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DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: Its possible involvement in the formation of epigenetic field defect

Takayuki Ando[1,2], Takeichi Yoshida[1,3], Shotaro Enomoto[3], Kiyoshi Asada[1], Masae Tatematsu[4], Masao Ichinose[3], Toshiro Sugiyama[2] and Toshikazu Ushijima[1]*

[1]*Carcinogenesis Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan*
[2]*Third Department of Internal Medicine, Toyama University, Toyama, Japan*
[3]*Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan*
[4]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, Chikusa, Nagoya, Japan*

Accumulation of aberrant DNA methylation in normal-appearing gastric mucosae, mostly induced by *H. pylori* infection, is now known to be deeply involved in predisposition to gastric cancers (epigenetic field defect), and silencing of protein-coding genes has been analyzed so far. In this study, we aimed to clarify the involvement of microRNA (miRNA) gene silencing in the field defect. First, we selected three miRNA genes as methylation-silenced after analysis of six candidate "methylation-silenced" tumor-suppressor miRNA genes. Methylation levels of the three genes (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*) were quantified in 56 normal gastric mucosae of healthy volunteers (28 volunteers with *H. pylori* and 28 without), 45 noncancerous gastric mucosae of gastric cancer patients (29 patients with *H. pylori* and 16 without), and 28 gastric cancer tissues (13 intestinal and 15 diffuse types). Among the healthy volunteers, individuals with *H. pylori* had 7.8–13.1-fold higher methylation levels than those without ($p < 0.001$). Among individuals without *H. pylori*, noncancerous gastric mucosae of gastric cancer patients had 7.2–15.5-fold higher methylation levels than gastric mucosae of healthy volunteers ($p < 0.005$). Different from protein-coding genes, individuals with past *H. pylori* infection retained similar methylation levels to those with current infection. In cancer tissues, methylation levels were highly variable, and no difference was observed between intestinal and diffuse histological types. This strongly indicated that methylation-silencing of miRNA genes, in addition to that of protein-coding genes, contributed to the formation of a field defect for gastric cancers.

Key words: field for cancerization; microRNA; methylation; gastric cancer; *Helicobacter pylori*

Metachronous occurrence of gastric cancers is becoming an important issue as localized resection of early gastric cancers by endoscopic submucosal dissection (ESD) has become common.[1] The incidence of secondary primary gastric cancers after ESD reaches as high as 2.0% per year[2] whereas the incidence of gastric cancer in the general Japanese population is 0.14% per year.[3] This indicates that noncancerous gastric mucosae are already predisposed to developing gastric cancers, forming a field defect (field for cancerization). High incidences of metachronous cancers have been known not only for gastric cancers but also for bladder, liver, and esophageal cancers[4–6] and are becoming recognized for lung, breast and colorectal cancers.[7–9]

A molecular basis for the field defect has been considered as an accumulation of genetic and epigenetic alterations in normal-appearing tissues. Traditionally, cells with a genetic alteration were considered to form a physically continuous patch, producing a genetically altered field.[10] Recently, we found that aberrant DNA methylation of specific genes can be induced in as high as several percentage of cells in noncancerous gastric mucosae (thus in multiple independent gastric glands), and the degree of methylation is associated with gastric cancer risks.[11,12] Importantly, *Helicobacter pylori* infection, a major carcinogenic factor in the stomach, was shown to potently induce aberrant DNA methylation in gastric epithelial cells.[11] In addition to gastric cancers, the presence of aberrant DNA methylation in noncancerous tissues and possible association with cancer risks have been reported for liver,[13] colon,[14] esophageal,[15] breast[16] and renal cancers.[17]

Genes so far analyzed in noncancerous gastric mucosae are those methylated in gastric cancers, including tumor-suppressor genes, such as *CDKN2A*, *MLH1*, *CDH1*, *LOX* and *APC*,[2,11,18,19] and genes with little or no expression in normal gastric mucosae, such as *FLNc*, *HAND1* and *THBD*. The latter group of genes is methylated in parallel with tumor-suppressor genes but with higher frequencies, and is considered as a good marker to detect the presence of an epigenetic field defect.[11] In contrast with these protein-coding genes, involvement of microRNA (miRNA) silencing in field defect formation has not been clarified yet. Since the role of aberrant expression or reduction of various miRNAs in human multistep carcinogenesis is now clear,[20,21] there is a possibility that miRNAs silencing by aberrant DNA methylation is involved in field defect formation. Indeed, several tumor-suppressor miRNAs, including *miR-124a*,[22] *miR-137*, *miR-193a*[23] and *miR-127*,[24] are reported to be silenced by aberrant DNA methylation of their promoter CpG islands (CGI) in cancers.

In this study, we aimed to clarify whether or not miRNA silencing by DNA methylation can be involved in the formation of a field defect for gastric cancers. First, we searched for miRNAs that are reported to have tumor-suppressive functions and be controlled by DNA methylation, and confirmed methylation-silencing of these candidate genes. Then, we quantified their methylation levels in gastric mucosae of healthy volunteers, noncancerous gastric mucosae of gastric cancer patients, and primary gastric cancer tissues.

Material and methods

Cell lines and tissue samples

Six gastric cancer cell lines, AGS, KATOIII, MKN28, MKN45, MKN74 and NUGC3 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44 and HSC57 were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. GC2 was developed by M. T. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan. 5-Aza-2′-deoxycitidine (5-aza-dC) treatment was performed with AGS, HSC57 and MKN28. Cells were seeded on day 0, media was added with freshly prepared 5-aza-dC on days 1 and 3, and cells were harvested on day 5. The concentrations of 5-aza-dC were determined as minimum concentrations that deplete DNMT1.[25]

Gastric mucosae were obtained by endoscopic biopsy of antral regions from 56 healthy volunteers (25 male and 31 female; aver-

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***Correspondence to:** Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan.
Fax: +81-3-5565-1753. E-mail: tushijim@ncc.go.jp

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age age 53, ranging from 27 to 91) and 45 gastric cancer patients (35 male and 10 female; average age 66, ranging from 38 to 89). Gastric cancer tissues were obtained from 28 gastric cancer patients (21 male and 7 female; average age 66, ranging from 49 to 81; 13 intestinal and 15 diffuse types) who underwent gastrectomy due to gastric cancers. Gastric epithelial cells were obtained by the gland isolation technique from eight noncancerous gastric tissues. Informed consents were obtained from all the patients and healthy volunteers. Gastric mucosae, noncancerous mucosae and cancer tissues were frozen in liquid nitrogen immediately after biopsy or resection, and stored at −80°C until extraction of genomic DNA. High molecular weight DNA was extracted by the phenol/chloroform method. RNA was isolated with ISOGEN (Nippon Gene, Tokyo, Japan).

H. pylori infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan), or culture test (Eiken, Tokyo, Japan). All cancers were histologically diagnosed according to the Japanese classification of gastric carcinoma,[26] and classified according to the Lauren classification system.[27]

Sodium bisulfite modification, methylation-specific PCR (MSP), quantitative real-time MSP and bisulfite sequencing

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA) 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 as previously described,[28] and the modified DNA was suspended in 40 μl of Tris-EDTA buffer. An aliquot of 1 μl was used for methylation-specific PCR (MSP) and Quantitative real-time MSP (qMSP) with a primer set specific to methylated (M) or unmethylated (U) sequences.

For MSP, the fully methylated and unmethylated DNA was used to determine an annealing temperature that specifically amplifies only methylated or unmethylated DNA. A minimum number of PCR cycles to obtain visible bands was determined using the fully (un)methylated DNA, and four cycles were added for analysis of gastric cancer cell lines. The primers were designed just upstream of reported transcription start sites within the CGI (Table I; Fig. 1*a*), whose methylation statuses are now known to be critical for induction of gene silencing.[29,30]

qMSP was performed by real-time PCR using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Although the same primer set was used for qMSP, a specific annealing temperature in the presence of SYBR® Green I was redetermined using the fully methylated and unmethylated DNA. The number of molecules in a sample was determined by comparing its amplification with those of standard DNA that contained exact numbers of molecules (10^1–10^6 molecules). Based on the numbers of M molecules and U molecules for a genomic region, a methylation level of the region was calculated as the fraction of M molecules in the total number of DNA molecules (# of M molecules + # of U molecules). The standard DNA samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI), respectively, or by purifying the PCR products using the Wizard SV Gel and PCR clean-up system (Promega).

For bisulfite sequencing, an aliquot of 1 μl of the sodium bisulfite-treated DNA was amplified by PCR with the primers common to methylated and unmethylated DNA sequences (Table I). The PCR product was cloned into pGEM-T Easy vector (Promega), and 15 clones or more were cycle-sequenced for each sample.

Quantitative real-time reverse transcription (RT)-PCR

For quantitative RT-PCR, cDNA was synthesized from 10 ng of total RNA using TaqMan® MicroRNA-specific primers and a

TABLE I – PRIMERS AND CONDITIONS FOR MSP AND REAL-TIME MSP

Gene	M/U	Primer sequence		Length (bp)	Anneal (°C)		Number of cycles for MSP
		Forward (5′→3′)	Reverse (5′→3′)		MSP	Real-time MSP	
miR-124a-1	M	AGAGTTTTTGGAAGACGTCG	AAAAAAATAAAAAACGACGC	155	58	58	36
	U	AATAAAGAGTTTTTGGAAGATGTTT	CAAAAA AAA AAAATAAAAAACAACAC	166	58	58	36
miR-124a-2	M	GGTTTATGTATGTTTTTAGGCG	TCCGTAAAAATATAAACGATAG	93	59	56	32
	U	TAGGTTTATGTATGTTTTTAGGTG	CTATTCCATAAAAATATAAACAATACA	99	52	50	36
miR-124a-3	M	GATAGTATAGTCGGTTGAGCGTAGC	CCTCAAAACTAAAACGAACGACG	152	61	59	31
	U	TAGTTGGTTGAGTGTAGTGTTTTTG	CAAAACTAAAACAAACAAACAATC	142	61	59	36
miR-137	M	TAGGGGCGGGTTAGCG	TACCGCTACCGCTACTACC	99	57	–	36
	U	TTTTGGTGGTGGTGGTGGT	ACCCAAAAATACCATCACCA	113	63	–	35
miR-193a	M	GAGGTAGTTTGGTCGGAGCGTAC	GACCCCCGAAACCAACG	86	61	–	36
	U	ATTGATTTATATTTTTGAGAGTGTTG	TCCCAAACTAACATACACTCCA	153	58	–	35
miR-127	M	GTTTGGGGAGAGCGTAAACG	GTAAACGAACACCGCACCG	96	63	–	34
	U	GTTTTGTGAATTATTTGGTTTTG	TTACAAATATCCCTCCACCAC	176	58	–	38

Gene	Primers for bisulfite sequencing		Length (bp)	Anneal (°C)	Number of cycles
	Forward (5′→3′)	Reverse (5′→3′)			
miR-124a-1	AAGGATGGGGGAGAATAAAGAGTTT	CTCAACCAACCCCATTCTTAACATT	354	60	32
miR-124a-2	ATTAGATTTATAGGTTTATGTATGTTTTAGG	ACTCTTCTCTCCCTACCCATC	235	54	30
miR-124a-3	GAAAGGGGAGAAGTGTGGGTTTTT	CTCTTAACATTCACCGCGTACCTTAAT	268	54	32

M, Primers specific to methylated DNA; U, Primers specific to unmethylated DNA.

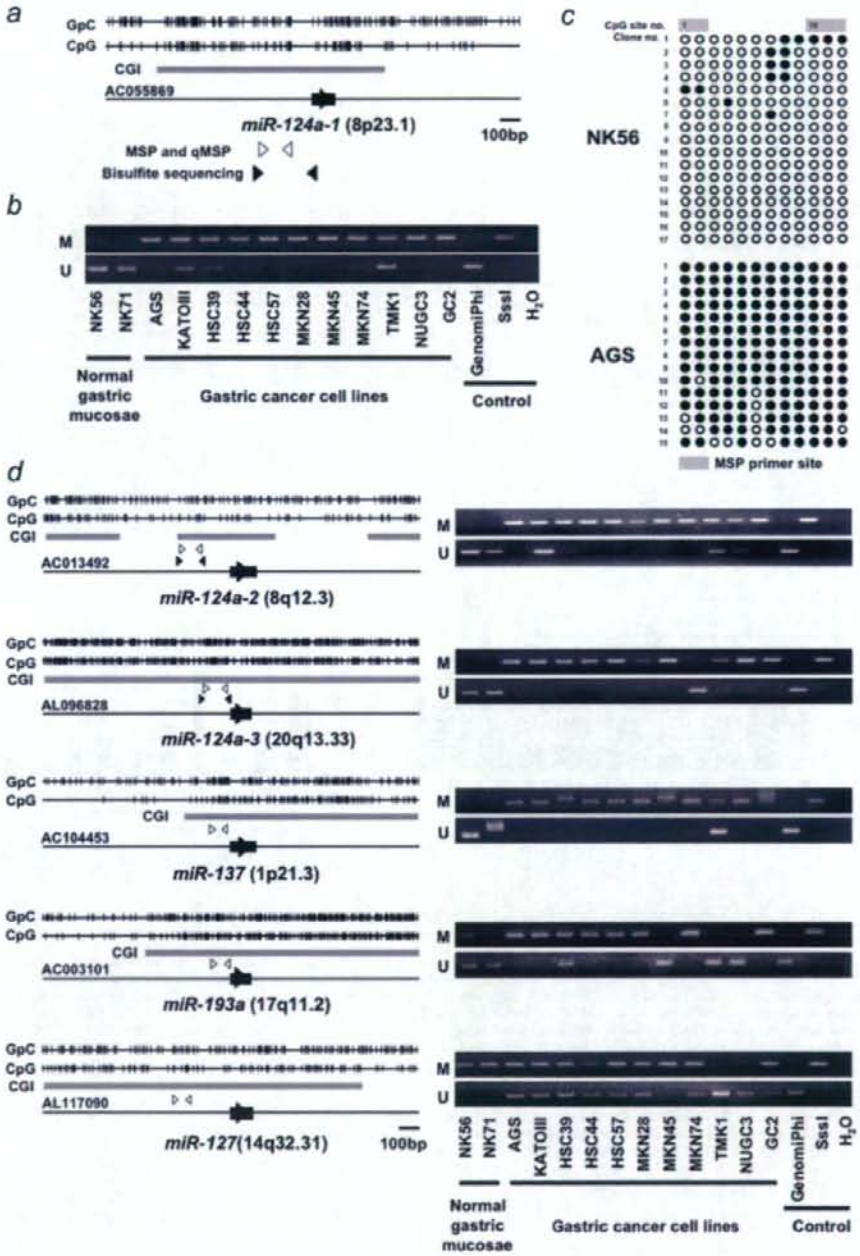

FIGURE 1 – Genomic structures and methylation statuses of the six miRNA genes in gastric cancer cell lines. (*a*) Structures of the *miR-124a-1* gene. Vertical ticks, individual GpC (top) and CpG sites (bottom); gray box, CGI; closed box, genomic location of *miR-124a-1*; open arrowheads, locations of the primers for MSP and real-time MSP; and closed arrowheads, locations of the primers for bisulfite sequencing. (*b*) Methylation statuses of *miR-124a-1* in normal gastric mucosae and 11 gastric cancer cell lines analyzed by MSP. SssI, genomic DNA methylated by *Sss*I methylase; GenomiPhi, genomic DNA amplified by GenomiPhi; and M and U, primer sets specific to methylated and unmethylated DNA, respectively. (*c*) The methylation status of a CGI around *miR-124a-1* analyzed by bisulfite sequencing. Twelve CpG sites were analyzed in NK56 normal and AGS gastric cancer cell lines, and 15 clones or more were sequenced for each sample. Closed circle, methylated CpG site; and open circle, unmethylated CpG site. (*d*) Genomic structures of five other miRNA genes and their methylation statuses in normal gastric mucosae and 11 gastric cancer cell lines analyzed by MSP.

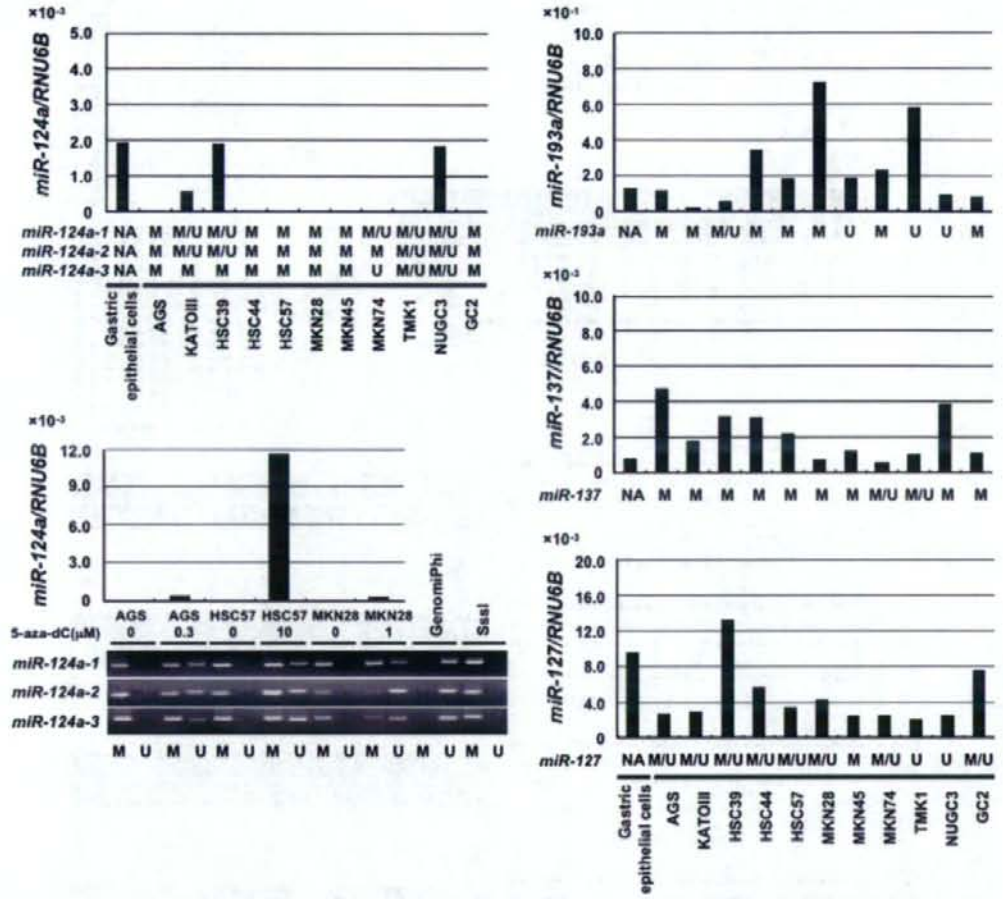

FIGURE 2 – Expression levels of miRNAs in gastric epithelial cells and gastric cancer cell lines. Expression levels were analyzed by quantitative RT-PCR, and normalized to *RNU6B* expression. Gastric epithelial cells were obtained by gland isolation technique from noncancerous tissues of eight gastric cancer patients, and average expression levels of the eight patient samples are shown. Results of MSP were duplicated from Figure 1 for convenience. M, M/U and U represent the presence of only methylated DNA, both methylated and unmethylated DNA, and only unmethylated DNA, respectively. NA, not applicable. Only *miR-124a* showed consistent repression in cell lines without unmethylated DNA molecules. After treatment by 5-aza-dC, *miR-124a* was re-expressed abundantly in HSC57, in association with demethylation, and in AGS and MKN28.

TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the ABI Prism 7300 Fast Real-Time PCR System (Applied Biosystems). Expression levels of target miRNAs were normalized to that of a small nuclear RNA *RNU6B* transcript.

Statistical analysis

A difference in mean methylation levels was analyzed by the *t*-test Welch method, and differences in methylation incidence in gastric cancer tissues were analyzed by the chi-square test. Correlation between the age and methylation levels of miRNA genes, and correlation between methylation levels of each gene were analyzed using Spearman's rank correlation coefficient. All the analyses were performed using SPSS (SPSS, Inc., Chicago, IL), and the results were considered significant when a p value less than 0.05 was obtained by two-sided tests.

Results

Identification of miRNAs silenced in gastric cancer cell lines

Six genes of four miRNAs (*miR-124a, miR-137, miR-193a* and *miR-127*) were reported to have a tumor-suppressive function and be controlled by DNA methylation in colon, bladder and oral cancers.[22–24] We first analyzed methylation statuses of their putative promoter regions in 11 gastric cancer cell lines and two normal gastric mucosae of healthy individuals without *H. pylori*. It was found that *miR-124a-1, miR-124a-2, miR-124a-3* and *miR-137* were unmethylated in the normal gastric mucosae, but were completely methylated (no unmethylated DNA molecules detected)

frequently in the cell lines (Figs. 1*b*–1*d*). *miR-193a* was partially methylated in one of the two normal gastric mucosae, and completely methylated frequently in gastric cancer cell lines. In contrast, *miR-127* was completely methylated in the normal gastric mucosae, but unmethylated in the gastric cancer cell lines.

We then examined the effect of methylation of the putative promoter regions on miRNA expression (*miR-124a* for *miR-124a-1*, *miR-124a-2* and *miR-124a-3* genes; *miR-137*; *miR-193a*; *miR-127*) in the 11 gastric cancer cell lines and gastric epithelial cells obtained by the gland isolation technique (Fig. 2). *miR-124a* was consistently unexpressed in six cell lines with simultaneous methylation of its three isoforms (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*), but was expressed in the gastric epithelial cells. In contrast, *miR-137*, *miR-193a*, and *miR-127* were expressed even in cell lines with complete methylation. This showed that these three miRNA genes were not silenced by their "promoter" methylation, and indicated that, in contrast, *miR-124a* was silenced by promoter methylation of its three isoforms.

Methylation-silencing of *miR-124a* was further confirmed by analyzing its re-expression in association with its promoter demethylation after treatment with a demethylating agent, 5-aza-dC, in three cell lines (AGS, HSC57 and MKN28). Re-expression and appearance of unmethylated DNA molecules were observed in all the three cell lines, HSC57 being prominent. This further orted that *miR-124a* was methylation-silenced.

The presence of miR-124a *methylation in primary gastric cancers*

Since methylation-silencing was identified only for *miR-124a*, we analyzed methylation levels of its three genes (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*), along with a representative protein-coding gene (*LOX*), in 28 primary gastric cancer tissues (13 intestinal and 15 diffuse types) by qMSP. The fact that densely methylated DNA molecules were being measured was confirmed by bisulfite sequencing (Supp. Info. Fig. 1). *miR-124a-1* showed a distribution of methylation levels similar to *LOX*, some having no methylation and the others having various levels of methylation (Fig. 3*a*). This was consistent with our previous finding that cancer samples could be essentially classified into two groups (cancers with and without methylation), and that the various degrees of methylation levels in methylation-positive cancer samples were mainly due to contamination of normal cells.[18] On the other hand, *miR-124a-2* and *miR-124a-3* showed a unimodal distribution of methylation levels, suggesting that they are susceptible to methylation induction in cancer tissues. Using a cut-off value of 6%, as in previous reports,[31,32] *miR-124a-1*, *miR-124a-2* and *miR-124a-3* were methylated in 11, 23 and 26 of the 28 samples, respectively. Between the two histological types, the incidences of methylation were the same for *miR-124a-1*, *miR-124a-2* and *miR-124a-3* (p = 0.95, 0.84 and 0.67) (Supp. Info. Fig. 2a).

We further analyzed an association between methylation and expression of *miR-124a* in an additional 19 gastric cancer samples. Using a cut-off value of 6%, eight samples had methylation of all the three *miR-124a* genes, and the other 11 samples had methylation of only one or two genes and retained at least one unmethylated gene. *miR-124a* was barely expressed in all the eight samples with methylation of the three genes whereas it was expressed in 5 of 11 cancer samples with at least one unmethylated gene (Fig. 3*b*).

Accumulation of methylation in H. pylori *positive gastric mucosae, and its association with gastric cancer risk*

Methylation levels of *miR-124a-1*, *miR-124a-2* and *miR-124a-3*, again along with *LOX*, were analyzed by qMSP in gastric mucosae of 56 healthy volunteers (28 volunteers with *H. pylori* and 28 without) and noncancerous gastric mucosae of 45 gastric cancer patients (29 patients with *H. pylori* and 16 without) (Fig. 3*b*). Among the healthy volunteers, the mean methylation levels of *miR-124a-1*, *miR-124a-2*, *miR-124a-3* and *LOX* in the *H. pylori*-positive individuals were 13.1-, 7.8-, 8.9- and 46.7-fold, respectively, as high as those in *H. pylori*-negative individuals. This showed that *H. pylori* infection was associated with aberrant methylation of not only protein-coding genes but also miRNA genes.

Next, methylation levels in gastric mucosae of healthy volunteers were compared with those of noncancerous gastric mucosae of gastric cancer patients. Since potent methylation induction by *H. pylori* can mask a difference in *H. pylori*-positive individuals, the comparison was made among *H. pylori*-negative individuals only (28 healthy volunteers and 16 gastric cancer patients) (Table II; Fig. 3*c*). The mean methylation levels of the three miRNA genes and *LOX* were much higher in noncancerous gastric mucosae of gastric cancer patients than those of gastric mucosae of healthy volunteers (15.5-, 7.2-, 13.3- and 24.7-fold, respectively). Between the two histological types, the mean methylation levels were not different (Supp. Info. Fig. 2b).

Correlations among methylation levels of miRNA genes and *LOX* were examined by calculating correlation coefficients. Correlations among the three miRNA genes were very strong, but correlations between a miRNA gene and *LOX* were weak or absent (Table III; Supp. Info. Fig. 3).

No effect of age and sex on methylation levels on miRNA genes

Methylation of various CGIs is reported to be correlated with age.[33,34] Also, males have twice as high an incidence of gastric cancers as females.[1] In *H. pylori*-negative healthy volunteers, methylation levels of *miR-124a-1*, *miR-124a-2 and miR-124a-3* were not correlated with age (Spearman correlation test: r = 0.19, 0.01 and 0.29; p = 0.35, 0.94 and 0.15), and not associated with sex (p = 0.05, 0.68 and 0.19). Also, in *H. pylori*-positive healthy volunteers, methylation levels were not correlated with age (r = 0.13, 0.18 and −0.1; p = 0.51, 0.35 and 0.51), and not associated with sex (p = 0.70, 0.20 and 0.67).

Discussion

The present study showed that significantly higher methylation levels of three miRNA genes (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*) were present in gastric mucosae of *H. pylori*-positive healthy volunteers, indicating that *H. pylori* infection can induce DNA methylation of miRNA genes, in addition to protein-coding genes. Moreover, it was also shown that methylation levels of the miRNA genes in noncancerous gastric mucosae of gastric cancer patients were higher than those in gastric mucosae of healthy volunteers among *H. pylori* negative individuals, indicating that miRNA silencing is involved in the formation of a field defect for gastric cancers. To our knowledge, the presence of miRNA silencing in a field for cancerization was shown here for the first time.

Recent studies demonstrated that expression of some miRNAs is regulated by epigenetic mechanisms.[24,35] From six miRNA genes that were reported to be silenced by promoter methylation and to have tumor-suppressor functions, we were able to confirm that three genes of *miR-124a* were methylation-silenced in gastric cancer cell lines. The other three genes, *miR-137*, *miR-193a* and *miR-127*, were expressed even in cell lines with complete methylation, and were unlikely to be silenced by promoter methylation in gastric cancers. Since methylation of putative promoter regions consistently represses transcription of their downstream genes,[29,30] the presence of the expression of the three genes in gastric cancer cell lines with complete methylation of their "promoter" CGI indicated that the three genes had additional or alternative promoters.

Lujambio *et al.*[22] discovered that *miR-124a* was silenced by promoter methylation after screening 320 miRNA genes. They also found that *miR-124a* down-regulates CDK6, a demonstrated oncogene involved in cell cycle progression and differentiation, and induces hypophosphorylation of RB.[22] Therefore, it is possible that *miR-124a* silencing is also involved in gastric carcinogenesis, and the presence of its silencing in noncancerous tissues

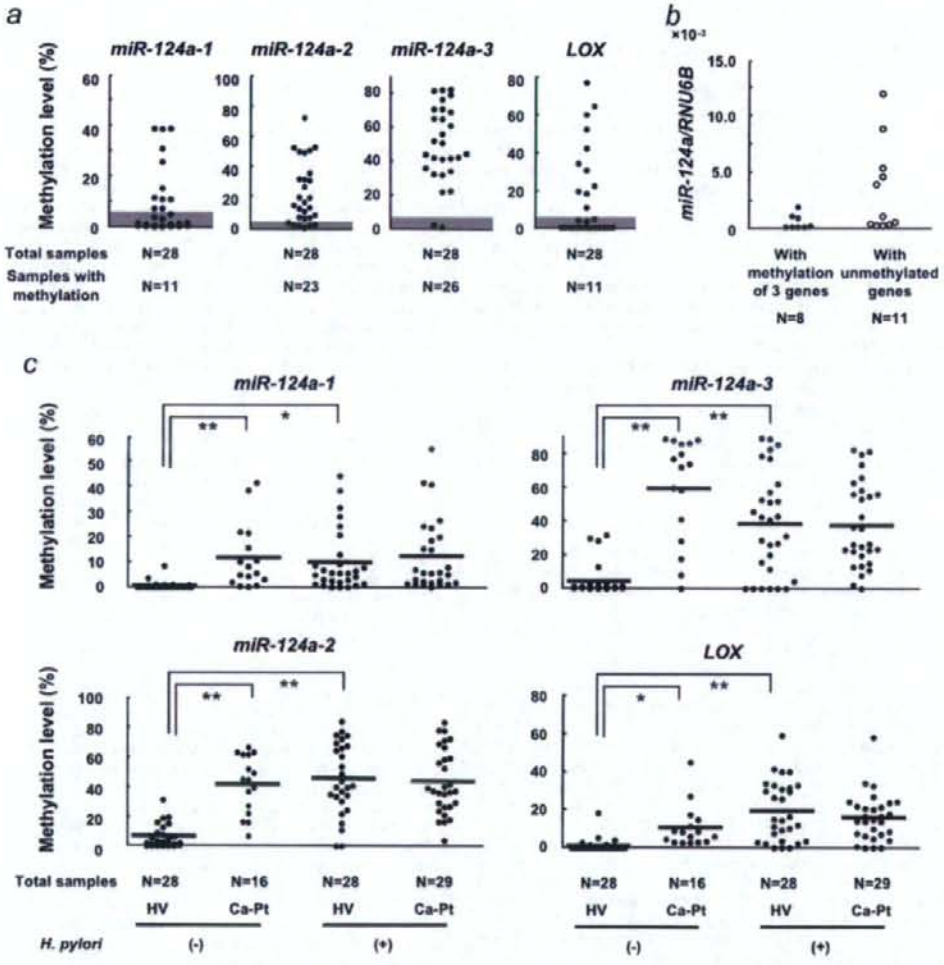

FIGURE 3 – Methylation levels of *miR-124a-1*, *miR-124a-2*, *miR-124a-3* and *LOX* in gastric mucosae of healthy volunteers, noncancerous mucosae of gastric cancer patients, and cancer tissues. (*a*) Distribution of methylation levels in gastric cancers. Gray areas show samples with methylation levels below the cut-off value of 6%. (*b*) Expression of *miR-124a* in eight cancer samples with methylation (three *miR-124a* genes, methylation positive) and 11 cancer samples with at least one unmethylated gene (*miR-124a-1* or *miR124a-3*). Five of the 11 cancers with unmethylated genes had high *miR-124a* expression levels. (*c*) Distribution of methylation levels in gastric mucosae of healthy volunteers (HV) and noncancerous mucosae of gastric cancer patients (Ca-Pt). A horizontal line represents a mean methylation level for each group. Among the healthy volunteers, *H. pylori*-positive individuals had 7.8–46.7-fold as high methylation levels as *H. pylori*-negative individuals ($*p < 0.005$; $**p < 0.001$). Among the *H. pylori*-negative individuals, noncancerous gastric mucosae of gastric cancer patients had 7.2–24.7-fold as high methylation levels as gastric mucosae of healthy volunteers ($*p < 0.005$; $**p < 0.001$).

TABLE II – MEAN METHYLATION LEVELS AND STANDARD DEVIATIONS OF THE FOUR GENES IN GASTRIC MUCOSAE OF HEALTHY VOLUNTEERS AND GASTRIC CANCER PATIENTS

		N	*miR-124a-1*	*miR-124a-2*	*miR-124a-3*	*LOX*
H.pylori(−)	(1) Healthy volunteers	28	0.76 ± 1.70	5.75 ± 7.73	4.45 ± 9.29	0.43 ± 1.22
	(2) Gastric cancer patients	16	11.82 ± 12.94	41.66 ± 19.33	59.42 ± 30.71	10.64 ± 11.33
H.pylori(+)	(3) Healthy volunteers	28	9.96 ± 12.28	44.79 ± 23.96	39.66 ± 30.08	20.10 ± 15.72
	(4) Gastric cancer patients	29	12.28 ± 14.31	46.33 ± 28.36	37.48 ± 25.13	15.93 ± 12.58
p value	(1) *vs.* (3)		<0.001	$<10^{-8}$	$<10^{-5}$	$<10^{-6}$
	(1) *vs.* (2)		0.004	$<10^{-5}$	$<10^{-6}$	0.003
	(3) *vs.* (4)		0.51	0.77	0.77	0.28

TABLE III – CORRELATION AMONG METHYLATION LEVEL OF *miR-124A-1, miR-124A-2, miR-124A-3* AND *LOX*

	miR-124a-1		*miR-124a-2*		*miR-124a-3*		*LOX*	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
miR-124a-1	–	–	0.70	$<10^{-15}$	0.77	$<10^{-20}$	0.03	0.76
miR-124a-2	0.70	10^{-15}	–	–	0.72	$<10^{-16}$	0.37	$<10^{-3}$
miR-124a-3	0.77	$<10^{-20}$	0.72	$<10^{-16}$	–	–	0.20	0.04

r, correlation coefficient.

could be directly associated with predisposition to developing gastric cancers.

As repeatedly shown by epidemiological studies, the majority of *H. pylori*-negative individuals with a gastric cancer are considered to have past exposure to *H. pylori*.[36] Methylation levels of protein-coding genes, including *LOX*, in the gastric mucosae of individuals with past infection (gastric cancer patients without *H. pylori*) were lower than those of individuals with current infection (both healthy volunteers and gastric cancer patients) in our previous study.[11] Actually, incidences of aberrant methylation and methylation levels of *CDH1* are reported to decrease after the eradication of *H. pylori*,[19,37] showing that DNA methylation in gastric mucosae decreases when *H. pylori* infection discontinues. Interestingly, methylation levels of the three miRNA genes in the gastric mucosae of individuals with past infection by *H. pylori* (gastric cancer patients without *H. pylori*) did not decrease compared with those of individuals with current infection (healthy volunteers and gastric cancer patients). Since aberrant methylation induced in stem cells is expected to persist even after *H. pylori* infection discontinues, DNA methylation of these miRNA genes might be relatively more easily induced in gastric stem cells than those of protein-coding genes.

In conclusion, our data indicated that DNA methylation of certain miRNA genes was associated with *H. pylori* infection, in addition to protein-coding genes, and involved in the formation of field defect for gastric cancers.

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

Takeshi Nakajima[1,2], Satoshi Yamashita[1], Takao Maekita[3], Tohru Niwa[1], Kazuyuki Nakazawa[3] and Toshikazu Ushijima[1]*

[1]*Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[2]*Endoscopy Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[3]*Second Department of Internal Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama-city, Wakayama 641-0012, Japan*

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

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

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

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

Material and methods

Tissue samples and DNA/RNA extraction

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

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

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***Correspondence to:** Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81 3 5565 1753. E-mail: tushijim@ncc.go.jp

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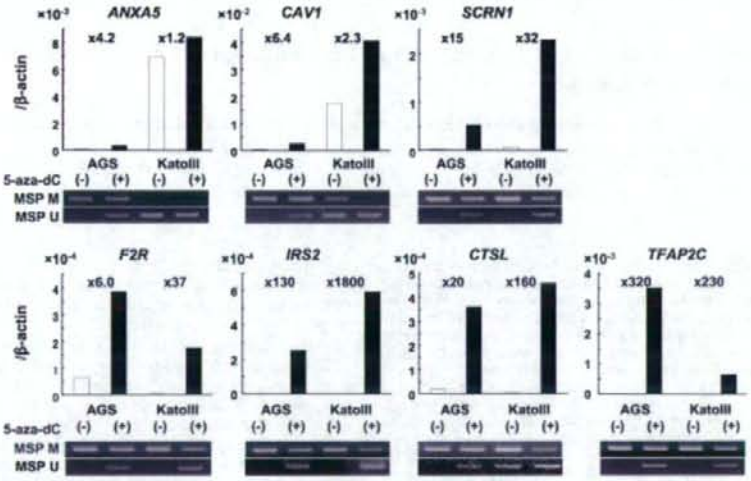

FIGURE 1 – Gene silencing due to methylation of the regions analyzed. mRNA expression and methylation were analyzed by real-time RT-PCR and MSP, respectively, in gastric cancer cell lines (AGS and KATO-III) before and after 5-aza-dC treatment. The fold increases after 5-aza-dC treatment is shown for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

Cell lines and 5-aza-dC treatment

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

Bisulfite treatment and methylation-specific PCR

Bisulfite treatment was performed as previously described.[9] Briefly, DNA samples (1 μg each) digested by *Bam*HI were denatured in 0.3 N NaOH at 37°C for 15 min. The samples underwent 15 cycles of 30-sec denaturation at 95°C and 15-min incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), and desulfonated in 0.3 N NaOH. DNA was ethanol precipitated and dissolved in 40 μL of TE buffer.

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

Quantitative reverse transcription PCR

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

Western blot analysis

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

Results

Confirmation of gene silencing due to promoter CpG islands

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

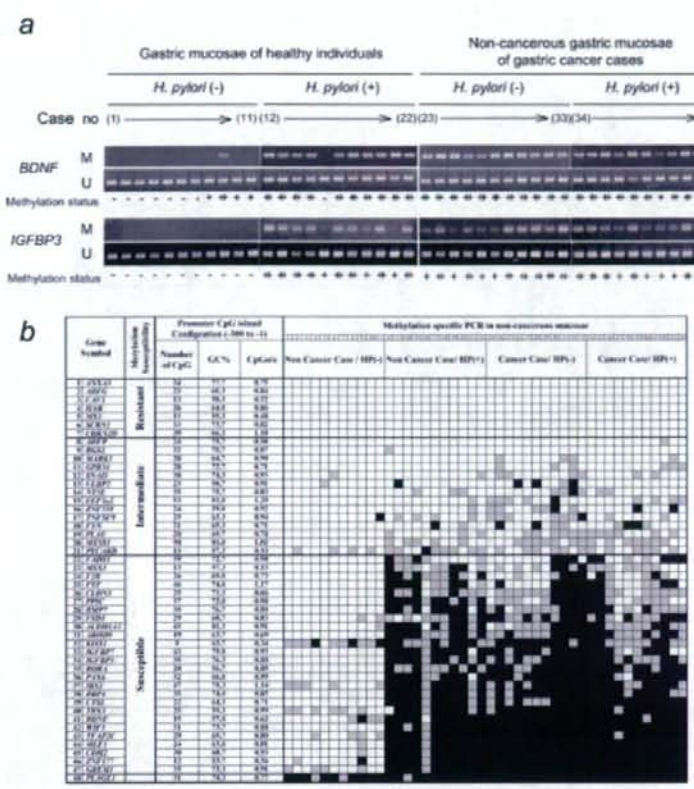

FIGURE 2 – Methylation profile of the 48 genes in noncancerous gastric mucosae. (*a*) Representative results of MSP. Samples 1–11, gastric mucosae of healthy individuals without *H. pylori* infection; 12–22, those with *H. pylori* infection; 23–33, noncancerous gastric mucosae of gastric cancer cases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

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

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

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

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

Lack of association between CpG island configuration and methylation susceptibility

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

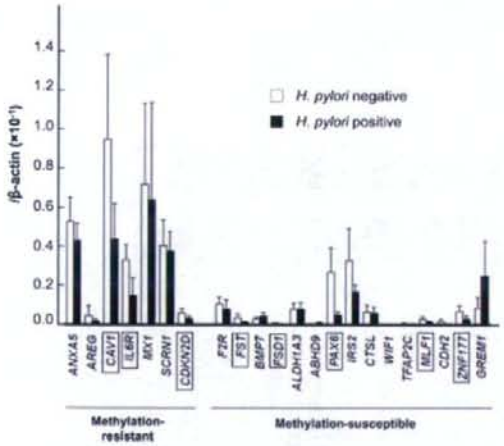

FIGURE 3 – The mRNA expression levels of genes resistant and susceptible to methylation induction. mRNA expression levels of 22 genes (7 resistant and 15 susceptible genes) in the noncancerous gastric mucosae of young healthy individuals with (closed columns) and without (open columns) *H. pylori* infection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

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

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

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

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

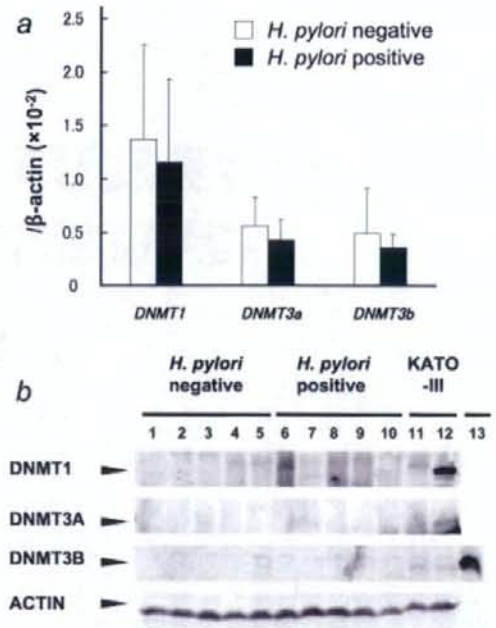

FIGURE 4 – The mRNA and protein expression levels of three DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) in noncancerous gastric mucosae of young healthy individuals with and without *H. pylori* infection. (*a*) mRNA expression levels of *DMNTs*. Closed columns, individuals with *H. pylori*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

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

Expression levels of DNA methyltransferase

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

Discussion

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

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

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

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

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

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

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Association between frequent CpG island methylation and *HER2* amplification in human breast cancers

Kotoe Terada[1,2], Eriko Okochi-Takada[1], Sadako Akashi-Tanaka[3], Kazuaki Miyamoto[4], Kiyomi Taniyama[4], Hitoshi Tsuda[5], Kiyoshi Asada[1], Michio Kaminishi[2] and Toshikazu Ushijima[1,*]

[1]Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [2]Department of Surgical Metabolic Care and Endocrine Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan, [3]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [4]Institute for Clinical Research, National Hospital Organization Kure Medical Center/ Chugoku Cancer Center, Hiroshima 737-0023, Japan and [5]Pathology Laboratory, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

*To whom correspondence should be addressed. Tel: +81 3 3547 5240; Fax: +81 3 5565 1753;
Email: tushijim@ncc.go.jp

The presence of frequent methylation of CpG islands (CGIs), designated as the CpG island methylator phenotype in some cancers, is associated with distinct clinicopathological characteristics, including gene amplification, in individual tumor types. Amplification of *HER2* in human breast cancers is an important prognostic and therapeutic target, but an association between *HER2* amplification and frequent CGI methylation is unknown. To clarify the association, we here quantified methylation levels of promoter CGIs of 11 genes, which are unlikely to confer growth advantage to cells, in 63 human breast cancers. The number of methylated genes in a cancer did not obey a bimodal distribution, and the 63 cancers were classified into those with frequent methylation ($n = 16$), moderate methylation ($n = 26$) and no methylation ($n = 21$). The incidence of *HER2* amplification was significantly higher in the cancers with frequent methylation (11 of 16) than in those with no methylation (2 of 21, $P = 0.001$). Also, the number of methylated genes correlated with the degree of *HER2* amplification ($r = 0.411$, $P = 0.002$). Correlation analysis with clinicopathological characteristics and methylation of *CDKN2A*, *BRCA1* and *CDH1* revealed that frequent methylation had significant correlation with higher nuclear grades ($P = 0.001$). These showed that frequent methylation had a strong association with *HER2* amplification in breast cancers and suggested that frequent methylation can be a determinant of various characteristics in a fraction of human breast cancers.

Introduction

Aberrant DNA methylation is deeply involved in the development and progression of human cancers (1–4). Methylation of CpG islands (CGIs) in promoter regions is a major mechanism for inactivation of tumor suppressor genes. At the same time, maintenance of appropriate DNA methylation levels is known to be important for maintenance of genome integrity. DNA hypomethylation can lead to genomic instability and increased tumor incidence in mice (5,6) and is associated with loss of heterozygosity in human cancers (7,8). On the other hand, aberrant DNA methylation precedes loss of heterozygosity in human liver cancers (9).

The presence of frequent methylation of CGIs in a cancer was first described in colorectal cancers and designated as the CpG island methylator phenotype (CIMP) (10). Depending upon tumor tissue types, the presence of frequent CGIs methylation, or CIMP, can be clearly observed and is associated with distinct clinicopathological features. For example, by careful selection of marker genes and their quantitative methylation analysis, CIMP in colorectal cancers was shown to be strongly associated with *BRAF* mutations (11). In neuroblastomas, both in Japan and Germany, CIMP was observed as a distinct entity associated with poor prognosis and *MYCN* amplification (12,13). Remarkably, all the cases with *MYCN* amplification had frequent methylation, with only one exception. Cases with CIMP but without *MYCN* amplification had a better prognosis than those that had both and a worse prognosis than those that had neither. This complete containment of tumors with *MYCN* amplification within CIMP-positive tumors suggested that CIMP could precede gene amplification or that at least the presence of frequent aberrant DNA methylation was associated with gene amplification.

Gene amplification of *HER2*, which is a member of the epidermal growth factor receptor family (14), is very important in human breast cancers. Initially, *HER2* amplification was found to be present in 15–30% of newly diagnosed breast cancer cases and to be associated with increased metastatic potential and decreased overall survival (15). Suppression of *HER2* activity was shown to have antitumor activity, and antibodies against HER2 were developed as a therapeutic agent against breast cancers. Now, it is well known that a humanized antibody against HER2, such as trastuzumab, is very effective against breast cancers with *HER2* amplification (16,17). Nevertheless, inducers of *HER2* amplification remain unknown.

In this study, we aimed to clarify whether or not the presence of frequent CGI methylation was associated with *HER2* amplification in human breast cancers. For this end, from the genes silenced in human cancers (18,19), we selected genes whose silencing is unlikely to confer growth advantage and avoided selection bias of cells with methylation. Also, we performed quantitative methylation analysis of their putative nucleosome-depleted regions (20), which are most resistant to DNA methylation (21). Association between frequent CGI methylation and clinicopathological characteristics, including silencing of three tumor-suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*), was also analyzed.

Abbreviations: CGI, CpG island; CIMP, CpG island methylator phenotype; ESR, estrogen receptor; PCR, polymerase chain reaction; PGR, progesterone receptor.

Materials and methods

Patients and tissue samples

Sixty-three breast cancer tissue specimens were obtained from patients who underwent mastectomy or breast-conserving surgery (stage I 22 cases; stage II 26 cases; stage III 15 cases and stage IV 0 case). Informed consent was obtained from all the patients, and analysis was approved by the institutional review boards. Cancer tissues were frozen after resection and stored at −80°C until extraction of genomic DNA. High-molecular weight DNA was extracted by the phenol–chloroform method. Histological types were evaluated according to the criteria of the Japanese Breast Cancer Society (22).

Bisulfite modification and quantitative methylation-specific polymerase chain reaction

Completely methylated DNA and completely unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA) and 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 as described previously (23). The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for polymerase chain reaction (PCR) with a primer set specific to methylated or unmethylated sequences (supplementary Table 1 is available at *Carcinogenesis* Online). Using the completely methylated DNA and completely unmethylated DNA, an annealing temperature specific for each primer set was determined. Real-time PCR was performed using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of DNA molecules with methylated sequences and

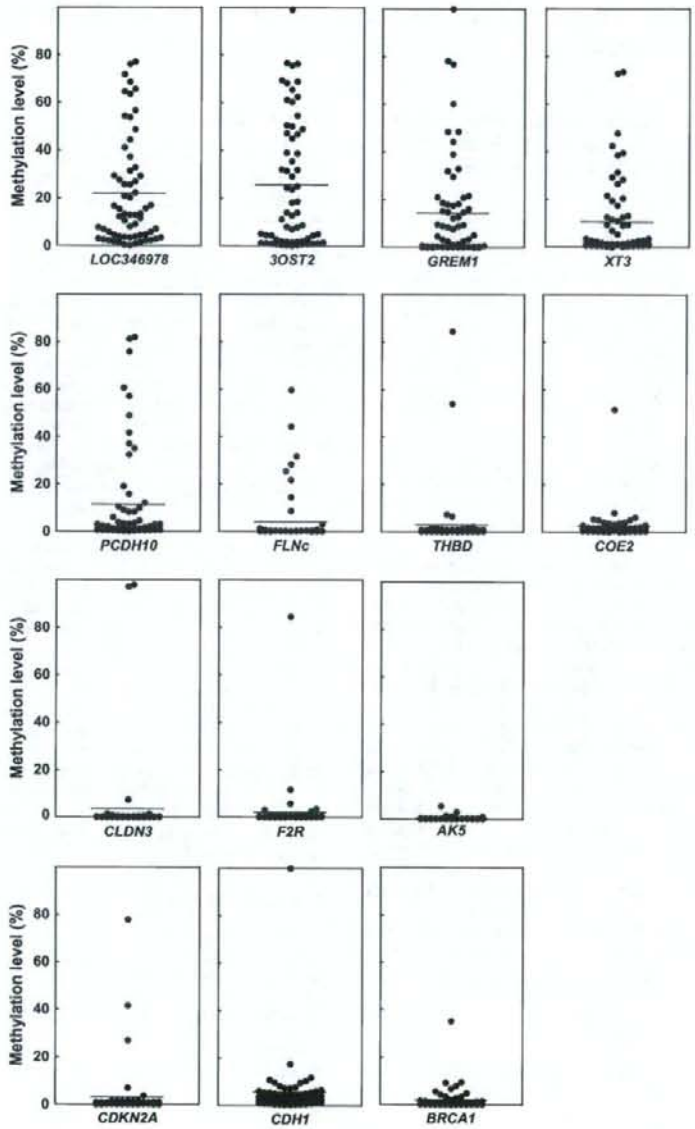

Fig. 1. Methylation levels in the 63 breast cancer samples. The numbers of DNA molecules methylated and unmethylated in promoter CGIs were obtained by quantitative methylation-specific PCR, and a methylation level was calculated as a fraction of methylated DNA molecules among the total DNA molecules. Some cancers had no methylation and the others had various levels of methylation. The methylation level in cancers was considered to represent the fraction of cancer cells in a sample and less occasionally the fraction of cells with methylation among cancer cells. We confirmed that we detected dense methylation of promoter CGIs by sequencing the quantitative methylation-specific PCR products obtained using primers specific to methylated DNA molecules (supplementary Figure 3 is available at *Carcinogenesis* Online).

that with unmethylated sequences in a test sample were measured by comparing its amplification with those of standard samples that contained 10–10^6 DNA molecules. The standard samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI) or by purifying their PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The 'methylation level' was calculated as the fraction of methylated DNA molecules among the total DNA molecules.

Fluorescence in situ hybridization analysis of the HER2 amplification

Fluorescence *in situ* hybridization was performed using a PathVysion kit (Abbot Molecular, Des Plaines, IL) with our modification (24). The *HER2* locus

and centromere of chromosome 17 (CEP17) were labeled by SpectrumOrange and SpectrumGreen fluorescence, respectively, and nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. *HER2* and CEP17 signals were counted in 60 nuclei under a fluorescence microscope. Cancers with *HER2*:CEP17 ratio ≥2 were determined as *HER2* amplification positive.

Analysis of 3OST2 expression on cell growth

MCF7 Tet-Off cell line was purchased from Clontech Laboratories (Mountain View, CA). Full-length *3OST2* complementary DNA, cloned from human mammary epithelial cells, was inserted into the multiple cloning site of pTRE2hyg vector (Clontech Laboratories). The MCF7 Tet-Off cell line was transfected with the vector, and a stable clone was obtained by selection using hygromycin. Growth curves were analyzed by counting the cell numbers for the parental cell line, stable clones transfected with *3OST2*-expressing vector and with empty vector (without doxycycline). Overexpression of *3OST2* complementary DNA was confirmed by real-time reverse transcription–PCR analysis.

Sequencing analysis of quantitative methylation-specific PCR products

Quantitative methylation-specific PCR products of seven genes, *3OST2*, *FLNc*, *GREM1*, *THBD*, *PCDH10*, *XT3* and *LOC346978*, were cloned into pGEM-T Easy Vector (Promega). For each sample, ~10 clones were cycle sequenced using T7 primer, 5′-TAATACGACTCACTATAGGG-3′ and an Applied Biosystems 310 sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis

Increasing or decreasing trends in no methylation, moderate methylation and then frequent methylation cancers were analyzed by the Mantel–Haenszel chi-square test. Differences between the frequent methylation *HER2*-positive cancers and moderate methylation (or no methylation) *HER2*-positive cancers were analyzed by the chi-square test. Correlation between the degree of *HER2* amplification and the number of methylated genes was analyzed using Pearson's correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL).

Results

Quantitative methylation analysis of breast cancers

From 20 and 14 genes that were methylated in human breast and gastric cancers, respectively (18,19), we selected 11 genes (*LOC346978*, *3OST2*, *GREM1*, *XT3*, *PCDH10*, *FLNc*, *THBD*, *COE2*, *CLDN3*, *F2R* and *AK5*) and quantified their methylation levels in 63 breast cancers. These genes, except for *3OST2* and *CLDN3*, were not expressed in normal human mammary epithelial cells (18,19,25,26), and their silencing was unlikely to confer growth advantage to cells. Also, introduction of *3OST2* complementary DNA into MCF7 cells did not cause growth suppression (supplementary Figure 1 is available at *Carcinogenesis* Online), and its silencing was unlikely to confer growth advantage. Therefore, the majority of the 11 genes were considered to be suitable to detect the presence of a cellular environment that tends to induce methylation of promoter CGIs. We also analyzed methylation of three tumor suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*) for clinicopathological analysis.

Quantitative methylation analyses of the 14 genes showed that some cancers had no methylation and the others had various levels

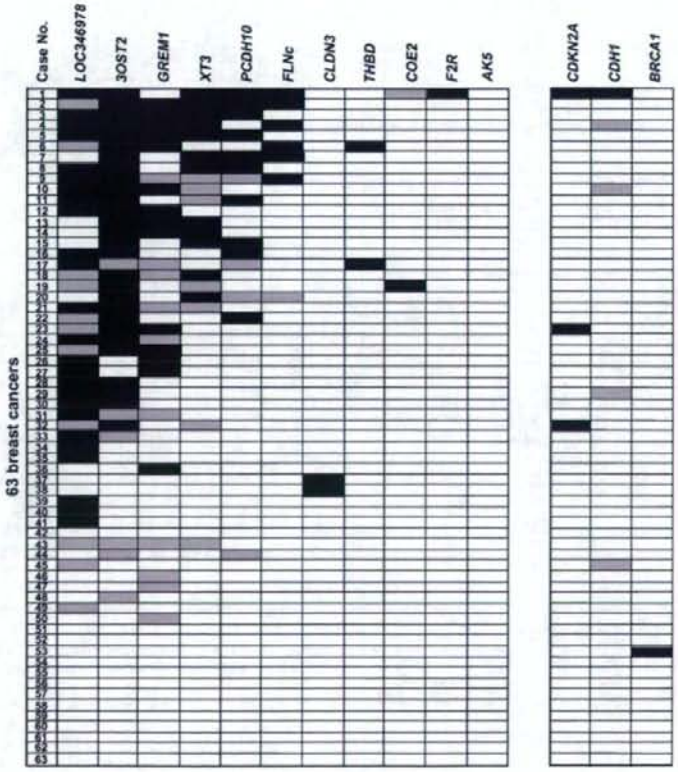

Fig. 2. Methylation profile of the 11 marker genes and three tumor suppressor genes in 63 breast cancers. Methylation in each sample was scored as positive or negative using two different cutoff values, and the 63 samples were aligned by the number of methylated CGIs. Methylation-positive samples using 10 and 20% as cutoff values are shown by gray and black boxes, respectively.

of methylation (Figure 1). Such distribution of methylation levels was typically observed for *FLNc*, *THBD*, *CLDN3*, *F2R* and *CDKN2A*. The presence of such distribution confirmed previous findings that cancer samples could essentially be classified into two groups: cancers with methylation of a specific gene and those without (11,19,27). Counting cancer cells in the tissue section samples showed that two samples with least cancer cells contained cancer cells with fractions of 19.8 ± 5.2% and 22.9 ± 0.3% (mean ± SD). Based on these data, we adopted two cutoff values 10 and 20% to score each cancer sample as positive or negative. When overall distribution of methylation was examined, similar patterns of cancers with methylation were observed using the two cutoff values (Figure 2). Using either value, the number of methylated genes in a cancer did not obey bimodal distribution and looked quite similar (Figure 3). Therefore, we adopted a cutoff value of 20% to score individual cancers as positive or negative for methylation.

Then, the 63 cancers were classified by the frequency of CGI methylation. To avoid biases due to a cutoff number of methylated genes, we classified the cancers into three groups, those with no methylation, moderate methylation and frequent methylation, using two different cutoff numbers for frequent methylation. Using a cutoff number of three methylated genes or more, 16, 26 and 21 cases were classified into cancers with frequent methylation, moderate methylation and no methylation, respectively. Using a cutoff number of four methylated genes or more, 8, 34 and 21 cases were classified into those with frequent methylation, moderate methylation and no methylation, respectively.

Association between frequent CGI methylation and the HER2 amplification

The presence of *HER2* amplification was analyzed by fluorescence *in situ* hybridization, and 24 of 63 (38%) cancers had *HER2* amplification (supplementary Figure 2 is available at *Carcinogenesis* Online). The extent of amplification ranged from 2.0- to 16.8-fold. Using a cutoff number of three for frequent methylation, the fractions of cancers with *HER2* amplification were 11/16, 11/26 and 2/21 in cancers with frequent methylation, moderate methylation and no methylation, respectively (Figure 4A). Using a cutoff number of four, it was 6/8, 16/34 and 2/21, respectively (Figure 4B).

When correlation between the degree of CGI methylation and fraction of cancers with *HER2* amplification was examined by trend analysis, a highly significant increasing trend was observed from cancers with no methylation, to those with moderate methylation and then to those with frequent methylation ($P < 0.001$ for both of cutoff numbers). When cancers with frequent methylation and those with no methylation were compared, the former had a significantly higher fraction ($P = 0.003$ and 0.001 for cutoff numbers of four and three, respectively). Also, the degree of *HER2* amplification showed a correlation with the number of methylated genes (correlation coefficient = 0.411, $P = 0.002$) (Figure 5 and supplementary Table 2 is available at *Carcinogenesis* Online). This demonstrated that frequent CGI methylation had an association with *HER2* amplification.

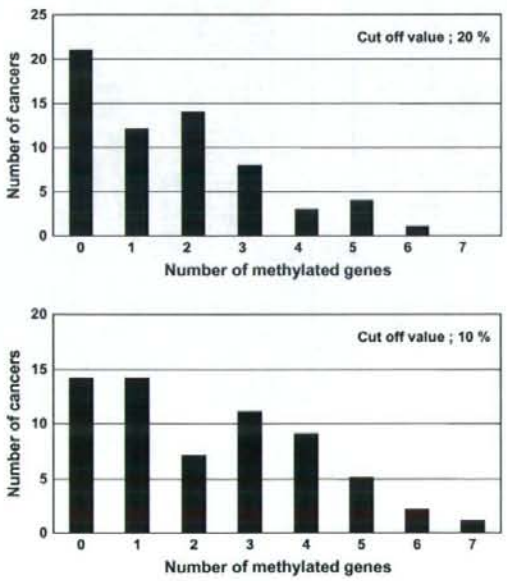

Fig. 3. Distribution of the number of methylated genes in a cancer. Similar distribution patterns were observed using two different cutoff values. The distribution was not bimodal, and involvement of multiple mechanisms in frequent methylation in breast cancers was suggested.

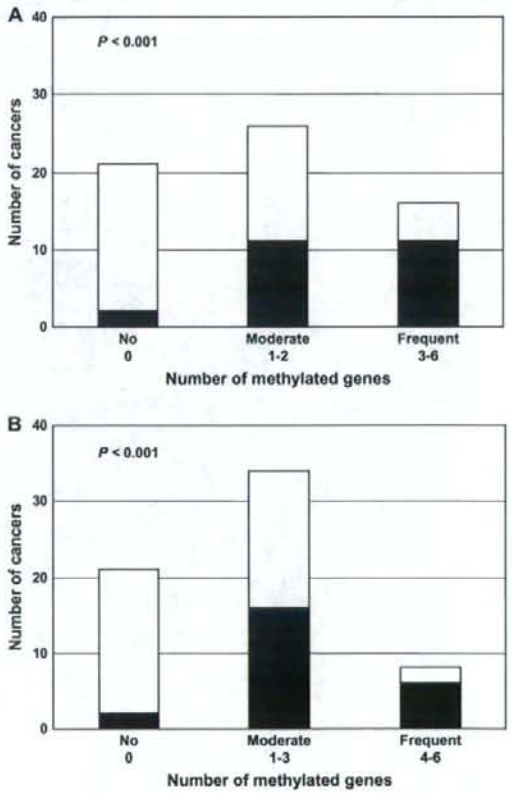

Fig. 4. The correlation between the degree of frequent methylation and *HER2* amplification. Two different cutoff numbers were used to define frequent methylation. These analyses adopted a cutoff value of 20% for methylation-positive. (**A**) Frequent methylation was defined as cancers with methylation of three or more genes. (**B**) Frequent methylation was defined as cancers with methylation of four or more genes. Whichever cutoff number was used, a clear increasing trend of *HER2*-positive cancers in no methylation, moderate methylation and then frequent methylation groups was observed ($P < 0.001$ for both of the two cutoff numbers). Closed and open boxes represent cancers with and without *HER2* amplification, respectively.

Association between frequent CGI methylation and other clinicopathological features, including methylation of tumor suppressor genes

The correlation between frequent CGI methylation and methylation of three tumor suppressor genes, *CDKN2A*, *CDH1* and *BRCA1*, was analyzed (Table I). However, none of the three genes showed any correlation (P = 0.557, 0.157 and 0.232, respectively). Regarding other clinicopathological characteristics, the degree of frequent CGI methylation correlated with higher nuclear grades (P = 0.001). The degree of frequent CGI methylation tended to show correlations with advanced pathological stage (P = 0.068) and post-menopausal status (P = 0.044). However, no association was observed with lymph node metastasis and negative expression of estrogen receptor (ESR) or progesterone receptor (PGR).

Discussion

The present study demonstrated for the first time that frequent CGI methylation in breast cancers had a highly significant association with *HER2* amplification. Regarding DNA methylation and HER2 overexpression, Fiegl *et al.* (28) previously found that methylation levels of four genes (*CDH13*, *PGR*, *HSD17B4* and *MYOD1*) and one gene (*BRCA1*), which were selected from 35 genes, correlated with HER2 expression positively and inversely, respectively (P = 0.01–0.04). Methylation levels of individual genes in cancers are affected by the content of cancer cells, and, also, the correlation observed in the study was considered to be due to interaction between a function of an individual gene and HER2 overexpression. In contrast, here, we focused on the abnormality in epigenetic regulation in cancers. To estimate its degree, we used marker genes that were unlikely to confer growth advantages even if methylated, scored their methylation as positive or negative and integrated the information from the 11 marker genes into the frequency of methylation in a cancer sample. The cancers were classified into three groups, namely those with frequent methylation, moderate methylation and no methylation. As a result, a very strong association between frequent methylation and *HER2* amplification ($P < 0.001$) was demonstrated. Also, the degree of frequent methylation showed a clear correlation with the degree of *HER2* amplification. *BRCA1* methylation did not correlate with the degree of frequent methylation or *HER2* amplification (P = 0.806).

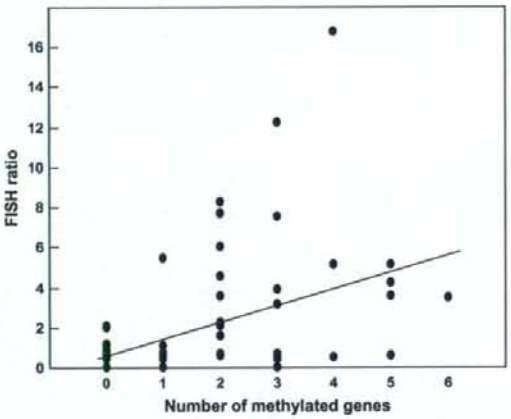

Fig. 5. The correlation between the number of methylated genes and degree of *HER2* amplification. The degree of *HER2* amplification showed a strong correlation with the number of methylated genes (correlation coefficient = 0.411, P = 0.002).

Table I. Association between frequent CGI methylation and clinicopathological features, including methylation of tumor suppressor genes

	No methylation	Moderate methylation	Frequent methylation	P value
CDKN2A methylation (+/−)	2/19	0/26	1/15	0.557
BRCA1 methylation (+/−)	1/20	0/26	0/16	0.232
CDH1 methylation (+/−)	0/21	0/26	1/15	0.157
Menopausal (pre/post)	12/9	9/17	4/12	0.044
Stage (I/II/III)	9/11/1	9/8/9	4/7/5	0.068
Lymph node metastasis (positive/negative)	6/15	13/13	7/9	0.308
ESR (positive/negative)	15/6	14/12	11/5	0.779
PGR (positive/negative)	17/4	14/12	9/7	0.100
Nuclear grade (1/2/3)	4/10/7	2/7/17	0/2/14	0.001

Frequent methylation was defined as breast cancers with methylation of three or more genes. Increasing or decreasing trends were tested by Mantel–Haenszel chi-square.

The association between frequent methylation and *HER2* amplification has clinical implications. It is known that *HER2* amplification status can show a discrepancy between primary and metastatic sites in a small fraction of patients (29). There is a possibility that HER2-negative breast cancers at initial diagnosis change into HER2 positive at their recurrence and that the presence of frequent methylation at the initial diagnosis can be used to predict such cases. Since accurate detection of HER2-positive cancers is very important to implement appropriate treatment, including trastuzumab (17), future studies to predict the *HER2* amplification status using frequent methylation and to clarify the mechanism of the association are warranted. Also, the effect of frequent methylation on long-term survival is important. So far, only 5 of 63 cases suffered from recurrence (one frequent methylation, three moderate methylation and one no methylation cases), and the effect cannot be statistically analyzed. Since the association between *HER2* amplification and poor survival (without trastuzumab) is well established, the effect of frequent methylation on long-term survival seems worth being analyzed in the future.

Some breast cancers with *HER2* amplification belonged to the moderate methylation or no methylation groups although the majority of cancers with *HER2* amplification belonged to the frequent methylation group. This was in contrast with the case of neuroblastomas, where all the neuroblastomas with *MYCN* amplification had frequent methylation, CIMP, with only one exception (12,13). Therefore, the relationship between frequent methylation and *HER2* amplification in breast cancers seems more complex than the relationship between CIMP and *MYCN* amplification in neuroblastomas. Not only frequent methylation could lead to *HER2* amplification through chromosomal instability (9), which was our initial expectation, but also *HER2* amplification could lead to frequent methylation or they might have common inducers.

The degree of frequent methylation also correlated with higher nuclear grades. It also tended to show association with advanced stages and post-menopausal status. It has been reported that *CDH1* methylation was associated with negative ESR and PGR expressions (P = 0.06 and 0.09, respectively) and that frequent methylation of seven tumor suppressor genes was associated with poor differentiation (30). It has also been reported that PGR expression was negatively associated with *ESR1*, *TGFBR2*, *PPTGS2* and *CDH13* methylation (P = 0.01–0.04) (31) and that ESR and PGR expressions were positively and negatively associated with *HIN-1/RASSF1A* and *RIL/CDH13* methylation, respectively (32). Taken together, the frequent methylation in breast cancers was weakly associated with advanced stages, negative PGR and ESR expressions and poor differentiation (higher nuclear grades). Nevertheless, the correlation between frequent methylation and *HER2* amplification was much stronger than these associations in our study. It was considered that quantitative analysis of marker genes was advantageous to clarify the strong association.

The cutoff value of methylation levels to score cancer samples as positive or negative for methylation was determined based upon the fraction of cancer cells in two samples with their smallest contents (20%). To count methylation in a fraction of cancer cells, we also tested a cutoff value, 10%, but quite similar results were obtained (Figure 3). Regarding the cutoff number for frequent methylation, we tried three and four but observed a highly significant association using both numbers (Figure 4). This excluded a possibility that a false-positive association between frequent methylation and *HER2* amplification was observed due to arbitrary cutoff values or numbers. Also, we confirmed that we detected dense methylation of promoter CGIs by our quantitative methylation-specific PCR analysis by sequencing the PCR products. Almost all the CpG sites in the products were densely methylated (supplementary Figure 3 is available at *Carcinogenesis* Online). Finally, we confirmed that the methylation detected in cancer tissues originated from cancer cells. Methylation levels of nine genes that showed high methylation levels (≥10%) in some cancer samples were measured in 11 pairs of non-cancerous breast and cancer tissues (supplementary Figure 4 is available at *Carcinogenesis* Online). The methylation levels of all the genes were elevated only in cancer tissues, and the methylation we detected was considered to originate from cancer cells.

In summary, frequent methylation in breast cancers had a strong association with *HER2* amplification.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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