Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation

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Hypomethylation of the global genome, considered to be composed mainly of repetitive sequences, is consistently observed in cancers, and aberrant hypo- and hypermethylation of CpG islands (CGIs) in promoter regions are also observed. Since methylation alterations in unique promoter sequences and in other genomic regions have distinct consequences, we analyzed the relationship between the global hypomethylation and the hypomethylation of unique promoter CGIs using human gastric cancers. Seven of ten gastric cancer cell lines showed marked decreases in 5-methylcytosine content, which correlated with hypomethylation of the LINE1 repetitive sequence. Six of the seven cell lines showed hypomethylation in five or all of the six normally methylated CGIs in promoter regions of six genes, and this was associated with induction of aberrant expression. The remaining three cell lines without global hypomethylation showed promoter hypomethylation in one or none of the six CGIs. Frequent promoter hypomethylation, however, did not correlate with frequent promoter hypermethylation. In primary gastric cancers too, global hypomethylation was associated with hypomethylation of LINE1 repetitive sequence and promoter hypomethylation. Of 93 gastric cancers, 33 cancers with frequent promoter hypomethylation and 27 cancers with frequent promoter hypermethylation constituted different groups. These findings represent experimental evidence that frequent hypomethylation of normally methylated promoter CGIs is associated with global hypomethylation, and that these hypomethylations occur independently of frequent promoter CGI hypermethylation. (Cancer Sci 2004; 95: 58–64)

Aberrant methylation of various genomic regions is present in cancers. Firstly, global hypomethylation, the decrease of 5-methylcytosine content in the genome,[1)] is known to involve coding regions of genes[2)] and repetitive sequences.[3,4)] Based on the facts that 80% of CpG dinucleotides are present in repetitive sequences and they are mostly methylated,[5,6)] global hypomethylation is considered to be mainly due to hypomethylation of repetitive sequences.

Secondly, hypomethylation is also observed in normally methylated CpG islands (CGIs) in promoter regions, and it induces aberrant expression of their downstream genes if transcription factors are available in cancer cells. Known normally methylated CGIs are very limited, and include some cancer-testis antigen genes, such as the *MAGE* genes.[7–9)] Hypomethylation of promoter CGIs is considered to have little effect on the content of 5-methylcytosine in the genome, and is distinct from hypomethylation of the repetitive sequences in the sense that it has a direct function on gene expression. While global hypomethylation is observed in most cancers,[1,10)] aberrant *MAGE* gene expressions were reported to occur in a smaller proportion of cancer cases (ranging from 0% to 86%, but generally from 10% to 40%).[11)] Therefore, a distinct regulatory mechanism may function for normally methylated promoter CGIs, and their hypomethylation may not be associated with global hypomethylation.

Thirdly, hypermethylation of normally unmethylated, i.e. ordinary, CGIs in promoter regions is well known. It can cause permanent silencing of tumor-suppressor genes, and can be causally involved in cancer development and progression.[12–14)] It is known that cancer cells generally harbor global hypomethylation and promoter hypermethylation simultaneously.[15)] However, the molecular mechanisms leading to these states are unclear, and it is not known whether common mechanism(s) are involved. It has been found that hypermethylation of *E-cadherin* promoter coexists with hypomethylation of satellite 2 DNA in breast cancers.[16)] In contrast, our previous study showed that gastric cancers with frequent hypermethylation form a different group from those with frequent hypomethylation of three *MAGE* genes.[17)]

In this study, global hypomethylation, repetitive sequence hypomethylation, promoter hypomethylation, and promoter hypermethylation were analyzed using the same set of gastric cancers to clarify their relationship and to gain insight into their molecular mechanisms.

Materials and Methods

Cell lines and tissue samples. Six gastric cancer cell lines, KATOIII, MKN28, MKN45, MKN74, NUGC3 and AGS, were purchased from the Japanese Collection of Research Bioresources (Tokyo) and from the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44 and HSC57, were donated by Dr. K. Yanagihara,[17)] and TMK1 was donated by Dr. W. Yasui.[18)] Ninety-three primary gastric cancer samples were obtained from 92 patients undergoing gastrectomy, with informed consent. For 28 of the 93 cancers, non-cancerous gastric epithelial tissues (normal samples) were also obtained by scraping off the non-cancerous mucosae. DNA was extracted by standard phenol/chloroform procedures, and total RNA was isolated with ISOGEN (Nippon Gene, Tokyo).

Analysis of 5-methylcytosine content. The 5-methylcytosine content was quantified by chromatographic separation of five deoxyribonucleotides, 5-methyl-dCMP, dCMP, dAMP, dGMP and TMP, as reported.[19–21)] Briefly, genomic DNA was degraded by treatment with DNase I and Nuclease P1. After filtration with a 0.45-μm filter, the sample was subjected to high-pressure liquid chromatography (HPLC). Peaks of the five deoxyribonucleotides were detected at 280 nm by a UV absorbance detector, and were compared with those of authentic samples. The 5-methylcytosine content was measured as the fraction of 5-methyl-dCMP in total deoxyribonucleotides. The

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HPLC analysis was performed two or three times for four samples, and the standard error was confirmed to be less than 0.01%.

Southern blot analysis. Southern blot analysis for the LINE1 element, as the most abundant repetitive sequence, was performed as previously reported.[4] Briefly, 3 μg of genomic DNA digested with *Hpa*II or *Msp*I was electrophoresed in a 1.3% agarose gel, and transferred to a nylon membrane (Hybond N; Amersham-Pharmacia, Uppsala, Sweden). After hybridization with 50 ng of probe DNA labeled with [α-^{32}P]dCTP, the membrane was washed for 10 min at 50°C five times and exposed to Kodak XAR film. Nucleotides −729 to −189 upstream of LINE1 ORF1 were cloned in pGEM-T Easy (Promega, Madison, WI), and used as the probe.

Bisulfite sequencing and methylation-specific PCR (MSP). One microgram of DNA, digested with *Bam*HI, was incubated with freshly prepared 0.3 *M* NaOH in a volume of 20 μl for 15 min. To this solution, 120 μl of freshly prepared 3.6 *M* sodium bisulfite (Sigma, St. Louis, MO) and 0.6 m*M* hydroquinone (Sigma) solution (adjusted to pH 5.0 with 10 *N* NaOH) were added, and the solution underwent 15 cycles of denaturation at 95°C for 30 s and incubation at 50°C for 15 min. The samples were desalted with Wizard DNA Clean-Up System (Promega) and desulfonated by incubation with 0.3 *N* NaOH for 5 min. The DNA was ethanol-precipitated and suspended in 20 μl of TE buffer.

For bisulfite sequencing, PCR was performed with primers common for the methylated and unmethylated DNA sequences using 1 μl of the solution as the template. The primers and PCR conditions are shown in Table 1. PCR products were cloned into pGEM-T Easy vector (Promega), and ten clones from each sample were cycle-sequenced with a BigDye Terminator kit (PE Biosystems, Foster City, CA) and an ABI automated DNA sequencer (PE Biosystems, Warrington, UK).

MSP was performed with primers specific for the methylated (M) or unmethylated (U) sequences using 1 μl of the bisulfite-

Table 1. Primers for bisulfite sequencing

Genes	Primer sequences	Length (position)	Accession #	Anneal	Strand
MG-A1	F: GGGTGGGTTAGGGTTGGTAGTA	238 bp	U82670	61°C	Bottom
	R: ATTCCCRCCAAAAAACATCC	(−95 to +143)			
MG-A2	F: TTTTGTAGGGTGGTTTAGGTT	318 bp	L18920	58°C	Bottom
	R: ACCAACACCATCTTCATACTTAC	(−198 to +120)			
MG-A3	F: TAGAGGTAGTATTGGATTATTTGAGG	296 bp	U82671	60°C	Bottom
	R: CCCATCACCATCTTCATACTTAC	(−185 to +111)			
MG-B2	F: TATTGAGGTGAGGATTTTTAGTGGA	237 bp	AC005185	63°C	Top
	R: AAAATTCACCCCTAACTAACCAAAC	(−162 to +75)			
MG-C1	F: GGATGGAAAGGAGGTTGATAAG	184 bp	AF064589	61°C	Top
	R: AAATACCTCAAATCCTTCAAACTC	(−162 to +22)			
MG-C2	F: GAAGGGTTGGAGGGTAGTTG	246 bp	HS142F18	62°C	Top
	R: AACTAACCAAATCTAAAACTCTTCC	(−165 to +81)			

MG, MAGE. F, forward; R, reverse. Position of the 5′ end of available exon 1 sequence was regarded as +1. Top/Bottom represented the bisulfite-treated DNA strand used in designing the primers.

Table 2. Primers for MSP

Genes	M/U	Primer sequences	Length (position)	Accession #	Anneal	Strand
MG-A1	M	F: TTCGGGTGTTCGGATGTGAC	110 bp	U82670	64°C	Top
		R: CCTAAATCAAATTCCTTCGACCG	(−78 to +32)			
	U	F: TTTGGGTGTTTGGATGTGAT	110 bp		56°C	Top
		R: CCTAAATCAAATTCCTTCAACCA	(−78 to +32)			
MG-A2	M	F: GCGTTTGTTTTTTTTCGTCGAC	108 bp	L18920	64°C	Bottom
		R: AAATCACGAACCCGAATATAACG	(−109 to −2)			
	U	F: GAAGTTATGGGTTTGGATGTGAT	107 bp		62°C	Top
		R: ACCTACTTCCCTCCACCAACA	(−110 to −4)			
MG-A3	M	F: TAGGATGTGACGTTATTGATTTGC	84 bp	U82671	63°C	Top
		R: ACGTCAAACCGTCGCTCG	(−87 to −4)			
	U	F: TTAGGATGTGATGTTATTGATTTGT	88 bp		57°C	Top
		R: CCAACATCAAACCATCACTCA	(−88 to −1)			
MG-B2	M	F: GTTAGAATAGTGACGTTCGGTAGC	120 bp	AC005185	63°C	Top
		R: AAATAAACCACATCCGCTCG	(−119 to +1)			
	U	F: ATGTTAGAATAGTGATGTTTGGTAGT	123 bp		59°C	Top
		R: AAAATAAACCACATCCACTCA	(−121 to +2)			
MG-C1	M	F: AGAGGAGGTTTCGTTTTACGTTATTC	109 bp	AF064589	64°C	Top
		R: AACTCCCAAAATAACCGCCG	(−106 to +3)			
	U	F: AGGAGGTTTTGTTTTATGTTATTT	107 bp		58°C	Top
		R: AACTCCCAAAATAACCACCA	(−104 to +3)			
MG-C2	M	F: TTTTTTCGTTAATTTGATTCGC	86 bp	HS142F18	58°C	Bottom
		R: CCGAATATACTTTCCCGACG	(−27 to +59)			
	U	F: GTTTTTGGATGTGTTTTTTTGAT	88 bp		58°C	Top
		R: CCCCCACTAACTTAATTCACA	(−31 to +57)			

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

modified DNA solution. MSP was performed for aberrant methylation of *Lysyl oxidase* (*LOX*), *HRAS-like suppressor* (*HRASLS*), *Filamin C* (*FLNc*), *HAND1*, *Thrombomodulin* (*TM*), *PPARγ angiopoietin related* (*PGAR*), *p16* and *hMLH1* as previously described.[14, 17, 22] MSP primers and PCR conditions are shown in Table 2. For normally unmethylated CGIs, a sample was regarded as methylation-negative when PCR product was obtained only with the U set, and was regarded as methylation-positive when PCR product was obtained with the M set or with both the U set and M set. For normally methylated CGIs, a sample was regarded as hypomethylation-negative when PCR product was obtained only with the M set, and was regarded as hypomethylation-positive when PCR product was obtained with the U set or with both the M set and U set.

Quantitative RT-PCR. Total RNA was treated with DNase I (Ambion, Austin, TX), and cDNA was synthesized from 3 μg of total RNA using a Superscript II kit (Life Technologies, Rockville, MD). Real-time PCR was performed using SYBR Green PCR Core Reagents (PE Biosystems) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of molecules of a specific gene in a sample was measured by comparing its amplification with the amplification of standard samples that contained 10^1 to 10^6 copies of the gene. The quantity of mRNA of each gene was normalized with that

Table 3. Primers for quantitative RT-PCR

Genes	Primer sequences	Length	Accession #	Anneal
MG-A1	F: CTGACCCAGGCTCTGTGA	93 bp	BC017555	57°C
	R: CTCCTTGGTGCTCCTCTGT			
MG-A2	F: AGGGAACTCTGGCATCTC	105 bp	L18920	55°C
	R: AGATCCTAGAACCACTGCAT			
MG-A3	F: TCGGTGAGGAGGCAAGGTT	97 bp	BC000340	62°C
	R: TGGAGACCCACTGGCAGAT			
MG-B2	F: GGGTGTATTCTCAGGACTGGT	146 bp	AF015766	58°C
	R: CTTCTTCCTCTGCTTCAGTGA			
MG-C1	F: TGAAGGACCTGAGGCATT	95 bp	NM_005462	55°C
	R: CTTTCTGGAGCACCTTGA			
MG-C2	F: TCCCACCATAGAGAGAAGAA	112 bp	AF196482	55°C
	R: GTGCTGACTTTAGGCTGTGT			
PCNA	F: ATGTCGATAAAGAGGAGGAA	105 bp	AF527838	55°C
	R: AGAGTGGAGTGGCTTTTGTA			

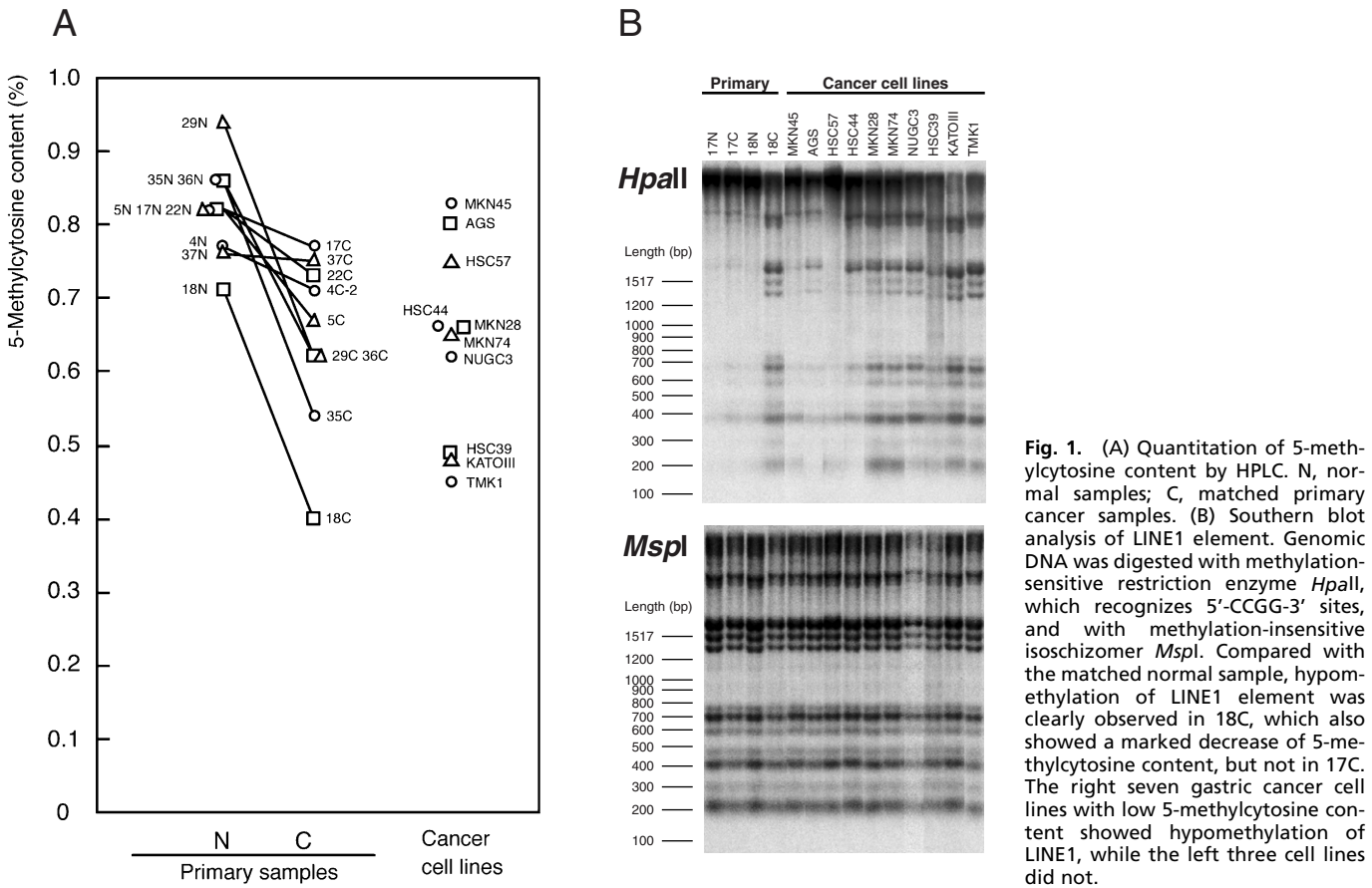

Fig. 1. (A) Quantitation of 5-methylcytosine content by HPLC. N, normal samples; C, matched primary cancer samples. (B) Southern blot analysis of LINE1 element. Genomic DNA was digested with methylation-sensitive restriction enzyme *Hpa*II, which recognizes 5′-CCGG-3′ sites, and with methylation-insensitive isoschizomer *Msp*I. Compared with the matched normal sample, hypomethylation of LINE1 element was clearly observed in 18C, which also showed a marked decrease of 5-methylcytosine content, but not in 17C. The right seven gastric cancer cell lines with low 5-methylcytosine content showed hypomethylation of LINE1, while the left three cell lines did not.

of *Proliferating cell nuclear antigen* (*PCNA*). The primers and PCR conditions are shown in Table 3.

5-Aza-2′-deoxycytidine (5-aza-dC) treatment. HSC44, HSC57 and MKN45 cells were seeded at a density of 3×10^5 cells/10-cm dish on day 0, and treated with 1–3 μ*M* 5-aza-dC (Sigma) for 24 h on days 1, 3 and 5. After each treatment, the cells were placed in fresh media, and harvested on day 6.

Results

Global hypomethylation and hypomethylation of the LINE1 repetitive sequence. The 5-methylcytosine content of normal samples was 0.82±0.07% (mean±SD), which is in accordance with previous reports[23] (Fig. 1A). Among the nine primary cancer samples, five samples showed a 5-methylcytosine content below the lowest level of the nine normal samples (0.71%), while four other samples did not. Among the ten gastric cancer cell lines, seven showed a 5-methylcytosine content below 0.71%.

Hypomethylation of the LINE1 repetitive sequence was analyzed by Southern blot analysis (Fig. 1B). A primary cancer sample with marked global hypomethylation, 18C, showed LINE1 hypomethylation compared with the matched normal sample, while a primary sample without, 17C, did not. The seven cancer cell lines with global hypomethylation showed clear LINE1 hypomethylation compared with the three cancer cell lines without global hypomethylation.

These data confirmed an association between global hypomethylation and hypomethylation of repetitive sequences, which has been generally accepted, but with limited experimental evidence.

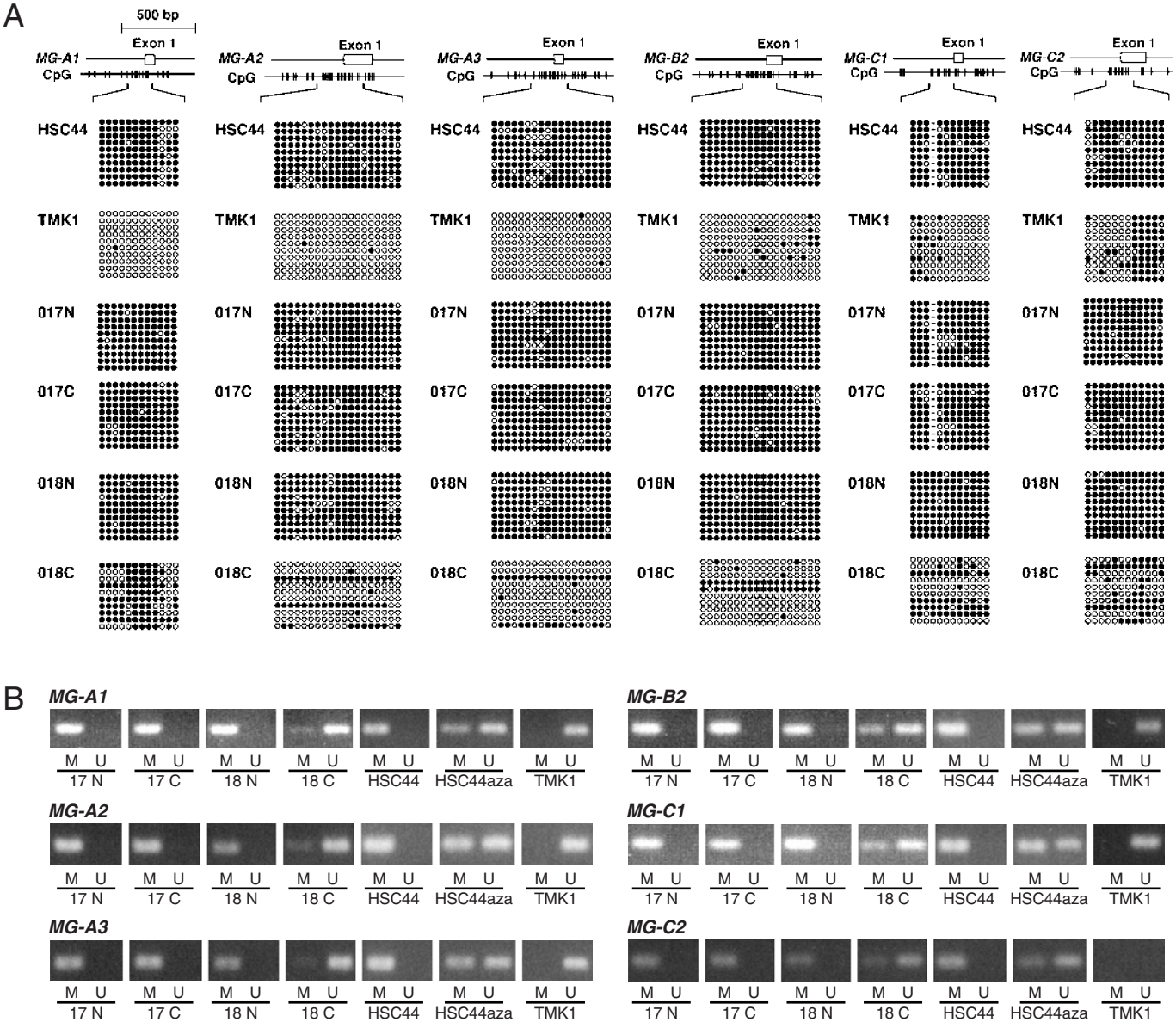

Fig. 2. The methylation status in the promoter CGIs of the six *MAGE* genes in two cancer cell lines (HSC44 and TMK1) and two pairs of primary samples (normal and cancer samples of cases 17 and 18). The methylation status analyzed by bisulfite sequencing (A) showed complete concordance with that analyzed by MSP (B), and MSP was adopted for the following analysis of all the ten cancer cell lines and 93 primary samples. The six CGIs were methylated in 28 normal samples analyzed, with three exceptions showing hypomethylation of *MAGE-B2*. In contrast, hypomethylation was frequently observed in cancer samples, as shown in Fig. 4.

Hypomethylation of the six *MAGE* genes and their aberrant expression. Methylation status of six normally methylated CGIs in the promoter regions of six genes, *MAGE-A1*, *-A2*, *-A3*, *-B2*, *-C1* and *-C2*, was analyzed by bisulfite sequencing in two gastric cancer cell lines and two pairs of primary samples (Fig. 2A). The promoter CGIs of all six genes were completely methylated in the two normal samples (17N and 18N). The methylated status was maintained for all the six genes in a cancer cell line, HSC44, and in a primary cancer sample, 17C. In contrast, aberrant hypomethylation was present for all six genes in a cancer cell line, TMK1, and a primary cancer sample, 18C. This methylation status was reproducibly detected by MSP (Fig. 2B), and MSP was adopted for the following analysis of all the ten cancer cell lines and 93 primary samples for the six genes.

Expression levels of the six *MAGE* genes were analyzed by quantitative RT-PCR. The correlation between the hypomethylation of the promoter CGIs detected by MSP and the expression of the downstream genes was confirmed for all six genes (Fig. 3). Further, treatment of HSC44 and HSC57 cell lines, in which all six *MAGE* genes were kept methylated, with 5-aza-dC led to demethylation of the six promoter CGIs and to their expression (Fig. 3).

Correlation between global hypomethylation and multiple promoter hypomethylation. The three cancer cell lines without global hypomethylation showed hypomethylation of one or none of the six normally methylated CGIs (Fig. 4A). In contrast, six of the seven cancer cell lines with global hypomethylation showed hypomethylation of five or all six CGIs. The 5-methylcytosine content of the six cancer cell lines with frequent promoter hypomethylation, 0.56±0.10%, was significantly lower than that of the four other cancer cell lines with rare promoter hypomethylation, 0.76±0.07% (P=0.008, t test). These data showed that frequent promoter hypomethylation largely correlates with global hypomethylation.

Hypomethylation and hypermethylation profiles in 93 primary gastric cancers. The hypomethylation profiles of the six *MAGE* genes were analyzed in 93 primary gastric cancers (Fig. 4B). Thirty-three cancers had aberrant hypomethylations in four or more of the six genes, 29 cancers had aberrant hypomethylations in one to three genes, and 31 cancers had no aberrant hypomethylation. This distribution is significantly different from that expected by hypothesizing a random occurrence of hypomethylation ($P<10^{-3}$, χ^2 test). Frequent promoter hypomethylation did not correlate with any clinicopathological factors examined, including histology, depth of cancer, or status of lymph node metastasis. Among the nine cancer samples whose 5-methylcytosine contents were measured (shown by asterisks in Fig. 4B), four primary cancers with frequent promoter hypomethylation showed much lower levels of 5-methylcytosine content (0.55±0.10%; P=0.0002, compared with the average level of 0.82±0.07% in normal samples by t test). In contrast, four primary cancers without promoter hypomethylation showed slightly lower levels (0.74±0.03%; P=0.06). The other primary cancer, 5C, showed an intermediate frequency of promoter hypomethylation (two of the six genes) and had an intermediate level of 5-methylcytosine content (0.67%).

Aberrant hypermethylation of CGIs in promoter regions was analyzed in eight genes, *LOX*, *HRASLS*, *FLNc*, *HAND1*, *TM*, *PGAR*, *p16* and *hMLH1* (Fig. 4). Among 93 primary cancers, 27 cancers showed aberrant hypermethylation in five or more of the eight genes, whereas 27 cancers did not show it in any of the eight genes. Cancers with frequent hypermethylation constituted a different group from those with frequent hypomethylation.

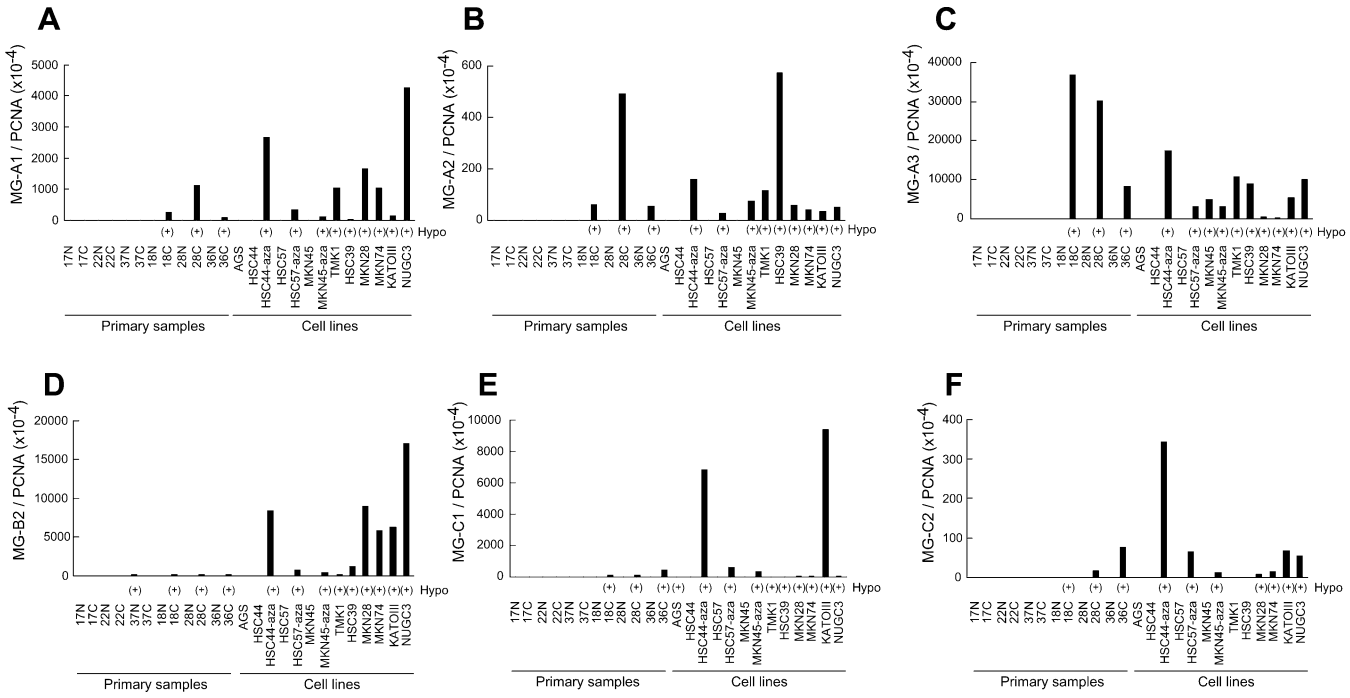

Fig. 3. Expression of the six *MAGE* genes and their hypomethylation status. In six pairs of primary samples and ten cancer cell lines, expression levels were analyzed by quantitative RT-PCR, and normalized to that of PCNA. Hypo, hypomethylation; aza, 5-aza-dC treatment. For each of the six genes, a good correlation between expression induction and hypomethylation was confirmed. No gene expression was detected in the normal samples, except for a small amount of *MAGE-B2* expression in 37N, which was associated with aberrant hypomethylation. Treatment with 5-aza-dC of HSC44 and HSC57 led to demethylation and expression of all the six *MAGE* genes. In MKN45, expressions of *MAGE-A1*, *-A2*, *-B2*, *-C1* and *-C2*, which were methylated, were induced by 5-aza-dC treatment, while expression of *MAGE-A3*, which was hypomethylated and expressed before 5-aza-dC treatment, was not affected.

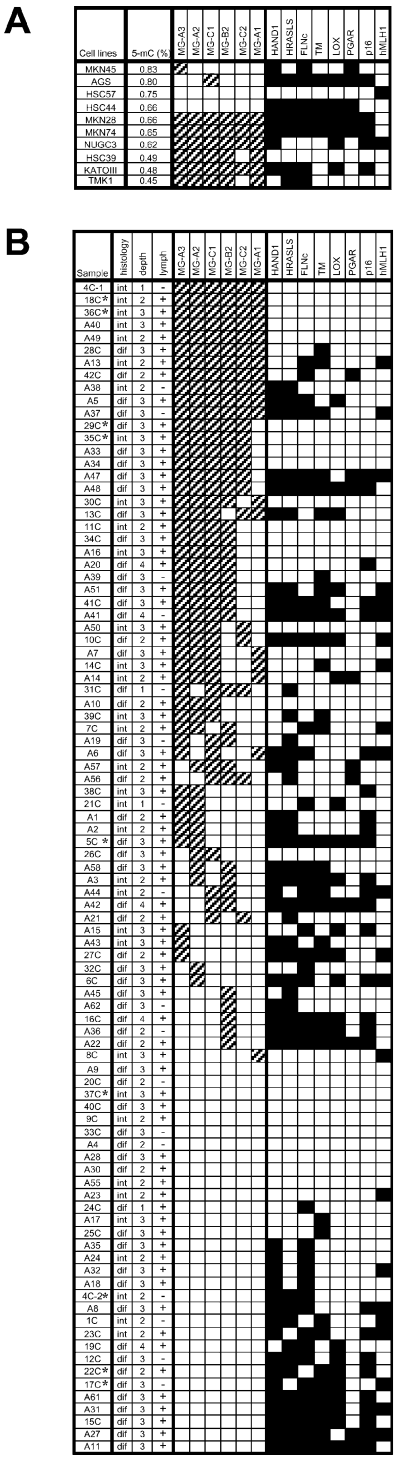

Fig. 4. Distribution of aberrant hypomethylation and hypermethylation in gastric cancer cell lines (A) and primary gastric cancer samples (B). dif, diffuse-type gastric cancer; Int, intestinal-type gastric cancer. depth, depth of cancer (1 for mucosa or submucosa, 2 for muscle propria or subserosa, 3 for serosa, 4 for invasion to other organs); lymph, status of lymph node metastasis. Hatched boxes for the left six genes, which are normally methylated, denote aberrant hypomethylations in cancers. Closed boxes for the right eight genes, which are normally unmethylated, denote aberrant hypermethylation in cancers. Asterisks (*) indicate the nine primary cancers whose methylcytosine content was measured (Fig. 1A). Cancers with frequent promoter hypomethylation and those with frequent hypermethylation constituted different groups. Cancers with frequent promoter hypomethylation showed no correlation with histology, depth of cancer, or status of lymph node metastasis.

Discussion

Genomic 5-methylcytosine content, hypomethylation of repetitive sequences, promoter hypomethylation, and promoter hypermethylation were simultaneously analyzed using human gastric cancers. It was demonstrated that frequent promoter hypomethylation correlated with global hypomethylation, but was independent of frequent promoter hypermethylation. Global hypomethylation was also confirmed to be consistently associated with hypomethylation of the LINE1 repetitive sequence, and this study has provided experimental evidence for this widely accepted idea.

Frequent promoter hypomethylation correlated well with global hypomethylation, with the exception of HSC44 cells, which had rare promoter hypomethylation (0/6) but low 5-methylcytosine content (0.66%). Previously, De Smet *et al*. found that *MAGE-1* promoter hypomethylation caused expression and that its promoter hypomethylation was associated with global hypomethylation.[24] This study examined multiple promoter CGIs so that demethylation pressure in promoter regions in a cell should have been accurately estimated, and confirmed the association between the frequent promoter hypomethylation and global hypomethylation.

Similar to our result on HSC44, De Smet *et al*. found two exceptional melanoma cell lines that did not express *MAGE-1*, but had global hypomethylation. One possibility is that global hypomethylation may affect the genome randomly, so that high copy number targets, such as repetitive sequences, are consistently hypomethylated whereas unique promoter regions are stochastically hypomethylated. Alternatively, we can hypothesize a promoter-specific protection mechanism(s) that could be disrupted when global hypomethylation exceeds a threshold. Considering that all six cell lines with global hypomethylation other than HSC44 showed promoter hypomethylation in five or all of the six genes simultaneously, we still cannot neglect this possibility. In either case, global hypomethylation generally correlated with frequent promoter hypomethylation.

No correlation was observed between promoter hypomethylation and promoter hypermethylation (Fig. 4). Frequent promoter hypermethylation was observed in three of six cancer cell lines with frequent promoter hypomethylation and in two of four cancer cell lines with rare promoter hypomethylation. In primary cancers, it was observed in seven (21%) of 33 primary cancers with promoter hypomethylation of four or more of six genes, in ten (34%) of 29 cancers with that of one to three genes, and in ten (32%) of 31 cancers with no promoter hypomethylation (Fig. 4). These data indicated that frequent promoter hypomethylation and frequent promoter hypermethylation could be present in the same cancers, but that they occurred independently.

The results of this study re-emphasize the importance of global hypomethylation, which can lead to promoter hypomethylation directly or perhaps by disrupting a protection mechanism of methylated promoter regions. Promoter hypomethylation might activate an unknown oncogene that is normally silenced by promoter methylation, though a transcription factor is available in the cell. Further research should be directed to clarify the molecular mechanisms of global hypomethylation, as well as those for promoter methylation.

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