

Aberrant promoter methylation of multiple genes during multistep pathogenesis of colorectal cancers

Takao Takahashi^{1,6}, Hisayuki Shigematsu¹, Narayan Shivapurkar^{1,2}, Jyotsna Reddy¹, Yingye Zheng³, Ziding Feng³, Makoto Suzuki¹, Masaharu Nomura¹, Meena Augustus⁴, Jing Yin⁵, Stephen J. Meltzer⁵ and Adi F. Gazdar^{1,2*}

¹Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX, USA

²Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA

³Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

⁴Avalon Pharmaceuticals, Germantown, MD, USA

⁵Department of Medicine, Division of Gastroenterology, University of Maryland and Greenebaum Cancer Center, Baltimore, MD, USA

⁶Department of Surgery, Gifu University School of Medicine, Gifu, Japan

Aberrant methylation of 5' gene promoter regions associated with gene silencing is an epigenetic phenomenon responsible for silencing of tumor suppressor genes in many cancer types. The aims of our study were to study the role of methylation of a large panel of genes during multistage pathogenesis and to correlate our findings with patient age and other clinico-pathological features. We investigated the aberrant promoter methylation profile of 19 genes in 92 colorectal cancers (CRCs) and corresponding nonmalignant epithelia (NME) ($n = 57$), and selected 15 genes for studying 26 colorectal adenomas (CAs). On the basis of our results, the genes could be divided into 3 groups. Group 1 consisted of 13 genes whose methylation was tumor-specific. For 8 of these genes, the methylation frequencies in CAs were similar to those of CRCs, but significantly different from the frequencies in NME. Group 2, consisting of 2 genes demonstrating little or no methylation, were present in any sample type. In Group 3, consisting of 4 genes, relatively frequent methylation was present in both CRCs and NME, and the differences between these specimen types were not significant. Methylation of Group 1 genes were tightly correlated with each other, and these genes demonstrated increased methylation frequencies in CRCs with increasing age. Methylation was not correlated with other clinico-pathological features. In general, methylation frequencies of CAs were intermediate between CRCs and NME. Our study constitutes the most comprehensive methylation profile of CRCs, demonstrates that methylation commences early during CRC pathogenesis and is an age-related phenomenon.

© 2005 Wiley-Liss, Inc.

Key words: methylation; tumor suppressor gene; colorectal cancer; colorectal adenoma; nonmalignant colonic epithelium

Introduction

Colorectal cancer (CRC) is the fourth most common cancer and second most common cause of cancer deaths in the United States.¹ The molecular pathogenesis of colorectal adenocarcinoma has been one of the most extensively studied and well characterized of all cancers.² Most colorectal adenocarcinomas develop from colorectal adenomas (CAs), and morphological and genetic progression in an adenoma-carcinoma sequence and in hereditary CRC syndromes are well described.^{3–5}

The majority of CRCs have truncating mutations or deletions of APC gene or mutations of the β -catenin gene. Point mutations of *K-ras* proto-oncogene and mutation of the *p53* gene are also common. In a second pathway to colorectal neoplasia, microsatellite instability is caused by alteration of a nucleotide mismatch repair gene, including *hMSH2*, *hMLH1*, *PMS1* or *PMS2*.⁶

Another molecular defect commonly present in CRCs is CpG island methylation.⁷ DNA methylation of the promoter regions has emerged as the major mechanism of inactivation of tumor suppressor genes (TSGs).⁸ In many cases, aberrant methylation of CpG island genes has been correlated with loss of gene expression, and DNA methylation provides an alternative pathway to gene deletion or mutation for the loss of TSG function.^{8–10} Markers for aberrant methylation represent a promising avenue

for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation.^{9,11,12} However, the gastrointestinal epithelium, especially of the colorectal, displays an unusual phenomenon, with methylation of certain genes demonstrating an age-related association.¹³

In the present study, we determined the methylation status profile of 19 TSGs including *RUNX3*, *3OST2* and *SOCS1* genes in nonmalignant colonic epithelia (NME), CAs and CRCs. The 19 genes were chosen for study because of their presumed or known roles in various cellular functions related to cancer development, including cell cycle regulation, tissue invasion and metastasis, JAK-STAT and TGF- β signal pathways, key components of retinoid activity, signal transduction, apoptosis, angiogenesis, putative cytokine, mitotic stress checkpoint, methyltransferase superfamily and O-sulfotransferase^{14–31} and were selected from the 6 ‘hallmarks of cancer’^{32,33} (Table I). The aims of our study were as follows: (i) to clarify the methylation status of TSGs not previously studied in detail in CRC and correlate methylation with gene expression; and (ii) to investigate the role of methylation during the multistage pathogenesis of CRC, comparing the tumor profile with that of CAs and NME. We correlated the findings with the clinico-pathological features of the tumors and with the age of the patients.

Material and methods

Cell lines

Twelve CRC cell lines (LoVo, LS174T, SW1417, SNU-C1, SW480, LS123, COLO320DM, RKO, HCT116, DLD-1, COLO201 and NCI-H630) were obtained from the American

Abbreviations: CRC, colorectal cancer; CA, colorectal adenoma; NME, nonmalignant colonic epithelium; PBMC, peripheral blood mononuclear cell; MSP, methylation-specific PCR; MI, methylation index; TSG, tumor suppressor gene; CDH13, H-cadherin; RASSF1A, RAS association domain family protein 1A; DcR1, decoy receptor 1; DcR2, decoy receptor 2; TIMP3, tissue inhibitor of metalloproteinase 3; CRBP1, cellular retinoid-binding protein 1; RIZ1, Rb-interacting zinc finger protein 1; RAR β , retinoic acid receptor β ; APC, adenomatous polyposis coli; SHP-1, hematopoietic cell specific protein-tyrosine-phosphatase SH-PTP1; SYK, spleen tyrosine kinase; SOCS1, suppressor of cytokine signaling 1; RUNX3, runt-related transcription factor 3; NORE1, novel potential RAS effector 1; 3OST2, heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2; CHFR, checkpoint with forkhead and ring finger domains; HIN-1, high in normal-1; HPPI1, hyperplastic polyps 1.

Grant sponsor: Early Detection Research Network; Grant numbers: 5U01CA8497102, CA85069, CA77057, CA95323, CA01808, CA098450.

*Correspondence to: Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-8593, USA.

Fax: +(214-648-4940). E-mail: adi.gazdar@UTSouthwestern.edu

Received 12 January 2005; Accepted after revision 24 June 2005

DOI 10.1002/ijc.21453

Published online 17 August 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – SUMMARY DATA OF GENES TESTED

Gene abbreviation	Gene name	Gene location	Function	Hallmark category ¹	Reference for methodology	Previous study in colon tumors
<i>P16^{INK4a}</i>	Cyclin-dependent kinase inhibitor 2A	9p21.3	Cell cycle regulator	Limitless replicative potential	14	Ca ² : 34-40 A ³ : 34
<i>CDH13</i>	H-cadherin	16q24	Tissue invasion and metastasis	Tissue invasion and metastasis	15	Ca: A: 41
<i>TIMP3</i>	Tissue inhibitor of metalloproteinase 3	22q12.3	Tissue invasion and metastasis	Tissue invasion and metastasis	16	Ca: 16
<i>SOCS1</i>	Suppressor of cytokine signalling 1	16p13.1	JAK-STAT Pathway	Self-sufficiency in growth signals	17	Ca: 42
<i>SHP-1</i>	Hematopoietic cell specific protein-tyrosine-phosphatase SH-PTP1	12p13.3	JAK-STAT Pathway	Self-sufficiency in growth signals	18	None
<i>SYK</i>	Spleen tyrosine kinase	9q22.2	JAK-STAT Pathway	Self-sufficiency in growth signals	19	None
<i>CRBP1</i>	Cellular retinol-binding protein 1	3q23	Key components in retinoid activity	Limitless replicative potential	20	Ca: 20
<i>RARβ</i>	Retinoic acid receptor β	3p24	Key components in retinoid activity	Limitless replicative potential	21	Ca: 38
<i>RASSF1A</i>	RAS association domain family protein 1A	3p21.3	Signal transduction	Self-sufficiency in growth signals	22	Ca: 39, 43, 44
<i>NORE1</i>	Novel potential RAS effector 1	1q32.1	Signal transduction	Self-sufficiency in growth signals	23	None
<i>APC</i>	Adenomatous polyposis coli	5q21.22	Signal transduction	Tissue invasion and metastasis	24	Ca: 38, 39, 45, 46, A: 45
<i>DcR1</i>	Decoy receptor 1	8p21.2	Apoptosis	Apoptosis	25	None
<i>DcR2</i>	Decoy receptor 2	8p22.2	Apoptosis	Apoptosis	25	None
<i>RUNX3</i>	Runt-related transcription factor 3	1p36.1	TGF- β signal pathway	Insensitivity to antigrowth signals	26	Ca: 47
<i>HPP1</i>	TMEFF2	2q32.3	TGF- β signal pathway	Insensitivity to antigrowth signals	27	Ca: 27, 48 A: 48
<i>HIN-1</i>	High-in normal-1	5q35.3	Putative cytokine	Insensitivity to antigrowth signals	28	None
<i>CHFR</i>	Checkpoint with forkhead and ring finger domains	12q24.23	Mitotic stress checkpoint gene	Limitless replicative potential	29	Ca: 49, 50 A: 50
<i>RIZ1</i>	Rb-interacting zinc finger protein 1	1p36	Methyltransferase superfamily	Limitless replicative potential	30	Ca: 37
<i>3OST2</i>	Heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2	16p12.2	O-sulfotransferase	Insensitivity to antigrowth signals	31	Ca: 31

¹Hallmark Categories are defined by Hanahan and Weinberg,³² and the category selection was from Widschwendter and Jones³³,²Ca, Colorectal cancers,³A, Colorectal adenomas.

Type Culture Collection (ATCC, Manassas, VA). They were grown in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO₂ at 37°C.

RT-PCR for gene expression of RUNX3, 3OST2 and SOCS1 in CRC cell lines

Expression of genes was analyzed by RT-PCR. Total RNA was extracted from cell lines with Trizol (Life Technologies), following the manufacture's instructions. The RT reaction was performed on 2 μ g of total RNA with Superscript II First-Strand Synthesis using the oligo (dT) primer system (Life Technologies). Primer sequences and conditions for RT-PCR product were as described previously.^{17,26,31} The housekeeping gene *GAPDH* was used as an internal control to confirm the success of the RT reaction. Total RNA from human NME was obtained from Clontech (Palo Alto, CA). NME and normal peripheral blood mononuclear cells (PBMC) from healthy volunteer were used as normal controls for RT-PCR. PCR products were analyzed on 2% agarose gels.

5-Aza-CdR treatment

Cell lines with known gene promoter methylation were incubated in culture medium with the demethylating agent 5-Aza-CdR at a concentration of 4 μ M for 6 days, with medium changes on days 1, 3 and 5.⁵¹

Clinical samples

Surgically resected specimens from 92 primary CRCs, 57 corresponding NME and 26 CAs were obtained from Department of Pathology at the University of Texas Southwestern Medical Center (Dallas, TX), Avalon Pharmaceuticals (Garmantown, MD) and the University of Maryland School of Medicine. (Baltimore, MD). We also obtained DNA from 14 PBMCs of healthy volunteers. Clinical staging data were available for 79 tumors: 12 were Dukes A, 20 were Dukes B, 32 were Dukes C and 15 were Dukes D. The mean age (both for CRC and NME samples) was 62 years (range 35–87), and the male:female ratio was 2.1:1. The adenomas consisted of equal numbers of tubular and villous adenomas, mean size 1.7 cm (range 0.3–5.0), and the mean patient age was 66 years (range 47–85).

Methylation assay

Genomic DNA was isolated from frozen tissue by digestion with 100 μ g/ml proteinase K followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation. DNA was treated with sodium bisulfite, as described previously.¹⁴ Treated DNA was purified by use of Wizard DNA Purification System (Promega, Madison, WI), desulfurated with 0.3 M NaOH, precipitated with ethanol and resuspended in water. Modified DNA was stored at -80°C until used. The methylation status of 19 genes was determined by methylation-specific PCR (MSP) assays. References for methodology and gene information are summarized in Table I. DNA from PBMC of a healthy volunteer treated with Sss1 methyltransferase (New England BioLabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Negative control sample without DNA was included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

Data analysis

Frequencies of methylation of groups were compared using χ^2 test or the Fisher exact tests. The methylation index (MI), a reflection of the methylation status of all of the genes tested, is defined as the total number of genes methylated divided by the total number of gene analyzed. To compare the extent of methylation for the panel of genes examined, we calculated the MIs for each case³², and then determined the mean for the different groups. Statistical analysis of MI between 2 variables was performed using the Mann-Whitney *U* nonparametric test. For statistical models with categorical covariates, we also performed trend tests. Tests

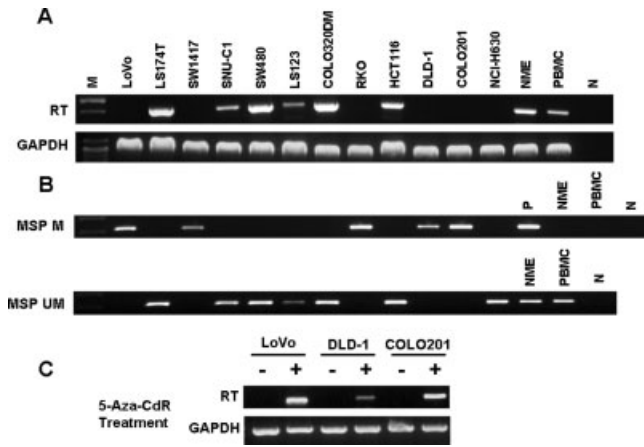


FIGURE 1 – Representative examples of RT-PCR (a), MSP (b) and 5-Aza-CdR treatment (c) of *RUNX3* in CRC cell lines. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. NME, nonmalignant colonic epithelium; PBMC, peripheral blood mononuclear cells; N, negative control (water blank). DNA from lymphocytes of a healthy volunteer treated with Sss1 methyltransferase and then subjected to bisulfite treatment was used as a positive control for methylated alleles. M, methylated form; UM, unmethylated form. In most instances except for NCI-H630, there was concordance between the presence of methylation and loss or downregulation of expression. (c), Representative examples of the effect of 5-Aza-CdR treatment on restoring gene expression in *RUNX3* methylated cell lines. Treatment with 5-Aza-CdR restored expression of *RUNX3*. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. +, with 5-Aza-CdR treatment; -, without 5-Aza-CdR treatment.

for trend were conducted by entering a single ordinal variable using the median of each category. For these tests, probability values of $p < 0.05$ were regarded as statistically significant. For correlation between TSGs, we used Pearson correlation coefficients test. For only this test, probability values of $p < 0.01$ were regarded as statistically significant.

Results

Aberrant promoter methylation and expression of *RUNX3*, *3OST2* and *SOCS1* in CRC cell lines

For 3 genes (*RUNX3*, *3OST2* and *SOCS1*) whose methylation status in CRC had not been previously studied in detail, we examined the correlation between aberrant promoter methylation and loss of gene expression using a panel of 12 CRC cell lines. Aberrant methylation of *RUNX3*, *3OST2* and *SOCS1* was found in 5 of 12 (42%), 12 of 12 (100%) and 6 of 12 (50%) lines respectively (Figs. 1b and 2b and Table II). Expression of the genes was examined by RT-PCR. The genes were expressed in NME and PBMC. However, loss or downregulation of *RUNX3*, *3OST2* and *SOCS1* gene expression were observed in 6 of 12 (50%), 12 of 12 (100%) and 5 of 12 (42%) CRC cell lines, respectively (Figs. 1a and 2a and Table II). The concordance rates between loss of gene expression and aberrant methylation of these genes were 92% (*RUNX3*), 100% (*3OST2*) and 75% (*SOCS1*).

5-Aza-CdR treatment

Three cell lines for *RUNX3* (LoVo, DLD-1 and COLO201), 4 cell lines for *3OST2* (LoVo, HCT116, COLO201 and LS123) and 3 cell lines for *SOCS1* (LoVo, COLO320DM and LS174T) that showed loss or downregulation of expression and methylation by MSP were cultured with 5-Aza-CdR. Expression of the 3 genes was restored after treatment in all methylated cell lines tested (Figs. 1c and 2c).

Frequency of methylation in primary CRCs, CAs and NME

We examined the methylation status of 19 genes in CRCs, CAs and NME (Fig. 3). The unmethylated form of p16, run as a control

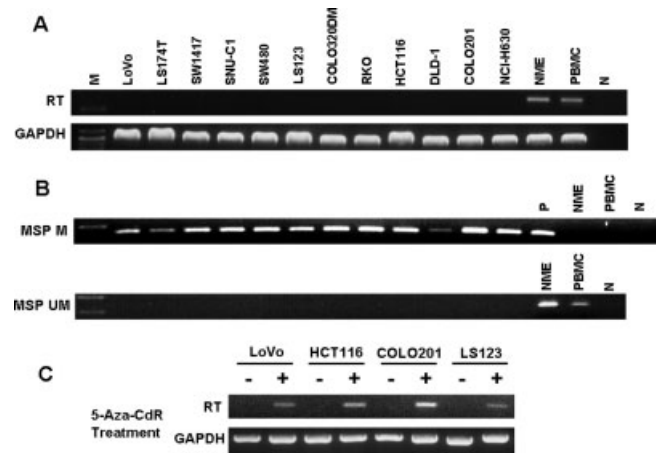


FIGURE 2 – Representative examples of RT-PCR (a), MSP (b) and 5-Aza-CdR treatment (c) of *3OST2* in CRC cell lines.

for DNA integrity, was detected in all of these samples. On the basis of the frequencies of methylation in CRCs, CAs and NME, the genes could be divided into 3 groups (Fig. 4a). Group 1 consisted of tumor-specific genes, *i.e.*, genes significantly more frequently methylated in CRCs compared to corresponding NME. Group 1 consisted of 13 of the 19 genes tested. It could be further divided into groups 1A and 1B. In group 1A, consisting of 8 genes, the methylation frequencies in CAs were similar to those of CRCs, but significantly different from the frequencies in NME. For group 1B (5 genes), the methylation frequencies of CAs were not significantly different from NME. For these genes, although the methylation frequencies of CAs were lower than CRCs, the differences were not significant, with the exception of *SOCS1*. Thus, for the 13 genes in Group 1, the frequencies of methylation in CAs were higher than those in NME for 11, with 8 of these differences being significant.

Group 2, consisting of 2 genes (*NORE1* and *SYK*), little or no methylation, were present in any sample type. However, DNA from PMBC of a healthy volunteer treated with Sss1 methyltransferase were methylation positive for 2 genes. In Group 3, consisting of 4 genes (*SHP-1*, *DcR1*, *RARβ* and *DcR2*), relatively frequent methylation was present in both CRCs and NME, and the differences between these specimen types were not significant (Fig. 4a). Because of these findings, we did not investigate the methylation status of these genes in CAs. However, we confirmed that the methylation status of 14 PBMCs were all negative for the group 3 genes.

For the 15 genes in groups 1 and 2, we calculated the MI, an index of overall methylation frequency, for each specimen type (Fig. 4b). The MI of CRCs (0.32) was significantly higher than the MI (0.05) of corresponding NME ($p < 0.0001$). The value for CAs was 0.22, highly significantly different from NME ($p < 0.0001$) and modestly different from the value for CRC ($p = 0.008$). The trend test was also statistically significant ($p < 0.001$).

Association between individual TSGs in CRCs

For the 13 genes in Group 1, we determined whether there was any association between the methylation status of individual genes, using Pearson's correlation coefficients test. Of interest, the methylation status of 11 of 13 genes of group 1A was correlated tightly with each other, in particular, methylation of *CDH13*, *CRBP1*, *SOCS1*, *RUNX3*, *CHFR* and *3OST2* was correlated with the methylation status of 3 or more other genes ($p < 0.01$).

Correlation between methylation and age in CRCs, CAs and NME

Because methylation frequencies of some genes may demonstrate an age-related effect in certain tissues, especially the colon,

TABLE II – *RUNX3*, *3OST2* AND *SOCs1* EXPRESSION AND METHYLATION STATUS IN COLORECTAL CANCER CELL LINES

Cell line	<i>RUNX3</i>		<i>3OST2</i>		<i>SOCs1</i>	
	Expression	Methylation	Expression	Methylation	Expression	Methylation
LOVO	–	+	–	+	–	+
LS174T	+	–	–	+	–	+
SW1417	–	+	–	+	+	–
SNU-C1	+	–	–	+	–	+
SW480	+	–	–	+	+	–
LS123	+	–	–	+	+	–
COLO320DM	+	–	–	+	–	+
RKO	–	+	–	+	+	–
HCT-116	+	–	–	+	+	+
DLD-1	–	+	–	+	+	+
COLO201	–	+	–	+	+	–
NCI-H630	–	–	–	+	–	–

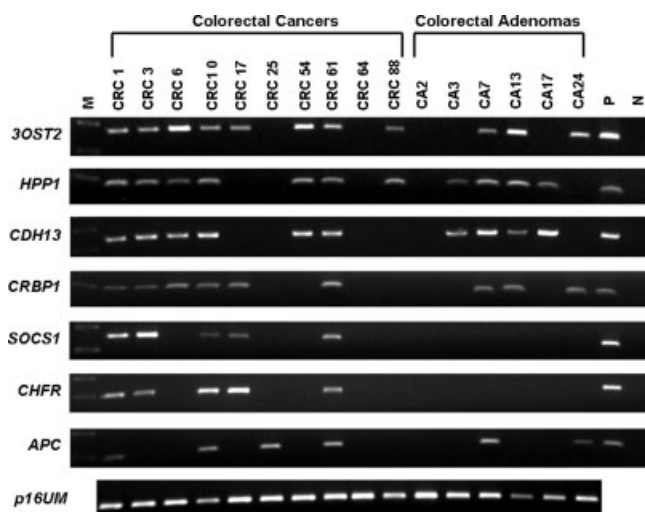


FIGURE 3 – Representative examples of MSP analyses of methylated form of 7 genes in primary CRCs and adenomas. The unmethylated form of p16 (p16UM) was run as an internal control for bisulfite treatment.

we compared the association between aberrant methylation of individual genes and the MI and age. Methylation frequencies (MI) showed a tendency that gradually increased with age in both CRCs (p for trend = 0.024) and NME (p for trend = 0.18) (Fig. 5). In tumors, there was a significant effect of age on methylation frequencies of 3 genes, *CDH13* ($p = 0.039$), *CRBP1* ($p = 0.011$) and *SOCs1* ($p = 0.013$), and for the MI ($p = 0.013$). For the 13 genes whose methylation status demonstrated tumor specificity (Group 1), the age-related effect on the MI was even stronger ($p = 0.005$). However, in NME and CAs, the MIs and methylation frequencies of several genes showed a tendency to increase with age, although these differences were not significant. Furthermore, Group 3 did not show remarkable effective with age.

Correlation between methylation and other clinico-pathological features in CRCs and CAs

Using a simple linear regression model or multivariate linear regression models, we examined the association between methylation status and clinico-pathological features including Duke's Stage, tumor location (right or left side), gender and degree of tumor differentiation. There was no association between any of these factors and methylation frequencies of individual genes or with the MI in CRCs. For CAs, there was no significant association between MI and histologic type or adenoma size.

Discussion

Previous studies have described the importance of DNA methylation in human cancers and have focused on regions of the genome that may have functional significance resulting from the extinction of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes, the methylation profiles of individual tumor types are characteristic.^{9,11,53} To date, several studies have demonstrated that various genes are hypermethylated and silenced in CRC. However, most of these studies have focused on the aberrant methylation of a single gene or cancer tissues only.

Recently, Hanahan and Weinberg described 6 hallmarks that a cell has to acquire to become malignant: (i) limitless replicative potential, (ii) self-sufficiency in growth signals, (iii) insensitivity to growth-inhibitory signals, (iv) evasion of programmed cell death, (v) sustained angiogenesis and (vi) tissue invasion and metastasis.^{32,33} For our methylation profile, we selected 19 genes from 5 of the 6 hallmark categories (Table I). These 19 genes are methylated frequently in 1 or more cancer types. For the 3 genes (*3OST2*, *SOCs1* and *RUNX3*) whose methylation status in CRCs had not been previously studied in detail, we confirmed that our methylation assay conditions were correlated with gene silencing in cell lines derived from CRCs. In addition to these genes, we confirmed that our methylation assay conditions were correlated with gene silencing in CRC cell lines for *RIZ1*, *CHFR* and *CDH13*⁴¹ (data not shown). Moreover, our results showed that expression of these genes was reexpressed after 5-Aza-CdR treatment in CRC cell lines that did not express these genes, and which were hypermethylated. Therefore, we confirmed that transcriptional repression of these genes was caused by hypermethylation. For the other genes, we or others have showed similar correlations in other cancers.^{21,22,54} We also demonstrated that PBMCs were unmethylated for all of the genes studied using our assay conditions.

We compared the methylation profiles of CRCs with those of CAs and NME using a panel of 19 known or potential TSGs. While 4 genes were frequently methylated in CRCs and NME, 13 genes showed tumor specificity (Group 1), with methylation frequencies ranging from 11% to 68% (median 32%). Only 2 genes were infrequently methylated in any tissue type (Group 2). Four genes were frequently methylated in CRCs and NME (Group 3). The MI was significantly higher in CRCs than in NME. Of considerable interest, for most genes, CAs demonstrated methylation frequencies and a MI that was intermediate between the values for CRCs and NME. Our findings are consistent with the concept that CAs represent an intermediate step in CRC pathogenesis. Other more limited studies on the methylation patterns of CAs are consistent with our observations and conclusions.^{55–58} Five of the Group 1 genes (*3OST2*, *HPP1*, *CDH13*, *CRBP1* and *APC*) were frequently methylated (>30%) in both CRCs and CAs. These genes may represent markers for risk assessment. In addition, methylation of *SOCs1* was present in 45% of CRCs, and

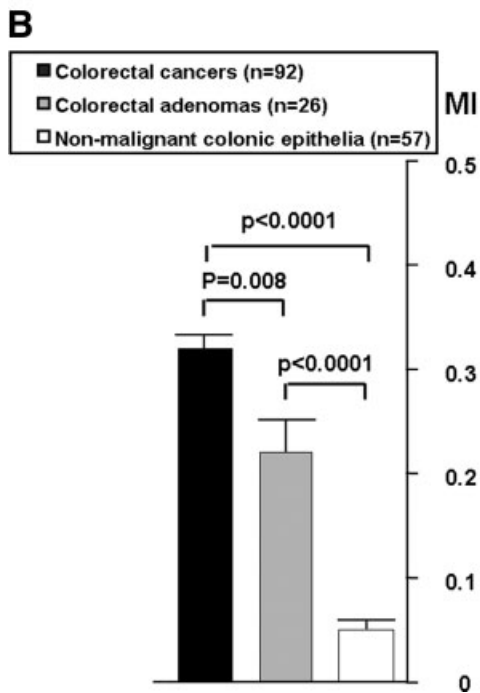
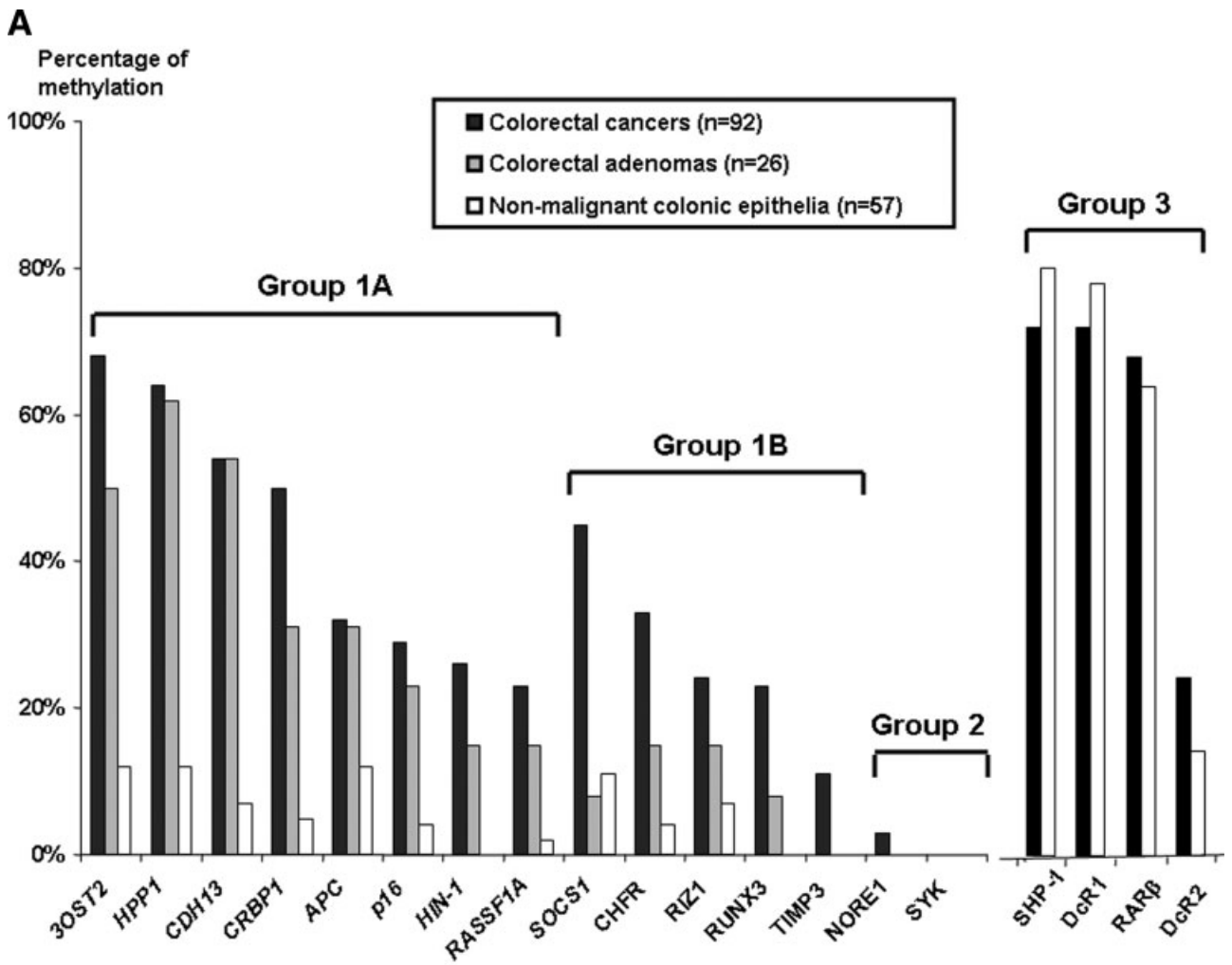


FIGURE 4.

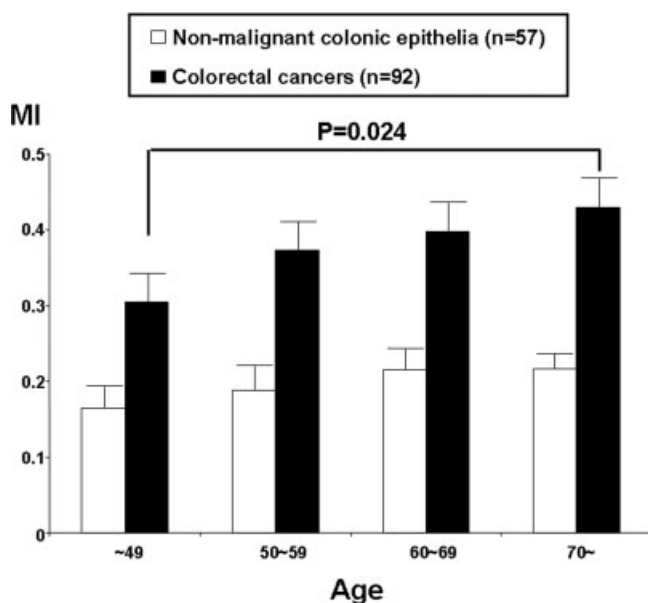


FIGURE 5 – Incidence of MI in CRC and NME samples in relation to patient age. Methylation frequencies gradually increased with age ($p = 0.024$ by trend test) in tumor tissues but not in NME.

in low frequencies in CAs and NME. Methylation of this gene was the most tumor-specific marker identified in the present study.

Methylation of several of the Group 1 genes was tightly correlated with each other in individual tumors. This phenomenon has been noted by others and has been labeled as CIMP (CpG island methylator phenotype).^{7,59-61} CIMP is characterized by simultaneous methylation of multiple genes including *p16*, *THBS1* and *hMLH1*. The mechanism of methylation of multiple CpG islands in individual tumors has been postulated to be either aberrant de novo methylation because of mutation in a DNA-methyltransferase or loss of protection against de novo methylation through the loss of a transactivating factor.⁶²

Another phenomenon noted previously has been the effect of aging on methylation of certain genes in NME.^{59,62} To date this phenomenon has been noted predominantly in gastrointestinal tissues. We noted age-related methylation of a subset of tumor-specific Group 1 genes, but only a weak tendency with the Group 3 genes that are frequently methylated in NME in a non-age dependent manner. Multivariate logistic regressions revealed that for CRC, age was a significant predictor ($p = 0.005$) and was independently associated with hypermethylation of CRCs (MI), but failed to reach significance in NME. Thus, methylation of Group 1 genes is both cancer-specific and age-related, and the latter finding may contribute to increasing cancer risk with increasing age.

Five genes (*3OST2*, *HPP1*, *CDH13*, *CRBP1* and *APC*) demonstrated high methylation frequencies in both CRCs and CAs, and may play an important role to development of CRC. The most frequently methylated gene in primary CRC samples was *3OST2*

(68%), and this gene was methylated in all 12 CRC cell lines tested. The *3OST2* gene encodes an enzyme involved in the final modification step of heparan sulfate proteoglycans (HSPGs), and its silencing is expected to result in abnormal modification of HSPGs and abnormal signal transduction. A previous study reported frequent methylation of this gene in breast cancers and in a limited number of CRCs.³¹

The *HPP1* gene was initially discovered because of its frequent hypermethylation in hyperplastic colon polyps, but it is also hypermethylated in CAs and carcinomas.⁴⁸ A member of the cell adhesion cadherin family, *CDH13*, is related to tumor invasion and metastasis, and was frequently methylated in CRCs and CAs as previously reported by us.⁴¹ *CRBP1* belong to the fatty acid-binding proteins, which are key components in retinoid activity. Ester *et al.* reported that hypermethylation of *CRBP1* was common in tumors and cancer cell lines, with the highest frequency in lymphoma and gastrointestinal cancers.²⁰ Selective methylation and silencing of the *IA* promoter of the *APC* gene have been frequently detected in several human tumors. *APC* is a well-studied molecule involving wnt signaling. *APC* somatic mutations are present at frequent of sporadic CRCs and appear very early in colorectal tumor progression.⁶³ In addition to somatic mutation, methylation is also frequent in CRCs.^{38,39,45} Our findings are consistent with these previous results and we confirmed that these genes may also play an important role in multistep pathway to CRC development.

The *RUNX3* gene, another tumor-specific gene, is an important target of TGF β superfamily signaling and plays a crucial role in mammalian development. *RUNX3* was reported to be frequently methylated in gastric cancers and testicular yolk sac tumors of infant.^{26,64} Recently, frequent methylation of *RUNX3* was published in CRC cell lines.⁴⁷ We clarified the methylation status of primary CRCs, CPs and NME in addition to CRC cell lines. The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, a putative TSG, is a member of a nuclear histone/protein methyltransferase superfamily. *RIZ1* inactivation has been commonly found in many types of human cancers.^{30,65}

Of the 19 genes we studied, 13 had been previously studied in CRCs (Table I). Our findings, with 1 exception (*SOCS1*), are consistent with these reports. *SOCS1*, a negative regulator of the JAK/STAT signaling pathway, has been reported to be infrequently methylated in CRCs.⁴² However, we found relatively frequent methylation in CRCs (but not in CAs). Our results may reflect geographic differences in the origins of the 2 tumor sets (USA and Japan).

The present study constitutes the most comprehensive methylation profile report available for CRCs, CAs and NME. We have identified groups of tumor-specific genes as well as genes that are frequently methylated in NME. We have confirmed and extended the observations of others that methylation of some genes are tightly correlated in tumors (the CIMP phenomenon) as well as genes that demonstrate age-related methylation. Of great interest, we have demonstrated that methylation of multiple genes is frequent in adenomas of various types and size, consistent with the concept that they represent intermediate steps towards most CRCs. Finally, we have identified genes that may prove to be useful as markers for risk assessment or early detection.

FIGURE 4 – Comparison of frequencies of aberrant methylation (a) and mean MIs (b) in CRCs, adenomas and nonmalignant epithelium samples. On the basis of the frequencies of methylation in CRCs, CAs and nonmalignant colonic epithelia (NME), the genes could be divided into 3 groups. Group 1 consists of tumor-specific genes. Group 2 showed infrequent methylation in both CRCs and NME. In Group 3, both CRCs and NME are frequently methylated. In Group 1, methylation frequencies of these genes in CRCs were statistically significantly higher than that in NME (p values ranged from <0.0001 to 0.014). We divided Group 1 genes into further 2 subgroups, Group 1A, (both CRCs and CAs were more significant frequently methylated than NME), Group 1B, (cancer-specific but there were no significant differences between CAs and NME).

References

- Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin* 2003;53:5–26.
- Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999;91:916–932.
- Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87:159–170.
- Fearon ER, Dang CV. Cancer genetics: tumor suppressor meets oncogene. *Curr Biol* 1999;9:62–65.
- Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000;119:854–865.
- Markowitz SD, Dawson DM, Willis J, Willson JK. Focus on colon cancer. *Cancer Cells* 2002;1:233–236.
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999;96:8681–8686.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–428.
- Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC, Schuller DE, Yu L, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000;24:132–138.
- Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum Mol Genet* 2001;10:687–692.
- Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61:3225–3229.
- Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002;21:5427–5440.
- Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res* 1998;58:5489–5494.
- Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;93:9821–9826.
- Sato M, Mori Y, Sakurada A, Fujimura S, Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. *Hum Genet* 1998;103:96–101.
- Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB, Graff JR. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* 1999;59:798–802.
- Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC, Herman JG. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* 2001;28:29–35.
- Oka T, Ouchida M, Koyama M, Ogama Y, Takada S, Nakatani Y, Tanaka T, Yoshino T, Hayashi K, Ohara N, Kondo E, Takahashi K, et al. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Res* 2002;62:63904.
- Yuan Y, Mendez R, Sahin A, Dai JL. Hypermethylation leads to silencing of the SYK gene in human breast cancer. *Cancer Res* 2001;61:5558–5561.
- Esteller M, Guo M, Moreno V, Peinado MA, Capella G, Galm O, Baylin SB, Herman JG. Hypermethylation-associated inactivation of the cellular retinol-binding-protein 1 gene in human cancer. *Cancer Res* 2002;62:5902–5905.
- Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, Maitra A, Heda S, Fong KM, Thunnissen F, Minna JD, Gazdar AF. Promoter methylation and silencing of the retinoic acid receptor- β gene in lung carcinomas. *J Natl Cancer Inst* 2000;92:1303–1307.
- Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle B, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 2001;93:691–699.
- Hesson L, Dallol A, Minna JD, Maher ER, Latif F. NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene* 2003;22:947–954.
- Tsuchiya T, Tamura G, Sato K, Endoh Y, Sakata K, Jin Z, Motoyama T, Usuba O, Kimura W, Nishizuka S, Wilson KT, James SP, et al. Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. *Oncogene* 2000;19:3642–3646.
- van Noessel MM, van Bezouw S, Salomons GS, Voute PA, Pieters R, Baylin SB, Herman JG, Versteeg R. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. *Cancer Res* 2002;62:2157–2161.
- Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* 2002;109:113–124.
- Sato F, Shibata D, Harpaz N, Xu Y, Yin J, Mori Y, Wang S, Oлару A, Deacu E, Selaru FM, Kimos MC, Hytioglou P, et al. Aberrant methylation of the HPP1 gene in ulcerative colitis-associated colorectal carcinoma. *Cancer Res* 2002;62:6820–6822.
- Krop IE, Sgroi D, Porter DA, Lunetta KL, LeVangie R, Seth P, Kaelin CM, Rhei E, Bosenberg M, Schnitt S, Marks JR, Pagon Z, et al. HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. *Proc Natl Acad Sci USA* 2001;98:9796–9801.
- Mizuno K, Osada H, Konishi H, Tatematsu Y, Yatabe Y, Mitsudomi T, Fujii Y, Takahashi T. Aberrant hypermethylation of the CHFR prophase checkpoint gene in human lung cancers. *Oncogene* 2002;21:2328–2333.
- Du Y, Carling T, Fang W, Piao Z, Sheu JC, Huang S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. *Cancer Res* 2001;61:8094–8099.
- Miyamoto K, Asada K, Fukutomi T, Okochi E, Yagi Y, Hasegawa T, Asahara T, Sugimura T, Ushijima T. Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. *Oncogene* 2003;22:274–280.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
- Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. *Oncogene* 2002;21:5462–5482.
- Guan RJ, Fu Y, Holt PR, Pardee AB. Association of K-ras mutations with p16 methylation in human colon cancer. *Gastroenterology* 1999;116:1063–1071.
- Wiencke JK, Zheng S, Lafuente A, Lafuente MJ, Grudzen C, Wrensch MR, Miike R, Ballesta A, Trias M. Aberrant methylation of p16INK4a in anatomic and gender-specific subtypes of sporadic colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 1999;8:501–506.
- Burri N, Shaw P, Bouzourene H, Sordat I, Sordat B, Gillet M, Schorderet D, Bosman FT, Chaubert P. Methylation silencing and mutations of the p14ARF and p16INK4a genes in colon cancer. *Lab Invest* 2001;81:217–229.
- Whitehall VL, Wynter CV, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, Jass JR. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. *Cancer Res* 2002;62:6011–6014.
- Yamamoto H, Min Y, Itoh F, Imsumran A, Horichi S, Yoshida M, Iku S, Fukushima H, Imai K. Differential involvement of the hypermethylation phenotype in hereditary and sporadic colorectal cancers with high-frequency microsatellite instability. *Genes Chromosomes Cancer* 2002;33:322–325.
- van Engeland M, Weijnenberg MP, Roemen GM, Brink M, de Bruine AP, Goldbohm RA, van den Brandt PA, Baylin SB, de Goeij AF, Herman JG. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Res* 2003;63:3133–3137.
- Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. *J Clin Oncol* 2003;21:3729–3736.
- Toyooka S, Toyooka KO, Harada K, Miyajima K, Makarla P, Sathyanarayana UG, Yin J, Sato F, Shivapurkar N, Meltzer SJ, Gazdar AF. Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. *Cancer Res* 2002;62:3382–3386.
- Fujitake S, Hibi K, Okochi O, Kodera Y, Ito K, Akiyama S, Nakao A. Aberrant methylation of SOCS-1 was observed in younger colorectal cancer patients. *J Gastroenterol* 2004;39:120–124.
- Wagner KJ, Cooper WN, Grundy RG, Caldwell G, Jones C, Wadey RB, Morton D, Schofield PN, Reik W, Latif F, Maher ER. Frequent RASSF1A tumour suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. *Oncogene* 2002;21:7277–7282.
- van Engeland M, Roemen GM, Brink M, Pachen MM, Weijnenberg MP, de Bruine AP, Arends JW, van den Brandt PA, de Goeij AF, Herman JG. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002;21:3792–3795.
- Esteller M, Sparks A, Toyota M, Sanchez-Céspedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG. Analysis of adenomatous polyposis coli promo-

- ter hypermethylation in human cancer. *Cancer Res* 2000;60:4366–4371.
46. Xiong Z, Wu AH, Bender CM, Tsao JL, Blake C, Shibata D, Jones PA, Yu MC, Ross RK, Laird PW. Mismatch repair deficiency and CpG island hypermethylation in sporadic colon adenocarcinomas. *Cancer Epidemiol Biomarkers Prev* 2001;10:799–803.
 47. Ku JL, Kang SB, Shin YK, Kang HC, Hong SH, Kim IJ, Shin JH, Han IO, Park JG. Promoter hypermethylation downregulates RUNX3 gene expression in colorectal cancer cell lines. *Oncogene* 2004;23:6736–6742.
 48. Young J, Biden KG, Simms LA, Huggard P, Karamatic R, Eyre HJ, Sutherland GR, Herath N, Barker M, Anderson GJ, Fitzpatrick DR, Ramm GA, *et al.* *HPPI*: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. *Proc Natl Acad Sci USA* 2001;98:265–270.
 49. Corn PG, Summers MK, Fogt F, Virmani AK, Gazdar AF, Halazoneitis TD, El-Deiry WS. Frequent hypermethylation of the 5' CpG island of the mitotic stress checkpoint gene *Chfr* in colorectal and non-small cell lung cancer. *Carcinogenesis* 2003;24:47–51.
 50. Toyota M, Sasaki Y, Satoh A, Ogi K, Kikuchi T, Suzuki H, Mita H, Tanaka N, Itoh F, Issa JP, Jair KW, Schuebel KE, *et al.* Epigenetic inactivation of *CHFR* in human tumors. *Proc Natl Acad Sci USA* 2003;100:7818–7823.
 51. Takahashi T, Shivapurkar N, Reddy J, Shigematsu H, Miyajima K, Suzuki M, Toyooka S, Zochbauer-Muller S, Drach J, Parikh G, Zheng Y, Feng Z, *et al.* DNA methylation profiles of lymphoid and hematopoietic malignancies. *Clin Cancer Res* 2004;10:2928–2935.
 52. Maruyama R, Toyooka S, Toyooka KO, Harada K, Virmani AK, Zochbauer-Muller S, Farinas AJ, Vakar-Lopez F, Minna JD, Sagalowsky A, Czerniak B, Gazdar AF. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res* 2001;61:8659–8663.
 53. Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* 2001;61:249–255.
 54. Virmani AK, Rathi A, Sathyanarayana UG, Padar A, Huang CX, Cunnigham HT, Farinas AJ, Milchgrub S, Euhus DM, Gilcrease M, Herman J, Minna JD. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. *Clin Cancer Res* 2001;7:1998–2004.
 55. Lee S, Hwang KS, Lee HJ, Kim JS, Kang GH. Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia. *Lab Invest* 2004;84:884–893.
 56. Rashid A, Shen L, Morris JS, Issa JP, Hamilton SR. CpG island methylation in colorectal adenomas. *Am J Pathol* 2001;159:1129–1135.
 57. Chan AO, Issa JP, Morris JS, Hamilton SR, Rashid A. Concordant CpG island methylation in hyperplastic polyposis. *Am J Pathol* 2002;160:529–536.
 58. Park SJ, Rashid A, Lee JH, Kim SG, Hamilton SR, Wu TT. Frequent CpG island methylation in serrated adenomas of the colorectum. *Am J Pathol* 2003;162:815–822.
 59. Toyota M, Issa JP. CpG island methylator phenotypes in aging and cancer. *Semin Cancer Biol* 1999;9:349–357.
 60. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci USA* 2000;97:710–715.
 61. van Rijnsoever M, Grieu F, Elsaleh H, Joseph D, Iacopetta B. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. *Gut* 2002;51:797–802.
 62. Issa JP. The epigenetics of colorectal cancer. *Ann N Y Acad Sci* 2000;910:140–153.
 63. Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum Mol Genet* 1992;1:229–233.
 64. Kato N, Tamura G, Fukase M, Shibuya H, Motoyama T. Hypermethylation of the RUNX3 gene promoter in testicular yolk sac tumor of infants. *Am J Pathol* 2003;163:387–391.
 65. Chadwick RB, Jiang GL, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. *Proc Natl Acad Sci USA* 2000;97:2662–2667.