A minor percentage (~10%) of gastric cancers are associated with EBV infection (14). It is known that when *H. pylori* infects the human stomach, it induces severe inflammation, including ulcers, then chronic inflammation, and finally gastric cancers within tens of years. Investigators have mainly discussed the carcinogenic mechanisms of *H. pylori* from

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the standpoint of induction of cell proliferation, mutations, and direct activation of cellular signaling (15–17).

However, tumor-suppressor genes such as *CDKN2A*, *CDH1*, *MLH1*, and *RUNX3* are inactivated more frequently by aberrant DNA methylation than by mutations, indicating that gastric cancer is an epigenetic disease (18). In addition to methylation silencing of driver tumor-suppressor genes, recent genome-wide analyses have revealed that hundreds of passenger genes are also methylated in gastric cancers (19). The fact that *H. pylori* infection induces epigenetic alterations provides the missing link between the causal role of *H. pylori* infection in gastric carcinogenesis and the deep involvement of epigenetic alterations in gastric cancers. Gastric cancer is a typical example of a disease in which infection, chronic inflammation, and epigenetic alterations are interconnected.

Induction of epigenetic alterations by *H. pylori* and the formation of field defects

The first hint that the presence of aberrant DNA methylation might be associated with *H. pylori* infection came from the observation that promoter methylation of *CDH1* was detected more frequently in the gastric mucosae of individuals with *H. pylori* infection than in those without the infection (20). By quantifying the methylation levels of 8 marker CpG islands, Maekita and colleagues (21) convincingly showed that individuals with *H. pylori* infection have much higher methylation levels in their gastric mucosae (5.4- to 303-fold) than those without ($P < 0.0001$). In addition to the 8 marker CpG islands associated with protein-coding genes, CpG islands of microRNA genes are also methylated in association with *H. pylori* infection (22, 23).

In one study, patients with gastric cancer who had previously had an *H. pylori* infection but were currently not infected had lower methylation levels of the 8 marker CpG islands in the gastric mucosae compared with patients who were currently infected with *H. pylori* (21). This suggests that the methylation level is very high when active *H. pylori* infection is present in the stomach and decreases to certain levels when the infection is discontinued. In other studies, various degrees of decrease were observed in individuals who received eradication therapy for *H. pylori* (24, 25), and the methylation level after the decrease was considered to represent the degree of epigenomic damage to the individual. This decrease of methylation could be due to a turnover of gastric epithelial cells with methylation or to the removal of 5-methylcytosine, which is present in individuals with active *H. pylori* infection.

Of importance, among individuals without current *H. pylori* infection, the methylation levels of the 8 marker CpG islands in gastric mucosae were shown to correlate with gastric cancer risk (21, 26). Patients with gastric cancers had 2.2- to 32-fold higher methylation levels in gastric mucosae compared with healthy individuals (21), and patients with multiple gastric cancers had significantly higher methylation levels than those with a single gastric cancer (26). This correlation strongly supports the notion that the accumulation of aberrant methylation in gastric mucosae produces an epigenetic field for cancerization, i.e., a field defect (Fig. 1; ref. 27).

Epigenetic field for cancerization

In the epigenetic field for gastric cancers, tumor-suppressor genes that are causally involved in gastric cancer development (i.e., driver genes), such as *CDKN2A*, *CDH1*, *MLH1*, and *RUNX3*, are methylated only at very low levels, showing that such events are present only in a very small fraction of cells (21). In contrast, many other genes that are unlikely to be causally involved in gastric carcinogenesis (i.e., passenger genes), such as *HAND1* (a transcription factor involved in heart morphogenesis), are methylated at high levels, showing that their methylation is present in a large fraction of cells. Most of the genes that are highly methylated in gastric cancers are either unexpressed or expressed only at low levels in normal cells (28). Generally, genes with low expression are susceptible to methylation induction (29), and it is considered that most of the genes that are methylated in the epigenetic field were methylated as a consequence of gastric carcinogenesis. In addition to accumulation of aberrant methylation, an epigenetic field involves hypomethylation of the Alu and Satα repeat sequences (30), which potentially can be involved in genomic instability.

Epigenetic field defects are present not only in gastric cancers but in other cancers as well (27). In the case of hepatocellular carcinoma, aberrant DNA methylation was frequently observed in noncancerous tissues of cancer patients compared with normal livers of patients with metastatic liver tumors (31). A quantitative analysis revealed increased methylation of multiple tumor-suppressor genes, such as *SOCS1*, *RASSF1A*, and *CDH1*, in HCV-infected, noncancerous liver tissues (32), suggesting the importance of an epigenetic field for HCV-associated hepatocarcinogenesis. In the case of esophageal adenocarcinoma, the presence of *APC* and *CDKN2A* methylation in Barrett's metaplasia has been reported (33), and such methylation was shown to be associated with progression of Barrett's metaplasia (34). Also in the case of esophageal squamous cell carcinoma, methylation of specific genes, such as *UCHL1* and *HOXA9*, in esophageal mucosae was associated with the risk of developing esophageal squamous cell carcinoma (35, 36). In ulcerative colitis, the driver gene *CDKN2A* and passenger genes such as *MYOD* and *ESR* were shown to be methylated in colonic mucosae, which are predisposed to colon cancers (37, 38). In addition, in the case of sporadic colorectal cancers, *MGMT* methylation in cancer tissues was associated with high levels of *MGMT* methylation in the background colonic mucosae (39). The presence of epigenetic field defects has also been indicated for breast (40), renal (41), and bladder cancers (42, 43).

Critical roles of specific types of inflammation in methylation induction

The association between *H. pylori* infection and high levels of DNA methylation in gastric mucosae in humans strongly indicates that *H. pylori* infection induces aberrant

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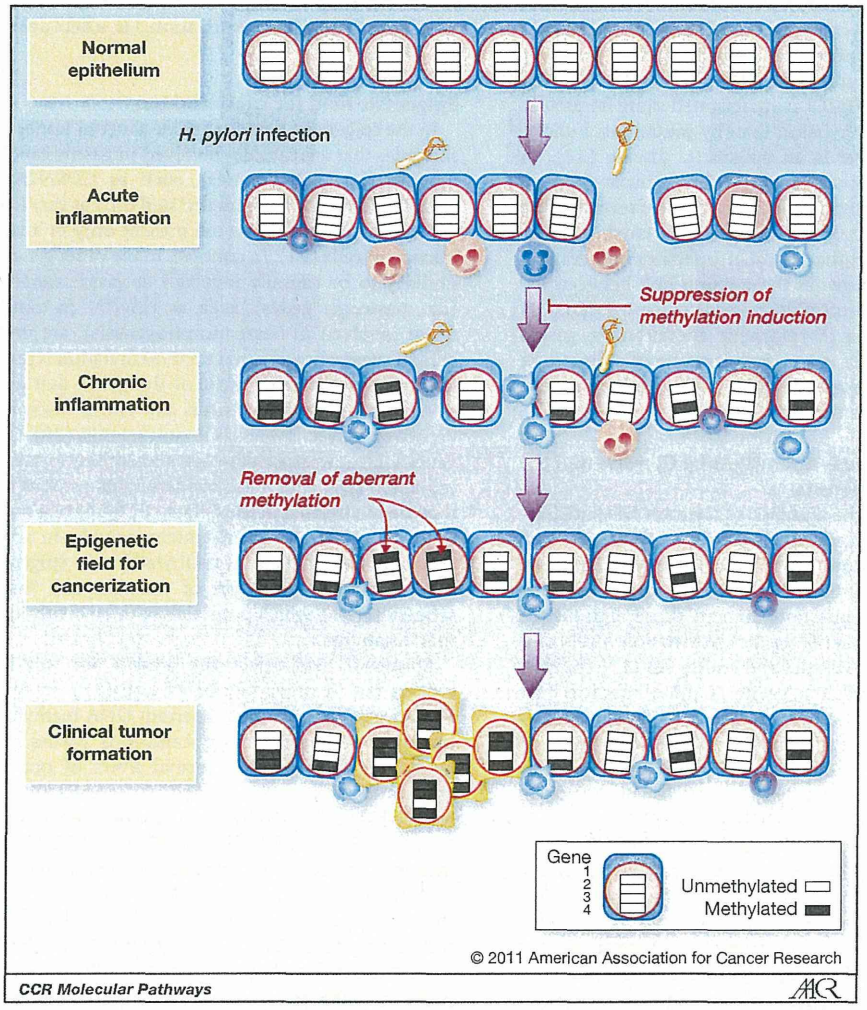

Figure 1. Formation of epigenetic field for cancerization by chronic inflammation triggered by *H. pylori* infection. *H. pylori* infection induces acute inflammation, followed by chronic inflammation, formation of an epigenetic field for cancerization, and development of gastric cancers. Aberrant methylation is induced in driver genes (schematically represented by gene 1) and passenger genes (genes 3 and 4). Specific genes are methylated in gastric mucosae with *H. pylori* infection, and driver genes usually have very low methylation levels. On the other hand, passenger genes that have low or no expression in normal cells usually have high methylation levels. The methylation level of some passenger genes reflects the degree of accumulation of epigenomic damage, and correlates with gastric cancer risk. Chronic inflammation triggered by *H. pylori* infection is critical for methylation induction, and if data from a mouse colitis model are combined, the importance of monocytes can be speculated. As translational targets, methylation levels of specific genes in normal-appearing tissues can be used as a cancer risk marker that reflects a person's life. The methylation signature has potential as a marker for past exposure to specific environmental factors. Suppression of induction of aberrant DNA methylation, and possibly removal of accumulated aberrant methylation can be used for cancer prevention (shown in red).

DNA methylation. This cause-consequence relationship was shown with the use of Mongolian gerbils, in which *H. pylori* infection-induced gastritis and gastric cancers can be recapitulated (44). Gerbils infected with *H. pylori* developed severe gastritis and had markedly increased methylation levels, showing the causal role of *H. pylori* infection in methylation induction (45). The methylation levels were clearly decreased after eradication of the *H. pylori*, in agreement with the decreased methylation levels observed in patients who received eradication therapy.

In the attempt to determine how *H. pylori* induces methylation, investigators have considered both direct and indirect actions of *H. pylori*. First, because *H. pylori* possesses multiple DNA methyltransferases (46) and a type IV secretion system [a syringe-like structure capable of delivering bacterial materials into a host cell (47)], *H. pylori* itself may

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induce methylation in epithelial cells by injecting its own DNA methyltransferases. Alternatively, studies in patients with ulcerative colitis showed that chronic inflammation played a role in methylation induction (37, 38), and chronic inflammation triggered by *H. pylori* infection may have been responsible for the methylation induction. Niwa and colleagues (45) addressed this issue by suppressing inflammation in gerbils with *H. pylori* infection using cyclosporin A, an immunosuppressant. Although colonization of *H. pylori* was not affected at all, methylation induction was markedly suppressed. This clearly shows that it was the inflammation triggered by the *H. pylori* infection, not the *H. pylori* itself, that was involved in methylation induction. A temporal analysis of the expression of inflammation-related genes in gastric mucosae of infected gerbils showed that the expression levels of *Cxcl2*, *Il1b*, *Nos2*, and *Tnf* paralleled the methylation levels.

Inflammation in the stomach can be induced not only by *H. pylori* infection but also by high concentrations of ethanol (EtOH) or saturated sodium chloride (NaCl) solution. A methylation analysis of gastric mucosae exposed to these kinds of inflammation showed that only inflammation triggered by *H. pylori* infection was capable of inducing aberrant DNA methylation (48). Histologically, *H. pylori* infection induced chronic inflammation with prominent lymphocyte and macrophage infiltration, whereas EtOH and NaCl treatment induced persistent neutrophil infiltration. Cell proliferation, which is known to be important for methylation induction (38), was most strongly induced in the NaCl group and was shown to be insufficient for methylation induction. Among inflammation-related genes, expression of *Il1b*, *Nos2*, and *Tnf* was increased specifically in gastric mucosae of gerbils with *H. pylori* infection. Therefore, it is considered that specific types of inflammation are necessary for methylation induction.

Chronic inflammation is characterized by infiltration of mononuclear cells, i.e., lymphocytes and monocytes. To clarify which cell type(s) plays the major role in methylation induction, Katsurano and colleagues (49) examined SCID mice, which lack both B and T lymphocytes. Because *H. pylori* cannot infect mice efficiently, they used a colitis model induced by dextran sulfate sodium (DSS). Even in SCID mice, DNA methylation and colon tumors could be induced at the same levels as in wild-type mice. This shows that lymphocytes are dispensable for methylation induction, and strongly suggests that monocytes are important. Expression of *Ifng*, *Il1b*, and *Nos2* was induced in both wild-type and SCID mice by DSS treatment.

If we hypothesize that the same effectors are working in gerbil stomachs infected by *H. pylori* and mouse colons treated with DSS, we can conclude that expression of *Il1b* and *Nos2* may be involved in methylation induction. Promoter polymorphisms of *IL1B* are reported to be associated with human gastric cancer susceptibility by increasing or decreasing IL1β production in response to *H. pylori* infection and thus the progression of gastric atrophy (50, 51). Increased production of NO *in vitro* is reported to increase the enzyme activity of DNA methyltransferases without changing their expression, and to induce DNA methylation of specific genes (52). In the human and gerbil stomachs infected by *H. pylori* and mouse colons treated with DSS, no changes in the expression of DNA methyltransferases 1, 3a, and 3b were observed (28, 45, 49). It is possible that a signal from chronic inflammation, possibly IL1β, and elevation of NO in epithelial cells lead to inappropriate localization of deregulated DNA methyltransferase(s) to methylation-susceptible CpG islands (see below) and induce aberrant DNA methylation as an infrequent event.

Methylation fingerprints produced by *H. pylori* infection

Target genes for methylation induction by *H. pylori* infection are present in gastric mucosae (28). Among 48 promoter CpG islands whose methylation was analyzed in gastric mucosae of individuals with and without *H. pylori* infection, some were consistently methylated in individuals with current or past infection and others were not methylated at all. Analysis of polyclonal tissues, unlike that of cancers, can provide information about multiple events that have taken place independently, and the presence of target genes was convincingly shown in gastric mucosae (29). Similarly, in the esophagus, specific genes were methylated in association with smoking history (35), and again the presence of target gene specificity for methylation induction was shown.

The target gene specificity is defined by epigenetic factors in the cells where methylation is induced (29, 53, 54) and in the genome architecture (55, 56). Epigenetic factors that promote methylation induction include low transcription and the presence of an H3K27me3 modification. In contrast, the presence of histone acetylation and RNA polymerase II (active or stalled) protects CpG islands from becoming methylated. A multivariate analysis revealed that the most influential factors are the promoting effect of H3K27me3 and the protective effect of RNA polymerase II (54). A genomic factor that promotes methylation induction is a distant location from repetitive elements (55, 56). It is currently speculated that infection by *H. pylori* induces H3K27me3 and removes RNA polymerase II at its target genes, and that these genes then become methylated.

Clinical-Translational Advances

Cancer risk marker that reflects life history

The importance of predicting cancer risks has been repeatedly emphasized because the ability to select high-risk individuals enables efficient cancer screening and reduces social costs (57–59). To this end, a massive effort has been made in association studies, and many cancer risk alleles for common cancers have been identified. Most of these risk alleles give odds ratios between 1.5 and 2.0 (51, 58, 59), and can be used to estimate cancer risk when a person is born.

At the same time, a person is exposed to various environmental carcinogenic factors, and the cancer risk of an adult will differ depending on what sort of life he or she has

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spent. Therefore, a cancer risk marker that incorporates information about life-to-date is important. At least some of a person's life history, such as smoking and infection by *H. pylori*, is imprinted on the epigenome and produces an epigenetic field for cancerization. The severity of the field can be measured as methylation levels of specific marker genes, and correlates with cancer risk. The odds ratios obtained by DNA methylation markers of gastric cancers, such as *THBD*, *FLNc*, and *miR-124a*, range from 2.4 to 22.1 [calculated based on our previous reports (21, 22)]. Passenger genes can be useful as marker genes because they are consistently methylated and have high methylation levels in noncancerous tissues (21, 27), whereas driver genes are only stochastically methylated and have low methylation levels (Fig. 1). Therefore, for evaluating the degree of epigenomic damage that has been done in the past, methylation of passenger genes is often superior to that of driver genes.

The presence of an epigenetic field defect is also known for other types of cancers, as mentioned above (27). Therefore, investigators are now developing methods to estimate epigenetic cancer risk, taking life history into account, in various types of cancer. For example, a multicenter study was conducted to evaluate the validity of methylation markers to predict progression of Barrett's esophagus, and methylation of *HPP1*, *CDKN2A*, and *RUNX3* were shown to be informative (34).

Marker for past exposure to specific environmental factors

H. pylori infection is associated with methylation of a specific set of genes, most of which are considered as passengers, in gastric mucosae (28). A history of smoking is associated with methylation of *UCHL1* and *HOXA9*, which are also considered to be passengers, in esophageal mucosae (35, 36). Once the specificity of methylation signatures to various carcinogenic agents is clarified, past exposure to such carcinogenic factors can be estimated by the methylation signature. The methylation signature has advantages over other exposure markers because it persists for a long time and does not require any record by humans. For example, past exposure to *H. pylori* infection can be estimated by serum antibody, but it persists only up to several years after *H. pylori* infection discontinues (60). The ability to estimate past exposure using a methylation signature would be very helpful from an epidemiological viewpoint.

Epigenetic cancer prevention

The presence of an epigenetic field for cancerization and the deep involvement of chronic inflammation in its formation provide targets for cancer prevention. Suppression of induction of aberrant DNA methylation is expected to lead to a decreased incidence of cancers. This concept is supported by animal models for macroscopic colon tumors (61, 62), lung tumors (63), and prostate cancers (64, 65). It was shown that in gerbil stomachs, administration of a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), decreased the incidence of gastric cancers induced by *H. pylori* infection and a mutagen, *N*-methyl-*N*-nitrosourea (unpublished). In addition to suppression of DNA methylation induction, suppression of H3K27me3, a premarker for DNA methylation induction, is also an attractive target. The histone methyltransferase that is responsible for this modification, EZH2, is known to be overexpressed in aggressive tumors (66) and precancerous lesions (67), and therefore inhibitors of EZH2, such as 3-deazaneplanocin A (66), may have preventive applications.

Anti-inflammatory drugs, especially nonsteroidal anti-inflammatory drugs (NSAIDs), are effective for prevention of at least some cancers, but their use is still limited to individuals with high risk (68). The use of NSAIDS is limited in part because of possible side-effects, such as peptic ulcer. To avoid such side-effects and suppress the pathways that are responsible for cancer development, researchers are actively investigating the mechanisms of cancer induction by chronic inflammation (69). Because induction of epigenetic alterations is one of these important mechanisms, suppression of specific components of inflammation that are responsible for induction of epigenetic alterations is expected to provide a good target for cancer prevention.

Lastly, DNA methylation is reversible by DNA demethylating agents, such as 5-aza-dC and 5-azacytidine (70). Currently available demethylating agents do not have a high specificity for aberrantly methylated genes, and can demethylate normally methylated sequences. Such sequences include normally methylated CpG islands and repetitive sequences originating from retrotransposons, and it is feared that DNA demethylating agents might induce demethylation of these retrotransposons. Therefore, for cancer prevention using current demethylating agents, we must carefully balance risk and benefit, and probably such agents are not widely indicated. However, many epigenetic drugs are being developed, and it is possible that some of the new demethylating agents will have a specificity or preference for aberrantly methylated promoter CpG islands, and can be used in a wider range of individuals in the future.

Conclusions

The fact that *H. pylori* infection induces aberrant DNA methylation in gastric mucosae provides the missing link between the major role of *H. pylori* infection in gastric cancers and the deep involvement of epigenetic alterations in gastric cancers. The severity of infection correlates with gastric cancer risk and can provide a unique cancer risk marker that reflects a person's life. *H. pylori* infection has been shown to induce methylation of specific genes, and there are underlying mechanisms. The methylation signature has potential as a marker for past exposure to *H. pylori* infection. Specific types of inflammation, such as that induced by *H. pylori* infection, are capable of inducing aberrant methylation, and monocytes appear to be involved in the induction. Suppression of methylation induction,

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specific inflammatory processes, and reversal of epigenetic alterations are targets for cancer prevention.

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ORIGINAL ARTICLE

Early-stage formation of an epigenetic field defect in a mouse colitis model, and non-essential roles of T- and B-cells in DNA methylation induction

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Epigenetic fields for cancerization are involved in development of human cancers, especially those associated with inflammation and multiple occurrences. However, it is still unclear when such field defects are formed and what component of inflammation is involved in induction of aberrant DNA methylation. Here, in a mouse colitis model induced by dextran sulfate sodium (DSS), we identified three CpG islands specifically methylated in colonic epithelial cells exposed to colitis. Their methylation levels started to increase as early as 8 weeks after DSS treatment and continued to increase until colon cancers developed at 15 weeks. In contrast to the temporal profile of DNA methylation levels, infiltration of inflammatory cells spiked immediately after the DSS treatment and then gradually decreased. Exposure of cultured colonic epithelial cells to DSS did not induce DNA methylation and it was indicated that inflammation triggered by the DSS treatment was responsible for methylation induction. To clarify components of inflammation involved, severe combined immunodeficiency (SCID) mice that lack functional T- and B-cells were similarly treated. Even in SCID mice, DNA methylation, along with colon tumors, were induced at the same levels as in their background strain of mice (C.B17). Comparative analysis of inflammation-related genes showed that *Ifng*, *Il1b* and *Nos2* had expression concordant with methylation induction whereas *Il2*, *Il6*, *Il10*, *Tnf* did not. These results showed that an epigenetic field defect is formed at early stages of colitis-associated carcinogenesis and that functional T and B cells are non-essential for the formation.

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Introduction

Epigenetic alterations are critically involved in human carcinogenesis (Jones and Baylin, 2007; Esteller, 2008). Especially, DNA methylation is stably inherited upon somatic cell replication (Riggs and Xiong, 2004) and methylation of promoter CpG islands (CGIs) consistently inactivates their downstream genes, including tumor-suppressor genes, such as *CDK2A*, *HIC1* and *SOCS1* (Ushijima, 2005). In spite of its importance, inducers of aberrant DNA methylation are still not fully understood. Among the few established inducers, importance of chronic inflammation, such as ulcerative colitis, hepatitis and gastritis due to *Helicobacter pylori* infection, has been shown by multiple studies (Hsieh *et al.*, 1998; Kondo *et al.*, 2000; Issa *et al.*, 2001; Niwa *et al.*, 2010; Hur *et al.*, 2011). In addition to chronic inflammation, aging and exogenous DNA, such as viruses, have been considered as inducers (Ushijima and Okochi-Takada, 2005).

Chronic inflammation-associated cancers are characterized by frequent occurrence of multiple cancers, suggesting that non-cancerous tissues exposed to chronic inflammation have already accumulated genetic and epigenetic alterations, forming a field for cancerization (Braakhuis *et al.*, 2003; Ushijima, 2007). Accumulation of genetic alterations is difficult to assess because their frequency in non-cancerous tissues is very low (Nagao *et al.*, 2001). In contrast, aberrant DNA methylation of various genes is accumulated at high levels in non-cancerous tissues exposed to chronic inflammation (Hsieh *et al.*, 1998; Kondo *et al.*, 2000; Issa *et al.*, 2001), and such accumulation levels are correlated with cancer risk (Schulmann *et al.*, 2005; Maekita *et al.*, 2006; Nakajima *et al.*, 2006; Garrity-Park *et al.*, 2010), forming an epigenetic field for cancerization (epigenetic field defect) (Ushijima, 2007). Methylation levels of marker genes, which show high methylation levels, are correlated with those of tumor-suppressor genes, which tend to show very low methylation levels (Maekita *et al.*, 2006; Ushijima, 2007).

However, it is still unclear when such an epigenetic field defect is formed during chronic inflammation-associated carcinogenesis, and how aberrant DNA methylation is induced by chronic inflammation. As inflammation involves multiple types of cells at different

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time points, it is important to investigate mechanisms of how such a field defect is formed *in vivo*. Such *in vivo* analysis has been hampered by the lack of appropriate animal models in which chronic inflammation and aberrant DNA methylation are involved, except for a *Helicobacter pylori* infection-induced gastritis model in Mongolian gerbils (Niwa *et al.*, 2010; Hur *et al.*, 2011). In mouse models, aberrant DNA methylation itself has been reported in lung (Vuillemenot *et al.*, 2004), skin (Fraga *et al.*, 2004), small intestine (Hahn *et al.*, 2008), and prostate (Yamashita *et al.*, 2008) cancers and in hematological malignancies (Yu *et al.*, 2005).

In this study, we focused on a mouse colitis model induced by dextran sulfate sodium (DSS), which share some aspects with human ulcerative colitis (Rosenberg *et al.*, 2009), as a possible model in which an epigenetic field defect is involved. After identification of CGIs aberrantly methylated in azoxymethane (AOM) and DSS-induced mouse colon cancers, we clarified when aberrant DNA methylation is induced in colonic epithelial cells during colitis and what cells are critically involved in the induction.

Results

Identification of CGIs methylated in colitis-associated mouse colon tumors

We started by identifying CGIs specifically methylated in mouse colon tumors associated with colitis. AOM/DSS-induced colon tumors and untreated normal colon epithelial samples were obtained from the animal experiment described in Figure 1a. Methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis was performed using a pool of the two tumors and a pool of two normal epithelial samples, and we isolated 23 candidate CGIs methylated in the tumors. Their methylation statuses were analyzed by methylation-specific PCR (MSP) of the samples used for the MeDIP-microarray analysis, three additional colon tumors and normal epithelial samples obtained from untreated mice. Fifteen of the 23 CGIs were specifically methylated in four or more of the five tumors (Figure 1b; Table 1). The presence of densely methylated DNA molecules was further confirmed by bisulfite sequencing for three CGIs (*Fosb*, *Hoxa5* and *Krt7*) in the five tumors (Figure 1c).

DNA methylation induction in colonic epithelial cells and its temporal profiles

DNA methylation of the 15 CGIs was then analyzed in epithelial samples exposed to AOM and/or DSS by quantitative MSP (qMSP) (Figure 1d and Supplementary Figure S1). Fourteen of them had significantly increased methylation levels in epithelial samples of AOM/DSS-treated and DSS-treated mice but not in those of AOM-treated mice. This showed that DNA methylation of these CGIs was associated with DSS treatment.

Epithelial samples might be contaminated with infiltrating blood cells although they were prepared by the crypt isolation technique. We therefore analyzed methylation levels of the 14 CGIs in peripheral blood, and three (*Fosb*, *Msx1* and *Sox11*) had low methylation levels (0–5.1 percentage of the methylated reference (PMR)) whereas the other 11 CGIs had high methylation levels (11–78 PMR; shown as numbers in parentheses in Figure 1d and Supplementary Figure S1). We further purified epithelial cells and blood leukocytes by fluorescence-activated cell sorting using Epcam (a pan-epithelial cell marker) and Cd45 (a pan-leukocyte marker), respectively, from colonic epithelial samples. At 15 weeks, methylation levels of the three CGIs with low methylation in peripheral blood (*Fosb*, *Msx1*, and *Sox11*) were significantly higher in the epithelial (Epcam-positive) cells of the DSS-treated mice than in those of untreated mice (7.3- to 19.3-fold; Supplementary Figure S3). These three CGIs were located inside the genes or in the 5′ far upstream region of the gene.

Using these three CGIs, temporal profiles of methylation induction were analyzed in the course of DSS-induced colitis (Figure 2a). All the three CGIs showed gradual increases of DNA methylation levels, starting at 8 weeks after DSS treatment and reaching 7.4- to 9.2-fold high levels, compared with untreated controls, at 15 weeks (Figure 2b). When the remaining 11 CGIs with high DNA methylation levels in peripheral blood were analyzed (Supplementary Figure S2), three (*Fut4*, *Hoxa5* and *Mex3a*) showed similar profiles to the above three CGIs and eight (*5730596B20Rik*, *Bcl6b*, *Epcam*, *Fmnl1*, *Irf2bp*, *Nav1*, *Rara* and *Sh2d3c*) showed a spiked increase immediately after the DSS treatment and a gradual decrease to control levels by 15 weeks.

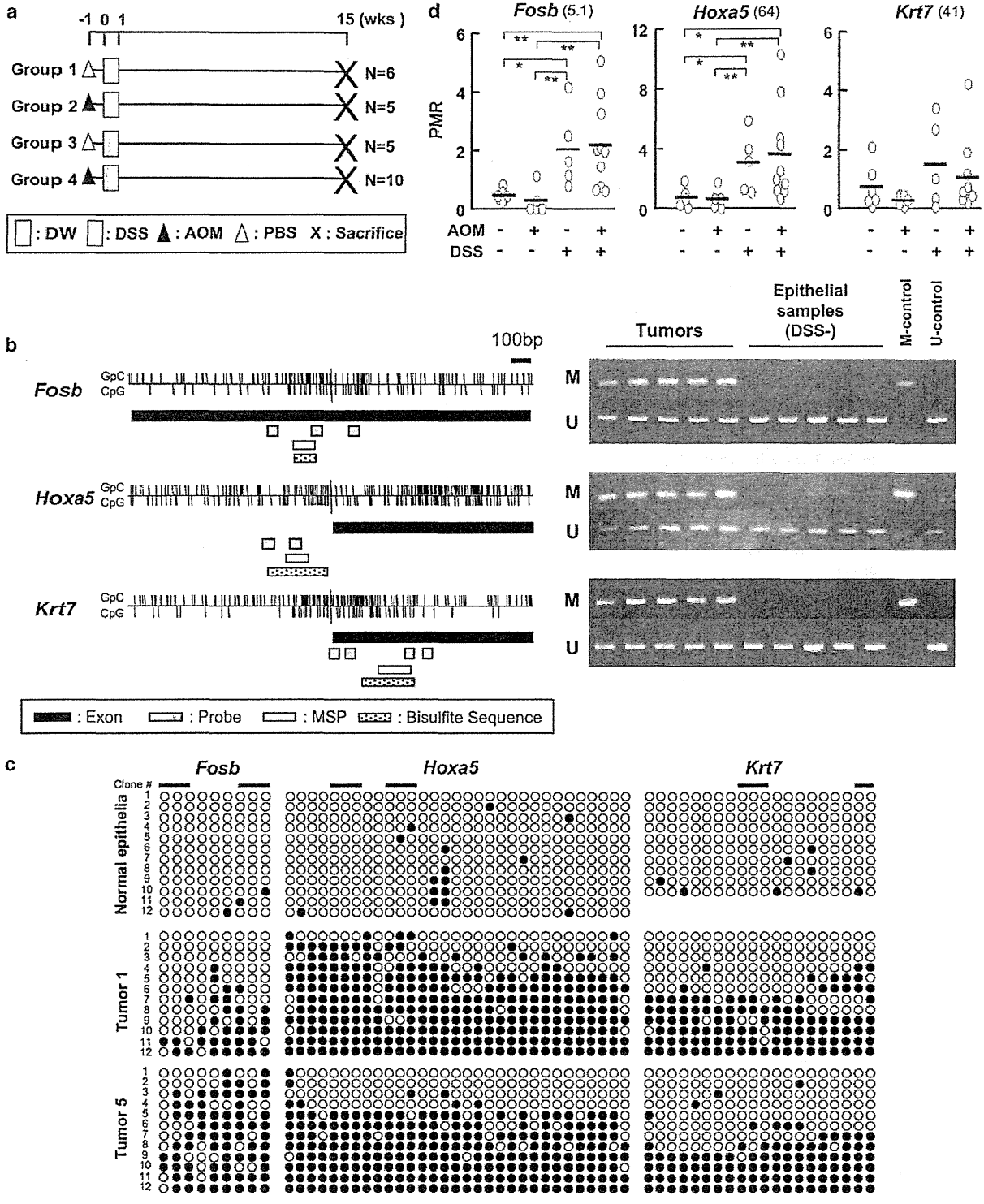
No induction of aberrant DNA methylation by treatment of colonic epithelial cells with DSS in vitro

DSS is known to have direct effects on epithelial cells, such as induction of cell growth arrest and production of cytokines (Ni *et al.*, 1996; Araki *et al.*, 2006), and this raised a possibility that DSS might directly induce aberrant DNA methylation in colonic epithelial cells. To address this possibility, immortalized normal mouse colonic epithelial cells (LIF-16) were treated with four concentrations of DSS that inhibited cell growth in a dose-dependent manner (Figure 3a). As the maximum dose (2%) did not affect cellular morphology (Figure 3b), the cells were cultured for 2, 5 and 8 weeks after 1 week of treatment with 2% DSS or in the presence of 2% DSS (Figure 3c). The methylation levels of the three CGIs (*Fosb*, *Msx1*, and *Sox11*) in the DSS-treated cells remained in the same range with those in untreated cells (Figure 3d). This showed that DSS itself was unlikely to induce DNA methylation in colonic epithelial cells.

Temporal profiles of inflammatory cell infiltration after the DSS treatment

In the previous study, we found that aberrant DNA methylation is induced in gastric epithelial samples exposed to specific kinds of inflammation (Hur *et al.*, 2011). Because a direct effect of DSS on methylation induction was unlikely, inflammation triggered by the

DSS treatment was suggested to be involved in the methylation induction. To identify specific inflammatory cells associated with DNA methylation induction, their infiltration was assessed by counting the number of infiltrating lymphocytes, macrophages, and neutrophils in colonic mucosae and submucosae (Figure 2c). Lymphocyte and neutrophil infiltration was most severe immediately after the DSS treatment, then gradually decreased, but was still present even at weeks 15. Macrophage infiltration stayed at high levels through-



out the experimental period. These results showed that inflammation was present in the DSS-treated colon throughout the experimental period despite DSS being transiently administered, and that dominantly infiltrating cells shifted from neutrophils/lymphocytes to macrophages. The number of inflammatory cells did not parallel the temporal profiles of gradually increasing DNA methylation levels of the six CGIs (*Fosb*, *Fut4*, *Hoxa5*, *Mex3a*, *Msx1* and *Sox11*).

Carcinogenicity and DNA methylation induction in severe combined immunodeficiency mice

As the infiltration of inflammatory cells in wild-type (BALB/c) mice after DSS treatment was extensive, identification of specific inflammatory cells and inflammation-related genes responsible for DNA methylation induction was difficult. Therefore, we adopted a strategy of analyzing carcinogenicity and DNA methylation induction in severe combined immunodeficiency (SCID) mice (Figure 4a), which lack functional T- and B-cells but have normal innate immunity (Bosma *et al.*, 1983). So far, there has been no report of aberrant methylation induction by DSS colitis in SCID mice. Tumor incidence and multiplicity showed no significant difference between SCID and its wild-type control, C.B17 (Figure 4b; Table 2), demonstrating that functional T- and B-cells are not essential for tumor induction in SCID mice. Methylation of *Fosb*, *Msx1* and *Sox11* was significantly induced in colonic epithelial samples of AOM/DSS-treated SCID mice to almost the same level as those in C.B17 mice (Figure 4c), demonstrating that functional T- and B-cells are dispensable for methylation induction.

Expression of inflammation-related genes in DSS-treated SCID mice

Infiltration of inflammatory cells and expression levels of inflammation-related genes were analyzed in the

Table 1 CGIs aberrantly methylated in mouse colon tumors

	Gene symbol	*Gene name*	*Accession numbers*	*Affected CGI region*[a]	*Chromosome*	*Position*[b]
1	*5730596B20Rik*	RIKEN cDNA 5730596B20 gene	NM_175261	52106690-52106900	6	5′ Proximal upstream
2	*Bcl6b*	B-cell CLL/lymphoma 6, member B	NM_007528	70042575-70042939	11	Inside
3	*Epcam*	epithelial cell adhesion molecule	NM_008532	87548510-87548927	17	Inside
4	*Fmnl1*	formin-like 1	NM_019679	103015371-103015514	11	3′ Downstream
5	*Fosb*	FBJ osteosarcoma oncogene B	NM_008036	18463460-18463668	7	Inside
6	*Fut4*	fucosyltransferase 4	NM_010242	14501158-14501694	9	Inside
7	*Hoxa5*	homeobox A5	NM_010453	52134331-52134506	6	5′ Proximal upstream
8	*Irf2bp1*	interferon regulatory factor 2-binding protein 1	NM_178757	18163624-18163938	7	Inside
9	*Krt7*[c]	keratin 7	NM_033073	101240421-101240949	15	Inside
10	*Mex3a*	mex3 homolog A	NM_001029890	88622212-88622469	3	Inside
11	*Msx1*	homeobox, msh-like 1	NM_010835	38109507-38109773	5	Inside
12	*Navl*	neuron navigator 1	NM_175447	137401177-137401323	1	Inside
13	*Rara*	retinoic acid receptor, alpha	NM_009024	98752420-98752549	11	5′ Far upstream
14	*Sh2d3c*	SH2 domain-containing 3C	NM_013781	32563730-32563900	2	Inside
15	*Sox11*	SRY-box-containing gene 11	NM_009234	27929531-27929660	12	5′ Far upstream

Abbreviation: CGI, CpG island.
[a]Region in the corresponding chromosome annotated by UCSC mm8 (NCBI Build 36, February 2006).
[b]Relative position to the gene (5′ far upstream, a region more than 300 bp upstream of the transcription start site; 5′ proximal upstream, a region within 300 bp upstream of the transcription start site; inside, a region in the exon or intron; and 3′ downstream, a region within 10 kbp downstream of the last exon).
[c]A gene whose methylation in non-cancerous mucosae was not significantly elevated.

Figure 1 Identification of CGIs methylated in mouse colon tumors and colonic epithelial samples exposed to DSS. (**a**) Experimental protocol of tumor induction by AOM and DSS in BALB/c mice. Tumor incidence of this experiment is summarized in Supplementary Table S1. (**b**) CpG map of a CGI, and its methylation in tumor samples (data shown for representative three CGIs). Vertical lines, individual CpG or GpC sites; open boxes, positions of MSP products; closed boxes, positions of exons; gray boxes, locations of probes in CGI microarrays; and dotted boxes, positions of bisulfite sequencing. MSP was performed using two pairs of colon tumors and normal epithelial samples without DSS treatment used for MeDIP-CGI microarray analysis and three additional pairs. M, MSP using primers specific to methylated DNA; U, MSP using primers specific to unmethylated DNA; M-control, fully methylated genomic DNA; and U-control, fully unmethylated DNA. (**c**) The presence of dense methylation in AOM/DSS-induced mouse colon tumors. Bisulfite sequencing of *Fosb*, *Hoxa5* and *Krt7* was performed in epithelial samples of an untreated mouse aged 22 weeks (group 1, weeks 15) and two AOM/DSS-induced colon tumors (group 4, weeks 15). Ten to 12 DNA molecules were analyzed per sample for a gene. Three other tumors not presented here showed similar methylation patterns. Bars, CpG sites interrogated by MSP primers; open circles, unmethylated CpG sites; and closed circles, methylated CpG sites. (**d**) DNA methylation levels in colonic epithelial samples from AOM/DSS, AOM, DSS and untreated groups. DNA methylation levels were quantified by qMSP. Bold horizontal bars indicate average. Numbers in parentheses show methylation levels in peripheral blood of mice aged 22 weeks. Methylation levels were shown to be increased by DSS treatment. $*P<0.05$; $**P<0.01$.