Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia

Sun Lee[1], Kyu Sang Hwang[2], Hyeon Joo Lee[2], Jung-Sun Kim[3] and Gyeong Hoon Kang[2]

[1]Department of Pathology, National Cancer Center, Goyang, Gyeonggi, Korea; [2]Department of Pathology, Seoul National University College of Medicine and Cancer Research Institute, Seoul, Korea and [3]Department of Diagnostic Pathology, University of Ulsan College of Medicine, Seoul, Korea

CpG island hypermethylation is a potential means of inactivating tumor suppressor genes, and many genes have been demonstrated to be hypermethylated and silenced in colorectal cancer. However, limited data is available upon the concurrent methylation of multiple genes in colorectal cancer and in its precursor lesion. To address changes in the methylation profiles of multiple genes during colorectal carcinogenesis, we investigated the methylation of 12 genes (*APC*, *COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *MGMT*, *p14*, *p16*, *RASSF1A*, *THBS1*, and *TIMP3*) in normal colon ($n = 24$), colon adenoma ($n = 95$), and colorectal cancer ($n = 149$), using methylation-specific PCR. The average number of these genes methylated per sample was 0.12, 1.8, and 3.0 in normal colon mucosa, adenoma, and carcinoma, respectively, showing a stepwise increase ($P < 0.001$). All the genes were methylated in colorectal cancer at frequencies varying from 51 to 9.4% and colon adenoma displayed methylation for the 11 genes, except for GSTP1, at frequencies varying from 40 to 1.1%. In contrast, normal colon mucosa demonstrated methylation for APC only, at a frequency of 12.5%. The total number of methylated genes per tumor showed a continuous, nonbimodal distribution in colon adenoma or cancer. CpG island hypermethylation exhibited a proclivity toward proximal colon cancer or adenoma, and the average number of genes methylated was higher in proximal colon cancer or adenoma than in distal colon cancer or adenoma, respectively (3.5 *vs* 2.6, $P = 0.018$ for cancer, and 2.5 *vs* 1.4, $P = 0.003$ for adenoma). In conclusion, concurrent CpG island methylation is an early and frequent event during colorectal carcinogenesis. It appears that CpG island methylation plays a more important role in proximal colon cancer development than in distal colon cancer development.
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There are two well-known pathways of colorectal carcinogenesis, that is, chromosomal instability (CIN) and microsatellite instability (MSI). The CIN pathway is characterized by alterations of chromosomal number and structure affecting proto-oncogenes and tumor suppressor genes.[1] The CIN phenotype shows a high frequency of allelic losses and abnormal DNA content by flow cytometry.[2] Whereas, the MSI pathway features alterations in repeated nucleotides within the coding sequences, which result in the inactivation of tumor suppressor genes.[3,4] In contrast to the CIN phenotype, the MSI phenotype shows near-diploidy and very low allelic loss frequencies.[2] Recently the CpG island methyla-tion phenotype has been added to these two phenotypes, and is characterized by the concordant methylation of the promoter regions of multiple genes that play a role in carcinogenesis.[5] Moreover, accumulating evidence has now linked promoter CpG island methylation with transcriptional silencing in human cells. Although the mechanism whereby CpG island methylation suppresses gene transcription has not been fully elucidated, it has been proposed that methylated promoter sequences are bound by MeCP2, methylated-DNA binding proteins, which recruit histone deacetylases. Eventually this process leads to a closed chromatin configuration,[6] in which the chromatin structure is inaccessible to transcription factors, thus insuring transcriptional suppression.

To date, several studies have demonstrated that various genes are hypermethylated and silenced in colorectal cancer. However, most of these studies have focused on the aberrant methylation of a single gene or upon cancer tissues only. If aberrant CpG

island hypermethylation of a specific gene contributes to the development of colon cancers, then this methylation change might be found in colon adenomas. A small number of studies have investigated the concordant methylation of multiple genes in colon adenoma.[7–9] Thus, our understanding of aberrant CpG island methylation in the adenoma-carcinoma sequence of colorectal tumorigenesis remains limited.

In the present study, we determined the methylation profiles of 12 genes, and temporal sequences of this methylation in non-neoplastic colonic mucosa, colon adenoma, and colorectal cancer. We also analyzed the results of DNA methylation in relation to the location of the tumor within the colon and with clinicopathological parameters. Genes were selected for their known involvement in carcinogenesis and for their frequent epigenetic inactivation in colorectal cancer or in other cancers. They included genes involved in cell cycle regulation (*COX-2*, *p14*, and *p16*), DNA repair or protection (*hMLH1*, *MGMT*, and *GSTP1*), signal transduction (*APC* and *RASS-F1A*), apoptosis (*DAP-kinase*), and angiogenesis (*THBS1*), and those related to metastasis and invasion (*E-cadherin* and *TIMP-3*).

Materials and methods

Tissue Samples and DNA Extraction

A total of 271 archival samples consisting of 149 sporadic colorectal carcinomas (mean age, 58 years; range 24–83 years; 70 male and 79 female subjects), 95 adenomas (mean age, 56 years; range 31–74 years; 34 male and 17 female subjects), and 24 normal colon mucosae (mean age, 57 years; range 22–79 years; 15 males and 9 females) were examined during the course of this study. The sporadic colorectal cancer samples were obtained from patients who had undergone a surgical resection for colorectal cancer at Asan Medical Center in Seoul from 1998 to 1999. Adenoma or normal colon tissue samples were obtained from patients, who underwent sigmoidoscopic or colonoscopic biopsy at Seoul National University Hospital from 2001 to 2002. A total of 24 normal colon samples were obtained from subjects without colorectal cancer or adenoma. Clinicopathological findings, including tumor location, size, histologic type and differentiation, depth of invasion, and regional lymph node status, were reviewed for all cancer samples. All of the adenoma samples were of low-grade dysplasia, no high-grade adenoma was not included. Adenoma locations were reviewed.

DNA was extracted from formalin-fixed, paraffin-embedded tissues. After tumor identification on hematoxylin–eosin-stained slides, tumoral areas, in which tumor cells occupied more than 50% of the tumor cell population, were scraped from the 10-μm thick paraffin sections. The collected materials were dewaxed by washing in xylene and then rinsed in ethanol three times. Dried tissues were digested with proteinase K and DNA was extracted using the classic phenol/chloroform/isoamylalcohol with ethanol precipitation method.

Methylation-Specific PCR (MSP)

For MSP assay, DNAs were subjected to bisulfite modification, as described previously.[10] This modification converts unmethylated cytosine to uracil and leaves 5-methyl cytosine unchanged. Briefly, 20 μl of genomic DNA was treated with 550 μl of a mixed solution of 3.5 M sodium bisulfite/1 mM hydroquinone (pH 5.0). After incubation at 55°C for 16 h, the treated DNA was purified and desulfonated with 0.3 N NaOH. The modified DNA obtained was ethanol-precipitated and dissolved in 50 μl of TE buffer.

The primer sequences of each gene promoter, for both methylated and unmethylated forms, have been described previously.[11] To amplify the bisulfite-modified promoter sequence of *p16*, *E-cadherin*, *COX-2*, or *hMLH1*, we used a PCR mixture containing 1 × PCR buffer (10 mM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl$_2$), deoxynucleotide triphosphates (each at 0.2 mM), primers (10 pmol each), and bisulfite-modified DNA (30–50 ng) in a final volume of 25 μl. For the amplification of *APC*, *DAP-kinase*, *GSTP1*, *MGMT*, *RASSF1A*, *p14*, *THBS1*, or *TIMP-3*, we used a PCR mixture containing 1X PCR buffer (16.6 mM (NH$_4$)$_2$SO$_4$, 67 mM Tris (pH 8.8), 6.7 mM MgCl$_2$, and 10 mM ß-mercaptoethanol), deoxynucleotide triphosphates (each at 1 mM), primers (10 pmol each), and bisulfite-modified DNA (30–50 ng) in a final volume of 25 μl. Reactions were hot-started at 98°C for 5 min before adding 0.75 U of *Taq* polymerase (Takara Shuzo Co., Kyoto, Japan). Amplifications were carried out in a thermal cycler (Perkin-Elmer, Foster City, CA, USA) for 33–35 cycles (40 s at 95°C, 50 s at variable temperatures according to primer, and 50 s at 72°C), and then given a final 10 min extension. The PCR products were electrophoresed on a 2.5% agarose gel and visualized under UV illumination after ethidium bromide staining.

Bisulfite Sequencing

The PCR products were gel-purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). 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 Dye Terminator Cycle Sequencing Kits (Perkin-Elmer, Foster City, CA, USA) and ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Immunohistochemistry

Immunohistochemical analyses for E-cadherin and MGMT were performed in 59-colon cancer samples. Tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, and then washed in water. Antigen retrieval was carried out using microwave irradiation in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity and non-specific protein binding was blocked by incubating with 3% H_2O_2 and then with 10% normal goat serum. Sections were incubated with antibodies to E-cadherin protein (dilution 1:100; ABC Co., Lexington, KY, USA) and MGMT (dilution 1:100; N99200; Ventana, San Diego, CA, USA) at 4°C overnight. After reacting the sections with bio-tinylated secondary anti-mouse antibodies, the antigen–antibody reactions were visualized using streptavidin–horseradish peroxidase conjugate (DAKO LSAB kit, Los Angeles, CA, USA) and diaminobenzidine as a chromogen. The slides were counterstained with hematoxylin.

Normal tissue adjacent to tumor was used as an internal positive control. All the epithelial cells of normal colonic crypts showed membranous staining for E-cadherin and nuclear staining for MGMT. Loss of expression was defined as membranous staining for E-cadherin and nuclear staining for MGMT were observed in less than a half of the tumor cells.

Statistical Analysis

SPSS 11.0 software was used throughout. To compare the methylation extents in the 12 promoter CpG islands tested, we determined the methylation index (MI) in each case. This was defined as the total number of genes methylated divided by the total number of genes tested. MI differences between groups were analyzed using the Student's *t*-test. Differences in the methylation frequencies of each gene in the groups were analyzed using the χ^2 test or Fisher's exact test. The Mann–Whitney *U*-test was used to examine methylation concordances at multiple loci. The *P*-values of < 0.05 were considered to be statistically significant.

Results

Figure 1 shows the MSP results of representative samples of colorectal carcinomas, adenomas, and normal colons. All cases showing PCR products for methylated DNA sequences also exhibited PCR products for unmethylated DNA sequences, which was probably caused by DNA contamination from stromal or inflammatory cells in the samples, or related to tumor heterogeneity regarding the methylation of specific CpG sites.

The average numbers of methylated genes were 0.12, 1.8, and 3.0 per 12 genes in normal colon, adenoma, and carcinoma samples, respectively, and

showed a stepwise increase ($P < 0.001$ and $P = 0.001$, between adenoma and normal colon mucosa, and between colorectal cancer and adenoma, respectively, student's *t*-test).

Distribution of CpG Island Hypermethylation

In total, 93% ($n = 139$) of the carcinoma samples ($n = 149$) showed promoter methylation of at least one gene, and this ranged from one to nine genes per sample, whereas 68.4% ($n = 65$) of adenoma samples ($n = 95$) showed promoter methylation of one to seven genes (Table 1). In contrast, 12.5% ($n = 3$) of normal colon samples ($n = 24$) showed promoter methylation of one gene only (*APC*). Table 1 summarized the total number of methylated CpG islands observed in each tumor or normal sample. The distribution of methylated CpG islands per tumor in colon adenomas or carcinomas was of the continuous pattern, but not of the bimodal pattern.

Bisulfite Sequencing

Sequencing analysis of one or two MSP samples for each gene in adenoma and carcinoma was performed, which exhibited that all of the cytosines at non-CpG sites were converted to thymines, validating the adequacy of the bisulfite modification. All of the sequenced methylated PCR 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 adenoma and carcinoma samples in either the number of methylated CpG sites or the methylation density of each CpG site for the particular gene. The vast majority of CpG sites of all of the tested genes exhibited methylation at a frequency greater than, or equal to, 50% (Figure 2).

Frequencies of Methylation of Individual Genes in Colorectal Cancer, Adenoma, and Normal Colon Samples

MSP results of the 12 genes are summarized in Table 2. In colorectal cancer samples, 12 genes were methylated at frequencies ranging from 9.4 to 51%. Adenoma samples showed variable methylation frequencies for 11 genes, ranging from 1.1 to 40%, except for *GSTP1*, which was not methylated in any adenoma sample.

Genes methylated in colorectal cancer at significantly higher frequencies than in adenoma were *APC*, *COX-2*, *DAP-kinase*, *GSTP1*, *hMLH1*, *p16*, *RASSF1A*, *THBS1*, and *TIMP-3*. For *E-cadherin*, *MGMT*, and *p14* genes, differences between colorectal carcinoma (CRC) and adenoma did not reach statistical significance. Adenoma samples had significantly higher methylation frequencies than
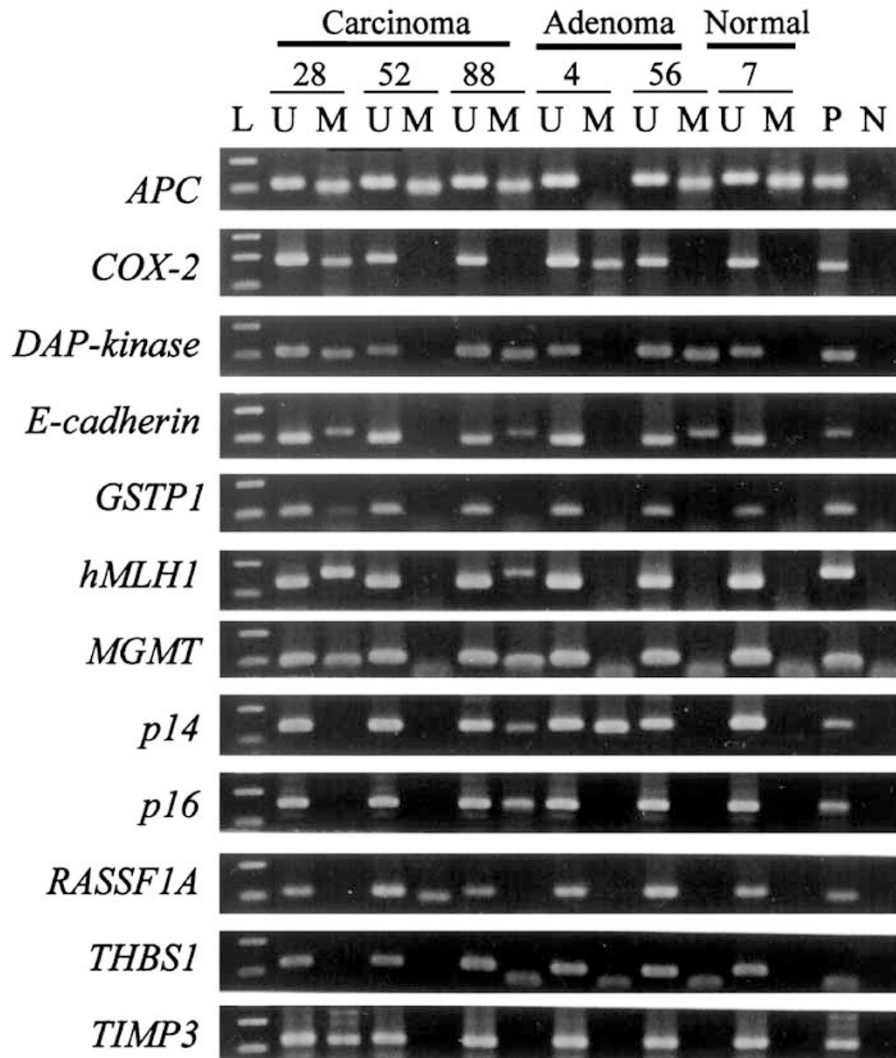
Figure 1 Representative samples of MSP analyses of DNA samples from colorectal carcinoma, adenoma, and normal colon mucosa. PCR products in the lanes marked U showed the presence of the unmethylated templates of each gene, whereas the products in the lanes marked M indicate the presence of methylated templates. L, size marker (100-bp DNA ladder); P, positive control; N, negative control. The positive control was a normal lymphocyte DNA treated with *Sss*1 methylase before bisulfite modification, and the negative control was distilled water without template DNA.

Table 1 The frequency of coincident number of gene hypermethylation in normal colon, colon adenoma, and colorectal carcinoma (CRC)

No. of genes methylated	0	1	2	3	4	5	6	7	8	9
Normal ($n = 24$)	21 (87.5)	3 (12.5)								
Adenoma ($n = 95$)	28 (29.5)	26 (27.4)	12 (12.6)	8 (8.4)	13 (13.7)	6 (6.3)		2 (2.1)		
CRC ($n = 149$)	13 (8.7)	27 (18.1)	30 (20.1)	27 (18.1)	18 (12.1)	15 (10.1)	11 (7.4)	3 (2)	4 (2.7)	1 (0.7)

Figures in parentheses are percentage of total.

normal colon samples for *APC*, *DAP-kinase*, *E-cadherin*, *MGMT*, and *p14* ($P < 0.05$).

Figure 3 shows the different patterns of methylation of the 12 genes during the adenoma–carcinoma sequence. The 12 genes were classified into four groups; (a) genes showing high methylation frequencies ($>30\%$) in adenoma, but significantly higher methylation frequencies in carcinoma (*APC* and *DAP-kinase*); (b) genes with high and similar methylation frequencies in both adenoma and carcinoma (*E-cadherin*, *MGMT*, and *p14*); (c) genes with a low methylation frequency ($<6\%$) in adenoma, but significantly higher methylation frequencies in carcinoma (*COX-2*, *GSTP1*, *hMLH1*, *p16*, *TIMP-3*,
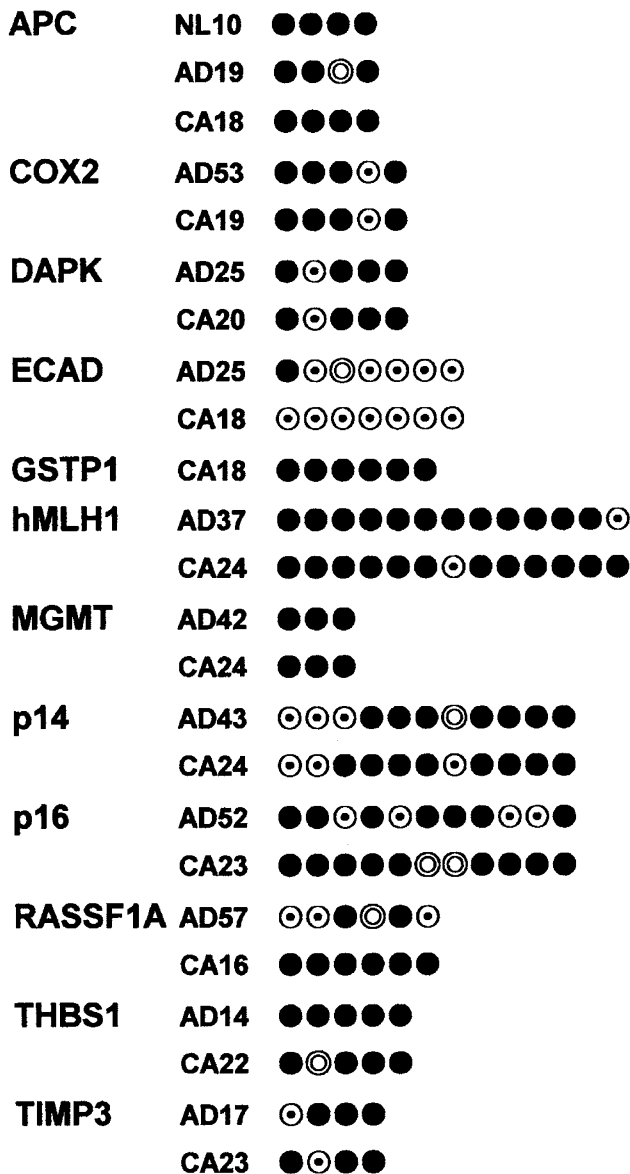
Figure 2 The methylation status of CpG sites on MSP products except for primer complementary sequences. Sequencing analysis of four cloned MSP products of each gene was performed. When four sequenced clones were all methylated at a CpG site, it was scored as 100%. ●, ◎, and ☉ represent methylation frequencies of 100, 75, and 50%. *ECAD*, E-cadherin; *NL*, normal colon mucosa; *AD*, adenoma; *CA*, carcinoma.

and *RASSF1A*); and (d) *THBS1*, which showed low and similar methylation frequencies in adenoma and colorectal cancer.

Clinicopathological Characteristics; Correlation with MSP Results

To clarify the clinical significance of methylation status of individual genes or the methylation extents of multiple CpG islands, we compared these molecular characteristics with the clinicopathologi-cal features of CRC patients. The results are detailed in Table 3. Right colon cancer had a higher methylation index than left colon cancer (0.29 *vs* 0.22, $P = 0.018$, two-tailed Student's *t*-test). In particular, *COX-2*, *DAP-kinase*, *E-cadherin*, and *hMLH1* genes were more frequently methylated in right colon cancer than in left colon cancer (37.5 *vs* 21.5%, $P = 0.039$, 60.7 *vs* 40.2%, $P = 0.018$, 63.6 *vs* 32.3%, $P < 0.001$, and 30.4% *vs* 12.9%, $P = 0.011$, respectively, χ^2 test) (Table 4). Adenoma samples also showed differences in terms of extent and frequency of methylation according to location. Right-sided colon adenomas had higher MI than left-sided colon adenomas (0.21 *vs* 0.12, $P = 0.018$, two-tailed Student's *t*-test). Adenoma also showed a right colon predominance for the methylation of *DAP-kinase*, *E-cadherin*, or *p14* (61.8 *vs* 19.7%, $P < 0.001$, 67.6 *vs* 24.6%, $P < 0.001$, and 44.1 *vs* 23%, $P = 0.039$, respectively, two-sided Fisher's exact test).

Colorectal cancer showed higher MI in older patients (≥ 58 years) than in younger patients (< 58 years). Other clinicopathological findings, including gender, tumor differentiation, gross type, depth of invasion, or pathologic stage, were not found to be associated with methylation status of individual genes or the methylation extent of multiple CpG islands.

Correlation between Gene Methylation Status and Their Protein Expressions

To determine the significance of promoter CpG island methylation on gene expression, we analyzed the protein expressions of E-cadherin and MGMT in CRC samples immunohistochemically (Table 5). We defined the expression status as positive or reduced for $\geq 50\%$, or $< 50\%$ of tumor cells with positive staining, respectively. An inverse correlation was found between methylation and protein expression for E-cadherin and for MGMT ($P = 0.011$ and $P < 0.001$, respectively, χ^2 test).

Concordance of CpG Island Methylation

CRC samples showed significant associations between the methylations of several genes (Table 6). Cases showing methylation for a specific gene showed higher numbers of methylated genes than cases showing no methylation of the same specific gene, suggesting that methylation occurs concordantly in specific samples.

Discussion

Here, we determined the CpG island methylation profiles of 12 genes in a large-scale CRC and adenoma study, and examined their chronological changes. None of the 12 genes, except *APC*, was

Table 2 Promoter methylation frequency for individual genes in colorectal cancer, adenoma, and normal colon

Genes	Frequency of methylation			P-value[a]		
	CRC[b] (n = 149)	Adenoma (n = 95)	Normal (n = 24)	CRC vs adenoma	Adenoma vs normal	CRC vs normal
APC	51%	35.8%	12.5%	0.025	0.021	<0.001
DAP-kinase	48%	34.7%	0	0.034	<0.001	<0.001
E-cadherin	43.9%	40%	0	NS[c]	<0.001	<0.001
COX-2	27.5%	5.9%	0	<0.001	NS	0.001
MGMT	25.5%	21.1%	0	NS	0.012	0.003
p14	24.2%	30.5%	0	NS	0.001	0.003
hMLH1	19.5%	1.1%	0	<0.001	NS	0.016
RASSF1A	16.1%	2.1%	0	<0.001	NS	0.048
p16	13.5%	4.2%	0	0.026	NS	0.080
GSTP1	10.1%	0	0	<0.001	—	0.026
THBS1	9.4%	2.1%	0	0.032	NS	0.229
TIMP3	9.4%	1.1%	0	0.011	NS	0.229

[a]Analyzed by two-sided Fisher's exact test.
[b]Colorectal cancer.
[c]Statistically not significant (*P*-value >0.05).

methylated in normal colon tissue samples, whereas 11 genes, excluding *GSTP1*, were methylated in colon adenoma. All 12 genes were methylated in CRCs, and generally at higher frequencies than in colon adenoma. A stepwise increase in the number of methylated genes was observed with lesion progression through the stages of multistep colorectal carcinogenesis. A temporal order was recognized in the methylation of the tested genes during the colorectal adenoma–carcinoma sequence. The methylation of *APC* was found to be an early event, because *APC* methylation was detected in normal colon mucosa. The methylations of *DAP-kinase*, *E-cadherin*, *MGMT*, and *p14* were inter-mediate, because their methylations were not detected in the normal colon but were observed in adenoma at frequencies of >20%. The methylations of *COX-2*, *GSTP1*, *hMLH1*, *p16*, *TIMP-3*, and *RASSF1A* were late events, because they were rarely methylated in adenoma but were significantly methylated in colorectal cancer. Of the intermediate events, the methylation frequencies of *E-cadherin*, *MGMT*, and *p14* were similar in adenoma and cancer, and could be considered related to tumor initiation, but not to tumor progression.

It has been suggested that proximal and distal colon tumors differ in terms of their genetic susceptibilities to neoplastic transformation.[12–14] Sporadic MSI-positive CRCs display a proclivity toward the proximal colon, whereas sporadic CRCs with chromosomal instability (CIN), although wide-spread, show a predilection for the distal colon.[12] Considering that the MSI-positive phenotype in sporadic CRCs is causally associated with promoter methylation of *hMLH1*,[15] and that sporadic MSI-positive tumors exhibit frequent concordant CpG island hypermethylation of multiple genes,[5,14,16] it is expected that CpG island hypermethylation might occur more prevalently in proximal colon tumor than in distal colon tumor. The studies of Hawkins *et al*[17] and of Yamashita *et al*[18] addressed the right-sidedness of CpG island hypermethylation within the colon. Consistent with these findings, the present study also found proximal colon-predominance of CpG island hypermethylation in colon cancer; identical results were observed in adenoma samples. These findings indicate that CpG island methylation plays a more important role in proximal colon tumorigenesis rather than in distal colon tumorigenesis. This supports the previous held opinion concerning the different mechanisms of the proximal and distal routes in colorectal carci-nogenesis. CpG island hypermethylation and the microsatellite instability pathway may play a key role in proximal colon tumorigenesis, whereas chromosomal instability favors distal colon tumor-igenesis.[19]

To determine whether CRC exhibits coordinated methylation at multiple loci, the Mann–Whitney test was used to compare frequencies of methyla-tions following the methylation of a specific locus. All individual loci showed a significant association with methylation at multiple loci, indicating that CpG island methylation is a nonrandom process, and suggesting the existence of an underlying defect. The concept of CpG island methylator phenotype (CIMP) has been proposed to explain such concordant methylation at multiple loci. CIMP was defined as the simultaneous methylation of multiple CpG island loci. However, in the present study, the number of methylated genes in colorectal adenoma or carcinoma showed a continuous pat-tern, which is far removed from the bimodal distribution described by Toyota *et al*.[5] Thus, it is difficult to delineate between tumors on the basis of an enhanced rate of CpG island hypermethylation. This continuous distribution pattern of CpG island hypermethylation has also been observed in other
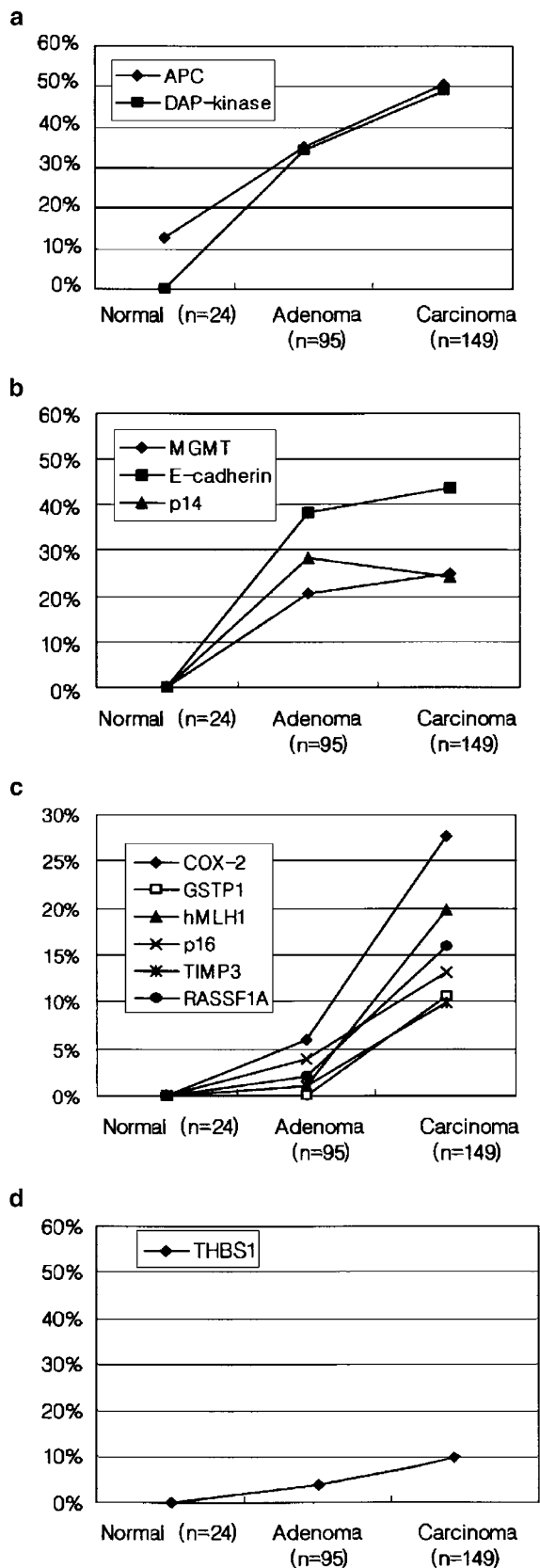
a

b

c

d

Table 3 Analysis of MI[a] in relation to clinicopathological findings in colorectal carcinomas

Characteristics	No. of cases (%)	Average of MI	P-value[b]
All tumors	149	0.22	
Age (mean, 58 years)			0.015
≥58 years	75 (50)	0.28	
< 58 years	74 (50)	0.21	
Gender			NS[c]
Male	70 (47)	0.23	
Female	79 (53)	0.27	
Gross type			NS
Fungating	34 (23)	0.21	
Ulcerofungating	98 (66)	0.24	
Ulceroinfiltrative	17 (11)	0.14	
Size (mean, 5.8 cm)			NS
>5.8 cm	80 (54)	0.25	
≤5.8 cm	69 (46)	0.25	
Tumor differentiation[d]			NS
WD	36 (24)	0.27	
MD	99 (66)	0.24	
PD	14 (9)	0.22	
Pathologic stage			NS
I	19	0.21	
II	80	0.27	
III	43	0.23	
IV	7	0.18	

[a]Methylation index (number of genes methylated/total number of genes tested).
[b]Analyzed by one-way ANOVA test.
[c]Statistically not significant (*P*-value > 0.05).
[d]WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
NS = not significant.

studies.[18,20] However, although CpG island hypermethylation is a potential mechanism for the inactivation of tumor-related genes, the concept of CIMP requires re-definition.

A number of studies have investigated the hypermethylation of multiple CpG islands in colon adenoma, but the number of genes examined was fewer than three, except for MINT loci.[7–9,21] In the present study, by using a panel of 12 genes we examined the methylation profile in colon adenoma. In all, 68.4% of adenoma samples showed the promoter methylation for at least one gene, and this ranged from one to seven genes; moreover, the concurrent CpG island hypermethylation of 40% or more of the tested genes were observed in 8.4% of the colon adenoma samples. Comparing the results of the present study with those of previous studies, the low methylation frequency of *hMLH1* found in

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Figure 3 Profiles of gene promoter hypermethylations with the multistep progression of colorectal carcinogenesis. *APC* and *DAP-kinase* showed a progressive increase in methylation frequency (**a**), whereas the *E-cadherin*, *MGMT*, and *p14* genes displayed high and similar methylation frequencies in both adenoma and carcinoma (**b**). *COX-2*, *GSTP1*, *hMLH1*, p16, *TIMP-3*, and *RASSF1A* showed low methylation frequencies (<6%) in adenoma, but significantly higher methylation frequency in carcinoma (**c**). *THBS1* exhibited low and similar methylation frequencies in adenoma and colorectal cancer (**d**).

Table 4 Frequencies of CpG island methylation of 12 genes in right and left colorectal cancer and adenoma

Genes	Frequency of methylation					
	CRC[a] (n = 149) (%)		P-value[b]	Adenoma (n = 95) (%)		P-value[b]
	Right colon (n = 56)	Left colon (n = 93)		Right colon (n = 34)	Left colon (n = 61)	
E-cadherin	63.6	32.3	< 0.001	67.6	24.6	< 0.001
DAP-kinase	60.7	40.2	0.018	61.8	19.7	< 0.001
APC	51.8	50.5	NS[c]	47.1	29.5	NS
COX-2	37.5	21.5	0.039	5.9	6.6	NS
hMLH1	30.4	12.9	0.011	0	1.6	NS
p14	28.6	21.5	NS	44.1	23	0.039
MGMT	26.8	24.7	NS	17.6	23	NS
p16	16.4	11.8	NS	5.9	3.3	NS
RASSF1A	10.7	19.4	NS	0	3.3	NS
GSTP1	8.9	10.8	NS	0	0	—
THBS1	7.1	10.8	NS	0	3.3	NS
TIMP3	7.1	10.8	NS	0	1.6	NS

[a]Colorectal cancer.
[b]Analyzed by two-sided Fisher's exact test.
[c]Statistically not significant (P-value > 0.05).
NS = not significant.

Table 5 Expression of E-cadherin or MGMT protein; correlation with the results of MSP

MSP[a] results	E-cadherin expression		MGMT expression	
	Negative	Positive	Negative	Positive
Negative	10	17	10	19
Positive	13	3	14	1
P-value[b]	0.011		< 0.001	

[a]Methylation-specific PCR.
[b]Analyzed by χ^2 test.

Table 6 Concordance of methylation of specific genes with that of the other genes

Gene	Average no. of 11 genes methylated except for the specific gene		P-value[a]
	Positive for methylation	Negative for methylation	
APC	2.6	1.3	< 0.001
COX-2	3.4	1.8	< 0.001
DAP-kinase	2.8	1.3	< 0.001
E-cadherin	3.0	1.2	< 0.001
GSTP1	3.8	2.1	0.002
hMLH1	3.9	2.0	< 0.001
MGMT	3.0	1.8	< 0.001
p14	3.3	1.6	< 0.001
p16	4.7	2.0	< 0.001
THBS1	3.4	2.1	0.006
TIMP3	3.9	2.1	0.001

[a]Analyzed by Mann–Whitney test.

the present study is consistent with studies by Bariol *et al*[8] and Park *et al*,[9] but the observed methylation frequencies of *p16* were discrepant, and was lower in the present study (5.9 *vs* 14–29%).[7–9] This difference may be related to ethnic, assay, or positive result criteria differences. In contrast to Rashid *et al*'s study,[7] which suggested a close association between CpG island hypermethylation and adenoma size or villous content, the study by Bariol *et al*[8] did not. Moreover, we found no association between adenoma size and CpG island hypermethylation (data not shown).

The present study used MSP for detecting methylated alleles. MSP is a qualitative assay and does not provide quantitative information about the methylated alleles. Thus, the methylation detected by MSP assay might not reflect gene expression, because the assay can detect one methylated allele among 1000 unmethylated alleles, and thus the vast majority of tumor cells may not harbor CpG island methylation of the given gene. Although many genes were methylated at higher frequencies in CRC than in adenoma, it is unknown whether this also applies to

the proportion of methylated alleles of these genes. Although the relationship between methylation detected by MSP, and expressional loss was strong for E-cadherin and MGMT, this result is not guaranteed to hold for the other genes. The present study shows that the methylated alleles of certain genes are present at the early stage, and that the number of genes with methylated alleles increases along the adenoma–carcinoma sequence.

Although a significant correlation between promoter CpG island hypermethylation and expressional decrease of *E-cadherin* or *MGMT* existed, there were cases with CpG island hypermethylation and protein expression of *E-cadherin* or *MGMT* or cases with absence of CpG island hypermethylation

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and absence of the expression of the particular gene. These discrepancies might be related to the tumor heterogeneity regarding the methylation of the particular gene. Tumor cells negative for DNA methylation of the particular gene may outnumber those positive for DNA methylation of the gene, thus giving expressional loss of the gene but positivity for the methylation assay. Another possibility is that there may be heterogeneity in methylation of specific CpG sites for *E-cadherin* or *MGMT* and the use of a few CpG sites to study methylation pattern can yield misleading results. Lastly, genetic alteration of *E-cadherin* may give expressional loss for cases without promoter CpG island hypermethylation of *E-cadherin*.

Although the correlative evidences between promoter CpG island hypermethylation and downregulation of gene expression have been accumulating, and the importance of CpG island hypermethylation in tumorigenesis has been increasingly recognized, there has been a dispute whether CpG island hypermethylation is a cause or secondary event of gene silencing.[22] Many correlative evidences between promoter CpG island hypermethylation and gene silencing do not give the cause and effect relationship and there is no definitive genetic evidence demonstrating mutation of the genes involved in DNA methylation, in cancers with aberrant CpG island hypermethylation. Furthermore, a recent study[23] has demonstrated that histone modifications and silencing of p16 gene can occur prior to CpG island methylation and another study showing that *p16* silencing can occur without CpG island hypermethylation in proliferating colonies of mammary epithelial cells that escape senescence.[24] Nevertheless, DNA methylation may still be an important means of ensuring transcriptional repression. The causal relationship to maintain the silent epigenetic state can be inferred from the facts that cancer cell lines with hypermethylation of promoter CpG islands of the particular genes (eg *MGMT*, *p15*, *RASSF1A*, etc) and no expression of the particular genes can be induced to express the particular gene with treatment of 5-aza 2′-deoxycytidine (azaC), a specific inhibitor of DNA methyltransferases and reversed to no expression of the particular genes with withdrawal of azaC. A recent study performing a functional analysis of promoter activity has demonstrated that *in vitro* hypermethylation of promoter CpG island of MGMT was able to suppress gene transcription.[25]

In conclusion, methylation analysis of a panel of genes provided gene-specific methylation patterns during colorectal carcinogenesis, and methylation profiles specific for colon adenoma and colorectal cancer. Our data indicate that aberrant CpG island hypermethylation occurs early and accumulates during multistep colorectal carcinogenesis, that a temporal order exist in the methylation of tumor-related genes, and that CpG island hypermethylation is not a dichotomatous trait, challenging the concept of CIMP. Moreover, our data show that CpG island hypermethylation plays a more important role in right-sided colon tumors than in left-sided colon tumors.

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