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Profile of Aberrant CpG Island Methylation Along the Multistep Pathway of Gastric Carcinogenesis

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SUMMARY: To date, several reports on methylation of various genes in gastric cancer (GC) have been published. However, most of these studies focused on cancer tissues or a single gene only and gave no information about the methylation status of specific genes in the premalignant stages or about the concurrent methylation of other genes in specific lesions. We attempted to investigate methylation of multiple genes in a large sample collection of GC ($n = 80$), gastric adenoma (GA) ($n = 79$), intestinal metaplasia (IM) ($n = 57$), and chronic gastritis (CG) ($n = 74$). We determined the methylation frequency of 12 genes, including *APC*, *COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *MGMT*, *p16*, *p14*, *RASSF1A*, *THBS1*, and *TIMP3* by methylation-specific PCR. Five different classes of methylation behaviors were found: (1) genes methylated in GC only (*GSTP1* and *RASSF1A*); (2) genes showing low methylation frequency (<12%) in CG, IM, and GA, but significantly higher methylation frequency in GC (*COX-2*, *hMLH1*, and *p16*); (3) a gene with low and similar methylation frequency (8.8–21.3%) in four-step lesions (*MGMT*); (4) genes with high and similar methylation frequency (53–85%) in four-step lesions (*APC* and *E-cadherin*); and (5) genes showing an increasing tendency with or without fluctuation of the methylation frequency along the progression (*DAP-kinase*, *p14*, *THBS1*, and *TIMP3*). The average number of methylated genes was 2.7, 3.6, 3.4, and 5.2 per 12 tested genes in CG, IM, GA, and GC, respectively. Our results suggest that tumor suppressor genes show a gene type–specific methylation profile and that aberrant CpG island methylation tends to accumulate along the pathway of multistep carcinogenesis. (*Lab Invest 2003, 83:635–641*).

In humans and most mammals, DNA methylation is the only known endogenous modification of DNA, affecting only the cytosine residue when it precedes a guanosine residue. DNA methylation has an important role in the transcriptional repression of imprinted genes (Barlow, 1995) and genes on the inactivated X chromosomes (Goto and Monk, 1998), maintaining the integrity of chromosomes (Erlich, 2000) or acting as a defense against highly repeated mobile elements (O'Neill et al, 1998). Aberrant methylation of CpG islands, which are normally protected from DNA methylation, is associated with DNA structural change and consequent gene inactivation. Aberrant methylation of promoter CpG islands is now recognized as an important mechanism for gene inactivation as an alternative to gene mutation or deletion in tumorigenesis (Baylin et al, 1998; Jones and Laird, 1999). Many tumors show simultaneous methylation of multiple genes involved in the tumorigenesis, including colon, hematologic, or uterine cervical cancers (Esteller et al, 2001; Toyota et al, 1999a, 2001; Virmani et al, 2001). Gastric cancer (GC) is one of the tumors with a high frequency of aberrant methylation (Kang et al, 2001; Toyota et al, 1999b).

An increasing number of genes that are inactivated by promoter CpG island hypermethylation have been reported in GC (Byun et al, 2001; Iida et al, 2000; Kang et al, 2000; Leung et al, 1999; Oue et al, 2001; Shim et al, 2000; Song et al, 2001; Tamura et al, 2000; Tsuchiya et al, 2000). These genes have been identified by demonstrating the close link between CpG island methylation of the specific gene and loss of mRNA or protein in addition to reversal of the expression by treatment with demethylating agents. However, most of these studies have focused on aberrant methylation in a single gene. There have only been a few studies investigating the methylation status of multiple genes in GC (Leung et al, 2001; Suzuki et al, 1999; Toyota et al, 1999a). Furthermore, most of these studies were restricted to cancer tissues only, so the methylation status of a specific gene in the premalignant stages of GC was not analyzed. If aberrant methylation of the specific gene contributes to tumor initiation, the methylation change is expected to be found in the premalignant or early stages of the cancer.

We have previously studied the methylation of five genes: *DAP-kinase*, *hMLH1*, *p16*, *THBS1*, and *TIMP3* in non-neoplastic and neoplastic gastric samples. We showed that CpG island methylation occurs early in multistep gastric carcinogenesis (Kang et al, 2001). In the present study, we extended the panel of tested genes from 5 to 12 and we studied the aberrant methylation of these loci for GCs and premalignant

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lesions. These genes are frequently methylated in cancers of the stomach or other organs and included the genes involved in cell cycle regulation (*p14*, *p16*, and *COX-2*), signal transduction (*APC* and *RASSF1A*), DNA repair or protection (*hMLH1*, *MGMT*, and *GSTP1*), apoptosis (*DAP-kinase*), and angiogenesis (*THBS1*) and those related to metastasis and invasion (*E-cadherin* and *TIMP3*). We analyzed the frequency of methylation of specific genes to determine the chronology and the extent of methylation during the multistep pathway of carcinogenesis from chronic gastritis (CG) to GC.

Results

We analyzed non-neoplastic and neoplastic gastric mucosa samples for the methylation status of CpG islands of the 12 genes using methylation-specific PCR (MSP). Tables 1 and 2 summarize the methylation frequency of each tested CpG island and the number of methylated genes in lesions of the each of the steps from CG to GC, respectively. Figure 1 displays representative examples of the MSP products analyzed by electrophoresis on an agarose gel. Methylation index was defined as the number of methylated genes divided by the number of tested genes. The average methylation index was 0.23, 0.3, 0.28, and 0.43 in CG, intestinal metaplasia (IM), gastric adenoma (GA), and GC, respectively. The methylation index was significantly higher for IM and GA than for CG ($p = 0.018$ and $p = 0.034$, respectively, two-tailed t test). A significant increase of methylation frequency from premalignant lesions (IM or GA) to GC, but a similar methylation index between IM and GA, was noted (Fig. 2).

Bisulfite genomic sequencing of the representative MSP samples for each gene was performed, which validated the adequacy of the bisulfite modification and indicated that all of the cytosines at non-CpG sites were converted to thymines. All of the sequenced MSP products showed extensive methylation of CpG sites between the MSP primer sequences as well as those CpG sites within the primer sequences. There were no differences between lesions at each step in either the number of methylated CpG sites or the methylation density of each CpG site. The vast majority of CpG sites, of all of the tested genes, exhibited methylation at a frequency greater than or equal to 75%.

Forty pediatric gastric tissue samples were examined for the methylation status of 12 genes. These samples were obtained from pediatric patients who complained of epigastric discomfort and who underwent an endoscopic biopsy. All of the samples were diagnosed as CG without IM. The patients ranged from 2 to 17 years old, with an average age of 11 years. Table 3 summarizes the MSP results. *COX2*, *DAP-kinase*, *GSTP1*, *hMLH1*, and *RASSF1A* were not methylated at all in the pediatric gastric samples. One of the 40 samples showed *p16* methylation. *MGMT* and *p14* were methylated at a frequency of 2.5%; *APC*, *E-cadherin*, *THBS1*, and *TIMP3* were methylated at frequencies of 15%, 15%, 10%, and 15%, respectively. The methylation index of pediatric CG was 0.05, and the maximum number of methylated genes was 4. The earliest age when these genes were methylated was 2 years, although most of the cases with methylation of two or more genes were observed in patients older than 14 years. When the methylation frequency of each gene was compared between the pediatric and adult CG samples, significant differences were noted in *APC*, *DAP-kinase*, *E-cadherin*, and *p14*. Thus, methylation of these genes may be considered aging related.

Discussion

We analyzed promoter hypermethylation changes of 12 genes in DNA from the lesions of the four steps from CG to IM, GA, and GC. These genes possess

Table 1. Methylation Frequency of Each Gene in CG, IM, GA, and GC

	CG ($n = 74$)	IM ($n = 57$)	GA ($n = 79$)	GC ($n = 80$)	p value
APC	48 (64.9%)	46 (80.7%)	57 (72.2%)	62 (77.5%)	NS[a]
COX2	1 (2.2%)	5 (8.8%)	3 (3.8%)	37 (46.3%)	<0.001
DAP-K	26 (35.1%)	28 (49.1%)	27 (34.2%)	45 (56.3%)	0.012
E-cadherin	63 (85.1%)	41 (71.9%)	46 (58.2%)	53 (67.5%)	0.003
GSTP1	0	0	0	13 (16.3%)	<0.001
hMLH1	0	4 (7%)	7 (8.9%)	16 (20%)	<0.001
MGMT	11 (14.9%)	5 (8.8%)	8 (10.1%)	17 (21.3%)	NS
p14	22 (29.7%)	18 (31.6%)	60 (75.9%)	50 (62.5%)	<0.001
p16	2 (2.7%)	4 (7%)	9 (11.4%)	35 (43.8%)	<0.001
RASSF1A	0	0	0	6 (7.5%)	0.001
THBS1	13 (17.6%)	28 (49.1%)	27 (34.2%)	45 (56.3%)	<0.001
TIMP3	17 (23%)	25 (43.9%)	22 (27.8%)	52 (65%)	<0.001
Average number of methylated genes	2.7[b]	3.6[c,d]	3.4[b,c]	5.2[d]	

[a] Not significant ($p > 0.05$).
[b] CG vs GA; $p = 0.034$; Student's t test.
[c] IM vs GA; $p = 0.527$; Student's t test.
[d] IM vs GC; $p < 0.001$; Student's t test.

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Table 2. Frequency of Coincident Number of Gene Hypermethylation Events in CG, IM, GA, and GC

No. of genes methylated	0	1	2	3	4	5	6	7	8	9	10	11	12
CG	3	22	17	6	11	6	8	1					
(n = 74)	(4.1%)	(29.7%)	(23%)	(8.1%)	(14.9%)	(8.1%)	(10.8%)	(1.4%)					
IM	4	8	8	6	11	8	8	2	2				
(n = 57)	(7%)	(14%)	(14%)	(10.5%)	(19.3%)	(14%)	(14%)	(3.5%)	(3.5%)				
GA	3	10	11	17	21	8	6	1	2				
(n = 79)	(3.8%)	(12.7%)	(13.9%)	(21.5%)	(26.6%)	(10.1%)	(7.6%)	(1.3%)	(2.5%)				
GC	3	4	5	13	6	13	8	13	6	5	3	1	
(n = 80)	(3.8%)	(5%)	(6.3%)	(16.3%)	(7.5%)	(16.3%)	(10%)	(16.3%)	(7.5%)	(6.3%)	(3.8%)	(1.3%)	

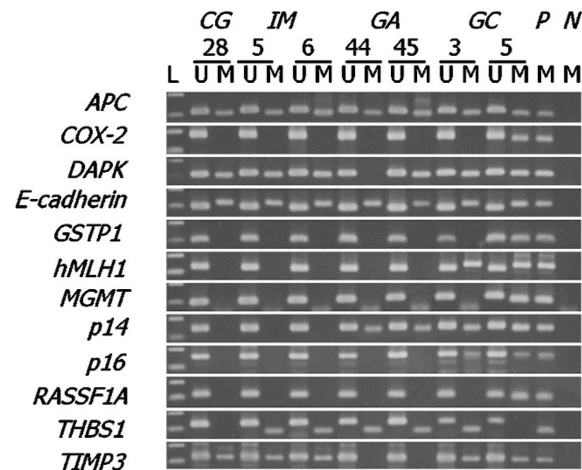

Figure 1.

Representative samples of methylation-specific PCR (MSP) analyses of DNA samples from chronic gastritis (*CG*), intestinal metaplasia (*IM*), gastric adenoma (*GA*), and gastric carcinoma (*GC*). The PCR products in the lanes marked *U* show the presence of unmethylated templates of each gene, whereas the products in the lanes marked *M* indicate the presence of methylated templates. *L* = size marker (100-bp DNA ladder); *P* = positive control; *N* = negative control; *DAPK* = DAP kinase. Positive control is normal lymphocyte DNA treated with Sss1 methyl transferase before bisulfite modification. Negative control is distilled water without template DNA.

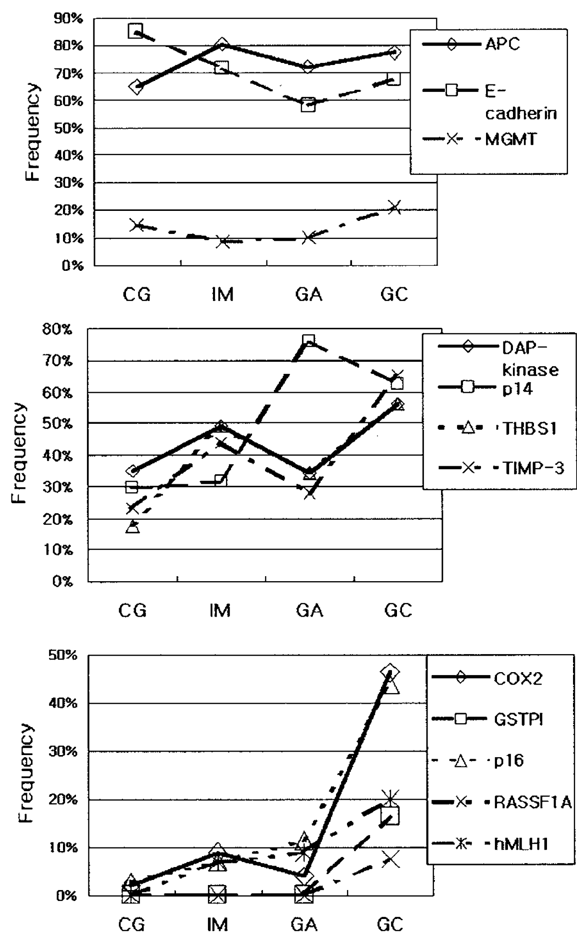

Figure 2.

Profiles of gene promoter hypermethylation along multistep gastric carcinogenesis. A gene with low and similar methylation frequency (8.8%-21.3%) in the four-step lesions (*MGMT*) and genes with high and similar methylation frequency (58–85%) in the four-step lesions (*APC* and *E-cadherin*) (upper). Genes showing an increasing tendency, with or without fluctuation, of methylation frequency along the progression (*DAP-kinase*, *p14*, *THBS1*, and *TIMP3*) (middle). Genes methylated in GC only (*GSTP1* and *RASSF1A*) and genes showing low methylation frequencies (<12%) in CG, IM, and GA, but a significantly higher methylation frequency in GC (*COX-2*, *hMLH1*, and *p16*) (lower).

CpG islands in their 5' region; their inactivation by aberrant methylation of these CpG islands and their recovery from silencing by demethylation in GC and other cancers have been demonstrated. The genes tested included those involved in the important molecular pathways of carcinogenesis, such as cell cycle regulation, signal transduction, DNA repair or protection, apoptosis, and angiogenesis. However, most of the previous studies focused on the aberrant methylation of these genes in GC samples only, but did not involve a study of the methylation status of the genes in CG, IM, or GA. Without analysis of the methylation status of these genes in DNA from stages before GC, it cannot be clearly indicated whether the aberrant methylation of a specific gene is cancer-related or not.

If hypermethylation-associated inactivation of a specific gene contributes to tumor initiation, aberrant methylation might be found in the early lesions of the multistep pathway of gastric carcinogenesis. In the present study, 10 genes, excluding *GSTP1* and *RASSF1A*, were found to be methylated in CG, IM, or GA. However, the methylation frequency of *APC*, *E-cadherin*, and *MGMT* was not different in lesions of each step, raising a question about the role of DNA

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Table 3. Comparison of Methylation Frequency of Each Gene Between Child and Adult CG

	Child CG (n = 40)	Adult CG (n = 74)	p value[a]
APC	6 (15%)	48 (64.9%)	<0.001
COX2	0	1 (2.2%)	NS[b]
DAP-Kinase	0	26 (35.1%)	<0.001
E-cadherin	6 (15%)	63 (85.1%)	<0.001
GSTP1	0	0	
hMLH1	0	0	
MGMT	1 (2.5%)	11 (14.9%)	0.054
p14	1 (2.5%)	22 (29.7%)	<0.001
p16	1 (2.5%)	2 (2.7%)	NS
RASSF1A	0	0	
THBS1	4 (10%)	13 (17.6%)	NS
TIMP3	6 (15%)	17 (23%)	NS
Average number of methylated genes	0.6	2.7	<0.001

[a] Analyzed by two-tailed Fisher's exact test.
[b] Not significant ($p > 0.05$).

methylation of these genes in the progression of the lesion along the multistep gastric carcinogenesis. *DAP-kinase*, *p14*, *THBS1*, and *TIMP3* showed an increasing tendency of methylation frequency along the progression from CG to GC. *COX-2*, *hMLH1*, and *p16* were rarely methylated in CG, IM, and GA, and their methylation frequency showed at least a 2-fold increase from the premalignant lesions to GC. Thus, these, in addition to *GSTP1* and *RASSF1A*, can be considered as cancer-related methylation genes.

In the present study, 12.2% of CG and 21% of IM samples showed concurrent methylation in at least seven of the tested genes. In these samples with a high frequency of methylation, the affected cells may have a growth-selective advantage imparted by the expressional loss of the methylated genes, which may predispose the cells to acquiring further genetic or epigenetic defects, which leads to neoplasia. If this is the case, a higher frequency of aberrant methylation would be expected in non-neoplastic gastric tissues for patients with GC. We investigated the methylation frequency of the 12 genes in 48 samples of non-neoplastic gastric mucosa from the patients with GC and found a methylation index of 0.48, with 60.1% of the samples showing concurrent methylation of at least six of the tested genes (data not shown). These figures are significantly higher than those for CG or IM samples from patients without GC. These results were consistent with those of the study by Waki et al (2002), which showed increased methylation frequencies in non-neoplastic gastric mucosa from GC compared with non-neoplastic gastric mucosa without association of GC. A longitudinal prospective study would be required to confirm that patients with a high frequency of methylation in their gastric mucosa are at a higher risk of developing cancer.

We have studied a large collection of gastric samples, from CG to GC, for the methylation status of a panel of genes, and we determined the timing and frequency of aberrant methylation for specific genes along the multistep pathway of gastric carcinogenesis. We found that tumor suppressor genes showed a gene type–specific methylation profile along the multistep pathway of gastric carcinogenesis and that aberrant CpG island methylation occurred in the early stages and tended to accumulate along the multistep pathway of gastric carcinogenesis.

Materials and Methods

DNA Preparation

We studied 80 archival samples of surgically resected GC, 79 archival samples of GA, and 131 samples of endoscopically obtained non-neoplastic gastric mucosae (57 IM and 74 CG). After identification of carcinoma, adenoma, or intestinal metaplasia on hematoxylin and eosin–stained slides and marking the respective lesion on paraffin blocks, the marked areas were scraped from the paraffin blocks. The collected materials were dewaxed in xylene and rinsed in ethanol. The dried tissues were digested in a lytic solution containing proteinase K, and the genomic DNA was purified with phenol/chloroform and by precipitation with ethanol.

Methylation-Specific PCR

Both normal and tumor DNAs were subjected to sodium bisulfite modification as described previously (Herman et al, 1996). In brief, 5 μg of DNA was denatured with 2 M NaOH, followed by treatment with 1 mM hydroquinone and 3.5 M sodium bisulfite and incubation for 16 hours at 55° C. After purification using a JETSORB gel extraction kit (Genomed, Bad Oeynhausen, Germany), the DNA was treated with 3 M NaOH and precipitated with three volumes of 100% ethanol and a one-third volume of 7.5 M NH_4Ac at −20° C. The precipitated DNA was washed with 70% ethanol and dissolved in distilled water. A panel of 12 genes were analyzed for their methylation status using MSP; the tested genes included *APC*, *COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *MGMT*, *p14*, *p16*, *RASSF1A*, *TIMP3*, and *THBS1*. The primer sequences of each gene, for methylated or unmethylated reac-

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Table 4. Primer Sequences and PCR Conditions for MSP Analysis

Primer name		Primer sequence (5′–3′) forward	Primer sequence (5′–3′) reverse	Product size (bp)	Annealing temperature (°C)	References
APC	m	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98	55	(Tsuchiya et al, 2000)
	7	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108	60	
COX-2	m	TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	161	61	(Akhtar et al, 2001)
	u	ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	171	61	
DAP-kinase	m	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60	(Katzenellenbogen et al, 1999)
	u	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60	
E-cadherin	m	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	115	57	(Herman et al, 1996)
	u	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	53	
GSTP1	m	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	91	59	(Esteller et al, 1998)
	u	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97	59	
MGMT	m	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	59	(Esteller et al, 2002)
	u	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59	
hMLH1	m	TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	153	60	(Kang et al, 1999)
	u	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60	
p14	m	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	60	(Esteller et al, 2000)
	u	TTTTTGGTGTTAAAGGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	60	
p16	m	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65	(Herman et al, 1996)
	u	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60	
RASSF1A	m	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	93	60	(Lo et al, 2001)
	u	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	105	60	
THBS1	m	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62	(Ueki et al, 2000)
	u	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62	
TIMP3	m	CGTTTCGTTATTTTTTGTTTTCGGTTTTC	CCGAAAACCCCGCCTCG	116	59	(Bachman et al, 1999)
	u	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	122	59	

m, methylated sequence; u, unmethylated sequence.

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tions, are described in Table 4. The 25-μl total reaction volume contained a PCR mixture of 1× PCR buffer (16.6 mM $(NH_4)_2SO_4$; 67 mM Tris, pH 8.8; 6.7 mM $MgCl_2$; 10 mM β-mercaptoethanol), dNTPs (each at 1 mM), primers (10 pmol each), and bisulfite-modified DNA (30–50 ng). The reactions were hot-started at 97° C for 5 minutes before the addition of 0.75 U of *Taq* polymerase (Takara Shuzo Company, Kyoto, Japan). The PCR conditions were as follows: 35 cycles of denaturation at 95° C for 40 seconds, annealing at the temperatures specified in Table 1 for 50 seconds, and finally a 50-second extension at 72° C. A final 10-minute extension at 72° C completed each PCR. The PCR products underwent electrophoresis on 2.5% agarose gels and were visualized after staining with ethidium bromide. Samples showing signals approximately equivalent to that of the size marker (7 ng/μl) were scored as methylated. Samples giving faint positive signals were repeated three times, and only those samples with consistent positive signals were regarded as methylated.

Sequencing Analysis

The PCR products were purified using a JETSORB gel extraction kit (Genomed) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, California). Plasmid DNA was extracted from individual clones by an alkaline lysis plasmid minipreparation. The inserted PCR fragments of the four individual clones, obtained from each sample, were sequenced with both M13 reverse and M13 (-20) forward primers using an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

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