Highly Methylated Genes in Colorectal Neoplasia: Implications for Screening

Hongzhi Zou,[1] Jonathan J. Harrington,[1] Abdirashid M. Shire,[1] Rafaela L. Rego,[1] Liang Wang,[2] Megan E. Campbell,[3] Ann L. Oberg,[3] and David A. Ahlquist[1]

[1]Division of Gastroenterology and Hepatology, [2]Department of Laboratory Medicine, and [3]Division of Biostatistics, Mayo Clinic, Rochester, Minnesota

Abstract

Discriminant markers are required for accurate cancer screening. We evaluated genes frequently methylated in colorectal neoplasia to identify the most discriminant ones. Four genes specifically methylated in colorectal cancer [*bone morphogenetic protein 3* (*BMP3*), *EYA2*, *aristaless-like homeobox-4* (*ALX4*), and *vimentin*] were selected from 41 candidate genes and evaluated on 74 cancers, 62 adenomas, and 70 normal epithelia. Methylation status was analyzed qualitatively and quantitatively and confirmed by bisulfite genomic sequencing. Effect of methylation on gene expression was evaluated in five colon cancer cell lines. *K-ras* and *BRAF* mutations were detected by sequencing. Methylation of *BMP3*, *EYA2*, *ALX4*, or *vimentin* was detected respectively in 66%, 66%, 68%, and 72% of cancers; 74%, 48%, 89%, and 84% of adenomas; and 7%, 5%, 11%, and 11% of normal epithelia ($P < 0.01$, cancer or adenoma versus normal). Based on area under the curve analyses, discrimination was not significantly improved by combining markers. Comethylation was frequent (two genes or more in 72% of cancers and 84% of adenomas), associated with proximal neoplasm site ($P < 0.001$), and linked with both BRAF and K-ras mutations ($P < 0.01$). Cell line experiments supported silencing of expression by methylation in all four study genes. This study shows *BMP3*, *EYA2*, *ALX4*, and *vimentin* genes are methylated in most colorectal neoplasms but rarely in normal epithelia. Comethylation of these genes is common, and pursuit of complementary markers for methylation-negative neoplasms is a rational strategy to optimize screening sensitivity. (Cancer Epidemiol Biomarkers Prev 2007;16(12):2686–96)

Introduction

Colorectal cancer is the second leading cause of cancer-related death in the United States, and currently, ~40% of affected individuals die from this cancer (1).Colorectal cancer mortality can be reduced by screen detection of premalignant adenomas and early stage cancers (2-5). An emerging approach to cancer screening involves the assay of tumor-specific DNA alterations in bodily fluids from cancer patients, such as stool, serum, and urine (6-15). It is important to select markers with high accuracy if efficiency and effectiveness are to be achieved in a cancer screening application. Due to the molecular heterogeneity of colorectal neoplasia, high detection rates will likely require a panel of markers.

Several methylated genes have been detected in the stool and serum/plasma samples from colorectal cancer patients (8, 9, 11, 14, 16-20). Whereas some methylated genes have been found in a majority of colorectal cancers, the yield of bodily fluid–based assays remains suboptimal (8-11, 13-20). It is unclear as to what extent biological or technical factors account for such observations.

A subset of colorectal cancers exhibiting gene methylation and associated with proximal tumor site has been described as the CpG island methylator phenotype (CIMP; refs. 21, 22). Reported prevalences of CIMP in colorectal cancer vary (21-28). CIMP has been associated with BRAF mutations and microsatellite instability (26-30), but the relationship to other gene alterations is less studied. The degree to which CIMP may influence tumor detection is incompletely understood.

This study was designed to (*a*) evaluate high-yield methylated genes as candidate markers for screening colorectal neoplasia, (*b*) explore the effect of combining gene markers on detection sensitivity, and (*c*) examine the relationship of aberrant promoter methylation to the expression of *bone morphogenetic protein 3* (*BMP3*), *EYA2*, *aristaless-like homeobox-4* (*ALX4*), and *vimentin* genes.

Materials and Methods

Approval of this study was obtained from the Institutional Review Board of Mayo Foundation.

Subjects. Two hundred and six colon tissues, including 74 cancers, 62 adenomas, and 70 normal colon epithelia, were collected at the Mayo Clinic and evaluated in two studies. Tissue study I comprised 104 tissues, including 43 cancers, 32 adenomas, and 29 normal epithelia. Tissue study II comprised 102 tissues, including 31 cancers, 30 adenomas, and 41 normal epithelia. Samples in study I included 22 frozen and 82 paraffin-embedded tissues; methylation markers were assayed

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Requests for reprints: David A. Ahlquist, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN 55905. Phone: 507-266-4338; Fax: 507-266-0350. E-mail: ahlquist.david@mayo.edu

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Table 1. Clinical characteristics of subjects

	Study	Cancer	Adenoma	Normal
No.	I	43	32	29
	II	31	30	41
	Total	74	62	70
Median age (range), y	I	66 (27-93)	67 (42-87)	67 (22-84)
	II	66 (34-90)	65 (37-86)	65 (31-82)
	Total	66 (27-93)	66 (37-87)	66 (22-84)
Sex (M/F)	I	20/23	17/15	13/16
	II	17/14	15/15	17/24
	Total	37/37	32/30	30/40
Location (proximal/distal)	I	21/22	19/13	
	II	12/19	18/12	
	Total	33/41	37/25	
Dukes stage (A/B/C/D)	I	2/21/19/1		
	II	6/9/15/1		
	Total	8/30/34/2		
Grade (1/2/3/4) or dysplasia (low/high)	I	1/10/28/4	25/7	
	II	0/4/26/1	21/9	
	Total	1/14/54/5	46/16	

qualitatively. Samples in study II were all frozen, and markers were assayed quantitatively. The demographic and clinical characteristics of these subjects are shown in Table 1.

Microdissection and DNA Extraction. Tissue sections were examined by a pathologist who circled out histologically distinct lesions to direct careful microdissection. Genomic DNA was extracted using Qiagen DNA minikit (Qiagen) or DNAzol (Invitrogen).

Conventional Methylation-Specific PCR. DNA was bisulfite treated using the EZ DNA methylation kit (Zymo Research) and eluted in 30 μL of elution buffer. One microliter of bisulfite-modified DNA was amplified in a total volume of 25 μL containing 1× PCR buffer (Applied Biosystem), 1.5 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate, 400 nmol/L of each primer, and 1.25 unit of AmpliTaq Gold polymerase (Applied Biosystem). Amplification included hot start at 95°C for 12 min, denaturing at 95°C for 30 s, annealing at certain temperatures for 30 s, extension at 72°C for 45 s for 35 cycles, and a final 10-min extension step at 72°C. Primer sequences and annealing temperatures were listed in Table 2, and primer locations were shown in Fig. 1. Bisulfite-treated human genomic DNA (Novagen) and CpGenomeTM universal methylated DNA (Chemicon) were used as positive controls for unmethylation and methylation, respectively.

Real-time Quantitative Methylation-Specific PCR. Bisulfite-treated DNA above was used as a template for methylation quantification with a fluorescence-based real-time PCR as described previously (31). Primers and probes were designed to target the bisulfite-modified methylated sequences of gene promoters (Fig. 1; Table 2). A region without CpG site in *β-actin* gene was also quantified with real-time PCR using primers and probe recognizing bisulfite-converted sequence as a reference of bisulfite treatment and DNA input (31). PCR reactions were done in a volume of 25 μL consisting of 600 nmol/L of each primer, 200 of nmol/L probe, 0.75 units of platinum Taq polymerase (Invitrogen), 200 μmol/L each of deoxynucleotide triphosphate, 16.6 mmol/L ammonium sulfate (Sigma), 67 mmol/L Trizma (Sigma), 6.7 mmol/L $MgCl_2$, 10 mmol/L mercaptoethanol, and 0.1% DMSO. One microliter of bisulfite-treated DNA was used in each PCR reaction. The gene methylation level was defined as the ratio of the fluorescence emission intensity value of target gene PCR product to that of *β-actin* PCR product multiplied by 1,000 (31).

Amplifications were done in 96-well plates in a real-time iCycler (Bio-Rad) under the following conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 62°C for 60 s. Bisulfite-treated CpGenomeTM universal methylated DNA (Chemicon) was used as positive control and serially diluted to create standard curve for all plates. Each plate consisted of bisulfite-treated DNA samples, positive and negative controls, and water blanks.

Selection of Tumor-Specific Methylated Markers. Forty-one genes were analyzed with methylation-specific PCR (MSP). These genes consisted of seven candidates identified in colorectal cancer by our group, including *EYA2, EYA3, BMP1, BMP2, BMP3, SIX2*, and *SIX6*, and 16 commonly methylated genes, including *p16, hMLH1, MGMT, CDH1, HIC1, GSTP1, RASSF1A, RUNX1, SLC5A8, SFRP1, vimentin, EYA4, BMP3b, TPEF, GATA4*, and *GATA5* (refs. 9, 32-46), as well as 18 methylated genes reported recently in the SW480 colon cancer cell line, including *ALX4, FOXF1, SHH, ZNF677, RASL11A, PAX6, ADAM12, KIAA0789, TGFB2, ZNF566, CDCA2, RPS27L, FLJ25439, TAZ, LOC283514, DAP, GATA3*, and a predicted gene (47). Methylated primers for the common methylated genes were from the literature, and the rest were designed by us with at least four CpGs and four Cs on each primer to discriminate methylated DNA sequence from unmethylated and wild-type ones.

The specificity of the primers to methylated sequence was first tested with bisulfite-treated universally methylated DNA, unmethylated human genomic DNA, and wild-type human genomic DNA. Primers that only amplified bisulfite-treated universally methylated DNA were further triaged in an age-matched independent set of colon tissues, including four cancers and four normal mucosa. Four genes, *BMP3, EYA2, ALX4*, and *vimentin*, were found to be methylated in three or more of the cancers but in none of the normal tissues (Fig. 2); thus,

these four methylation markers were selected for more extensive evaluation in the present investigation as described above. Primers for *BMP3, EYA2, ALX4*, and *vimentin* were presented in Table 2, and primers for other genes are available upon request. The schematic graphs of the 5′ regions of *BMP3, EYA2, ALX4*, and *vimentin* were shown in Fig. 1.

Bisulfite Genomic Sequencing. Methylation status of representative samples was confirmed by bisulfite genomic sequencing using primers (Table 2) that flank the MSP and/or real-time MSP regions of *BMP3, EYA2, ALX4*, and *vimentin* (Fig. 1). One microliter of bisulfite-modified DNA was amplified in a total volume of 25 μL containing 1× PCR buffer (Applied Biosystem), 3.0 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate, 400 nmol/L of each primer, and 1.25 unit of AmpliTaq Gold polymerase (Applied Biosystem). Amplification included 95°C for 10 min, denaturing at 95°C for 30 s, annealing at certain temperatures for 30 s,

Table 2. Primers used in this study

Gene	Primer	Primer sequence (5′→3′)	Product size (bp)	Annealing temperature (°C)	Note*
BMP3	Unmethylated	TTTAGTGTTGGAGTGGAGATGGTGTTTG AAACACAACCAAATACAACAAAATAACAA	146	60	
	Methylated	TTTAGCGTTGGAGTGGAGACGGCGTTC CGCGACCGAATACAACGAAATAACGA	143	68	
	Real-time MSP	AATATTCGGGTTATATACGTCGC CCTCACCCGCGCAAAACG 6FAM-TAGCGTTGGAGTGGAGACGGCGTTCG-TAMRA	87	62	
	Bisulfite sequencing	GAGGAGGGAAGGTATAGATAGA AATTAAACTCCAAACCAACTAAAAC	256	60	
	RT-PCR	CCCAAGTCCTTTGATGCCTA TGGTACACAGCAAGGCTCAG	147	62	
EYA2	Unmethylated	GGGAGGAGAAGGGGTTGGTTTTTTTG CCTAAAATAAACACCACTAACAATACTCACCA	209	60	
	Methylated	TTTCGGCGTAGGTAGTAGTCGC GACCTAAAATAAACGCCGCTAACGA	190	66	
	Real-time MSP	TTTTCGGCGTAGGTAGTAGTC GACGAAACCGAACTAACTACGA 6FAM-CGGTAACGGTAGAGATAGTAACGTGTTC-TAMRA	97	62	
	Bisulfite sequencing	GGTTTAGGGAGGAGAAGGGGT CCTCTACCCTTATACCTTCCTAAC	370	60	
	RT-PCR	GGACAATGAGATTGAGCGTGT ATGTCCCCGTGAGTAAGGAGT	90	60	Ref. (57)
ALX4	Unmethylated	TGTGTTTTTTATTGTGAGTTGTTGGTT ACAACAACAACTAAAACTACAAAATCAAC	295	60	
	Methylated	TGCGTTTTTTATTGCGAGTCGTCGGTC GACGACGACTAAAACTACGAAATCGACGA	293	68	
	Real-time MSP	TTGTAGAGGTTTCGTTTTTCGTC GCCTAAATTTCCCGTAAACTTTCGA 6FAM-CGTCGTCGTAGGTGAGAGCGTCGT-TAMRA	132	62	
	Bisulfite sequencing	GGATAGTAGGATTGTAGAGGT CTAAAACCCTAAAATCTCTAACTC	188	60	
	RT-PCR	AGACCCACTACCCAGACGTG GCCAGGACGGGTTCTGAAT	222	63	
Vimentin	Unmethylated	TTGGTGGATTTTTTGTTGGTTGATG CACAACTTACCTTAACCCTTAAACTACTCA	188	60	
	Methylated	TCGTTTCGAGGTTTTCGCGTTAGAGAC CGACTAAAACTCGACCGACTCGCGA	216	68	Ref. (9)
	Real-time MSP	GTTTTAGTCGGAGTTACGTGATTAC GAAAACGAAACGTAAAAACTACGA 6FAM-CGTATTTATAGTTTGGGCGACGCGTTGC-TAMRA	97	62	
	Bisulfite sequencing	GTAGTTATGTTTATTAGGTT CATTCAACTCCTACAACTC	342	55	
	RT-PCR	GGACCAGCTAACCAACGACA CTGGATTTCCTCTTCGTGGA	247	60	
β-actin	Real-time bisulfite PCR	TGGTGATGGAGGAGGTTTAGTAAGT AACCAATAAAACCTACTCCTCCCTTAA 6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA	Unknown	62	Ref. (31)
GAPDH	RT-PCR	CATCACCATCTTCCAGGAGCG TGACCTTGCCCACAGCCTTG	442	60	Ref. (50)
K-ras	PCR	AAGGCCTGCTGAAAATGACTGAAT CTGTATCAAAGAATGGTCCTGCACC	179	64	
BRAF	PCR	CCACAAAATGGATCCAGACA TGCTTGCTCTGATAGGAAAATG	173	60	

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
*Oligos were designed by us except those with references.

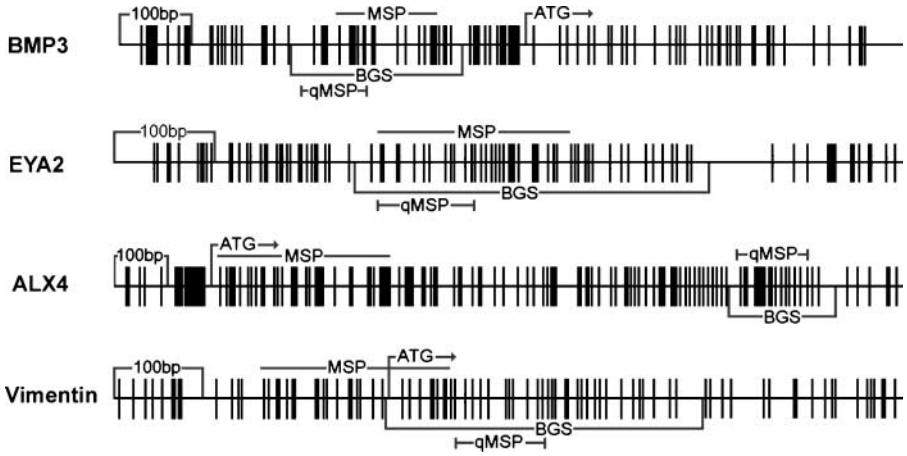

Figure 1. Schematic graph of the 5′ regions of *BMP3, EYA2, ALX4*, and *vimentin* genes. *Vertical bars,* CpG sites. Regions analyzed by MSP, quantitative MSP (*qMSP*), and bisulfite genomic sequencing, and the start codons were indicated.

extension at 72°C for 45 s for 40 cycles, and a final 10-min extension step. PCR products were cut from gels, purified using QIAquick gel extraction kit (Qiagen), and then ligated into pCR 2.1-TOPO cloning vector using a TOPO TA cloning kit (Invitrogen). For each cloning, six colonies were grown, extracted with Wizard Plus Minipreps DNA purification system (Promega), and then sequenced with ABI Prism 377 DNA sequencer (Perkin-Elmer) to get detailed methylation status of each CpG site.

Mutation Detection. Mutations on *K-ras* at codons 12 and 13 and on *BRAF* (V600E) were assayed by Sanger sequencing. Genomic DNA (100 ng) was amplified with primers flanking the mutant loci (Table 2). Five microliters of PCR products were incubated with 2 μL ExoSAP-IT (U.S. Biochemical Corporation) at 37°C for 30 min to get rid of residual deoxynucleotide triphosphates, primers, and possible dimmers and then directly sequenced in an ABI Prism 377 DNA sequencer (Perkin-Elmer).

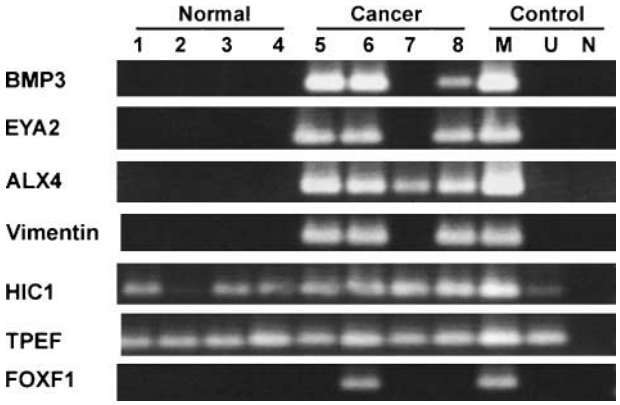

Figure 2. Tumor-specific methylated gene markers selected for study. Among 41 candidate genes, *BMP3, EYA2, ALX4*, and *vimentin* were methylated in at least three of four of the colorectal cancers, but in none of four normal colon tissues screened. HIC1, TPEF, and FOXF1 as representative of less specific or less sensitive markers for comparison. Universally methylated DNA and water were amplified as positive control and negative control, respectively.

Cell Lines and 5-Aza-Deoxycytidine Treatment. Five colon cancer cell lines, including SW480, SNUC4, HCT15, SW620, and WIDR, were used in this study. SW480, SW620, and WIDR were grown in DMEM supplemented with 10% fetal bovine serum, and SNUC4 and HCT15 were grown in RPMI 1640 supplemented with 10% fetal bovine serum. These cells were split to low density in 4-mL flasks, grown for 12 to 24 h, and then treated using 5 μmol/L 5-aza-deoxycytidine or mock treated with PBS for 96 h. Medium containing 5-aza-deoxycytidine and with PBS was changed every 24 h. The dose and timing of 5-aza-deoxycytidine were based on prior tests showing optimal reactivation of gene expression and published studies (48, 49).

Real-time Reverse Transcription–PCR. The mRNA expression of *BMP3, EYA2, ALX4*, and *vimentin* in these colon cancer cell lines with or without 5-aza-deoxycytidine treatment was quantified with real-time reverse transcription–PCR (RT-PCR). Briefly, RNA was extracted with RNeasy minikit (Qiagen). Reverse transcription was done on 2 μg of total RNA using Omniscript RT kit (Qiagen). One microliter of cDNA was amplified in a real-time iCycler (Bio-Rad) using a reaction volume of 25 μL containing 1× iQ SYBR Green Supermix (Bio-Rad) and 200 nmol/L of each primer under the following conditions: 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. Primers for each gene were designed on different exons to

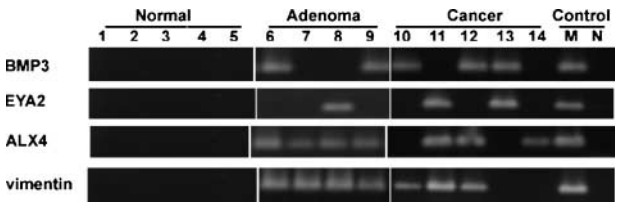

Figure 3. Neoplasm-specific methylation of *BMP3, EYA2, ALX4*, and *vimentin* genes. Methylation status was determined by conventional MSP using methylation-specific primers. Representative tissues from normal colon epithelia, adenomas, and cancers. Universally methylated DNA and water were amplified as positive and negative controls, respectively.

Table 3. Gene methylation associated with tumor location in cancer subjects

Study	Gene	Location	Methylation rate or level (median; range)	*P*
Study I	*BMP3*	Proximal	90% (19 of 21)	0.0002
		Distal	32% (7 of 22)	
	EYA2	Proximal	71% (15 of 21)	0.02
		Distal	32% (7 of 22)	
	ALX4	Proximal	95% (20 of 21)	0.004
		Distal	55% (12 of 22)	
	Vimentin	Proximal	95% (20 of 21)	0.01
		Distal	59% (13 of 22)	
Study II	*BMP3*	Proximal	34 (0-628)	0.01
		Distal	1 (0-302	
	EYA2	Proximal	155 (0-1082)	0.03
		Distal	2 (0-360)	
	ALX4	Proximal	458 (17-1182)	0.002
		Distal	25 (0-379)	
	Vimentin	Proximal	418 (0-1055)	0.005
		Distal	10 (0-276)	

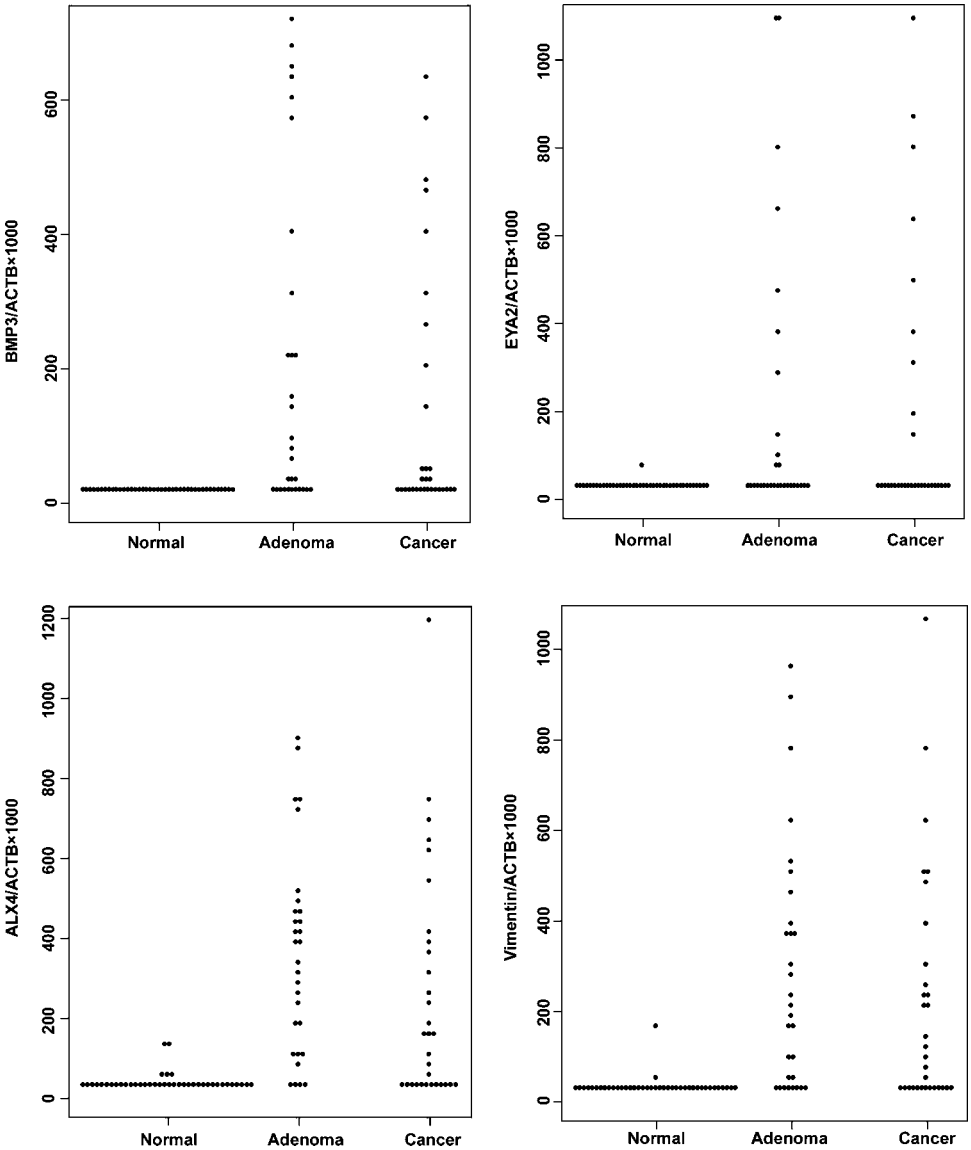

Figure 4. Methylation levels of *BMP3, EYA2, ALX4*, and *vimentin* measured by quantitative real-time MSP in colorectal cancer, adenoma, and normal epithelia. Each dot reresents a sample.

guarantee specific amplification of cDNA (Table 2). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference gene for normalizing the cDNA input (50). The mRNA expression ratios of the four genes were defined as the ratio fluorescence emission intensity value of target gene PCR products to that of *β-actin* PCR products multiplied by 1,000. Amplification was done in 96-well plates. Each plate consisted of cDNA samples and multiple water blanks, as well as positive and negative controls. Each assay was done in duplicate. Serial dilutions of positive controls were used to make standard curves for each plate. Melt curve was conducted for each reaction to guarantee that only one identical product was amplified, and the PCR products were further confirmed by agarose gel electrophoresis.

Statistical Analysis. χ^2 test was used to compare gene methylation frequencies between each of the three different tissue groups in study I, and Fisher exact test was used to analyze the association of gene methylation

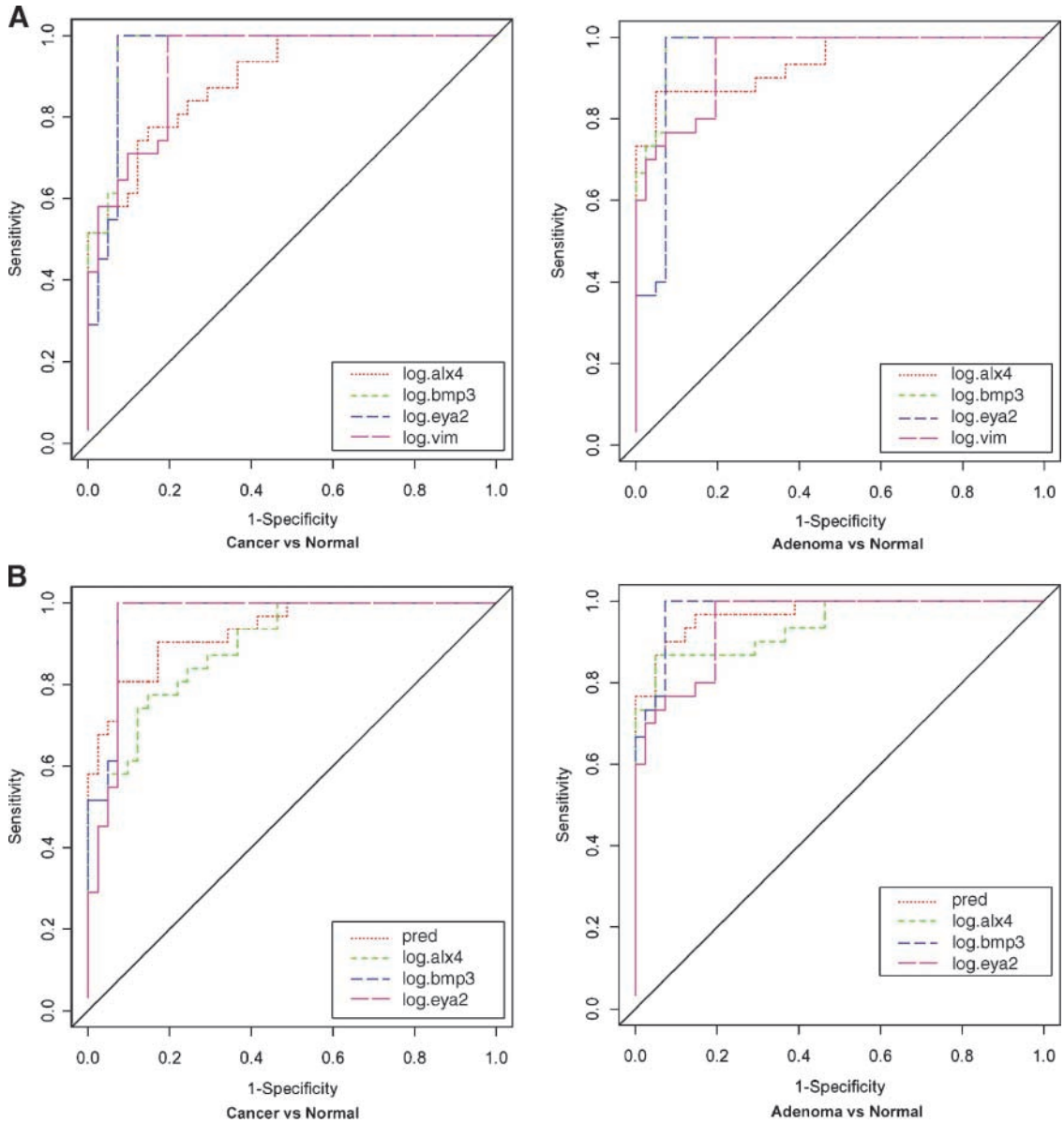

Figure 5. A, receiver operating curves for gene methylation levels in colorectal cancers or adenomas versus normal controls. For cancers versus normal controls, AUC values were 0.85, 0.9, 0.89, and 0.88 for *BMP3, EYA2, ALX4*, and *vimentin*, respectively; for adenomas versus normal controls, AUC values were 0.87, 0.79, 0.93, and 0.89 for *BMP3, EYA2, ALX4*, and *vimentin*, respectively. **B,** predicted receiver operating curves of best combinations of methylated markers in cancers or adenomas versus normal controls. AUC values were 0.92 for the predicted combination (*BMP3, EYA2*, and *ALX4*) in cancers and 0.94 for the predicted combination (*ALX4, BMP3*, and *vimentin*) in adenomas, which are not significantly higher than with single markers.

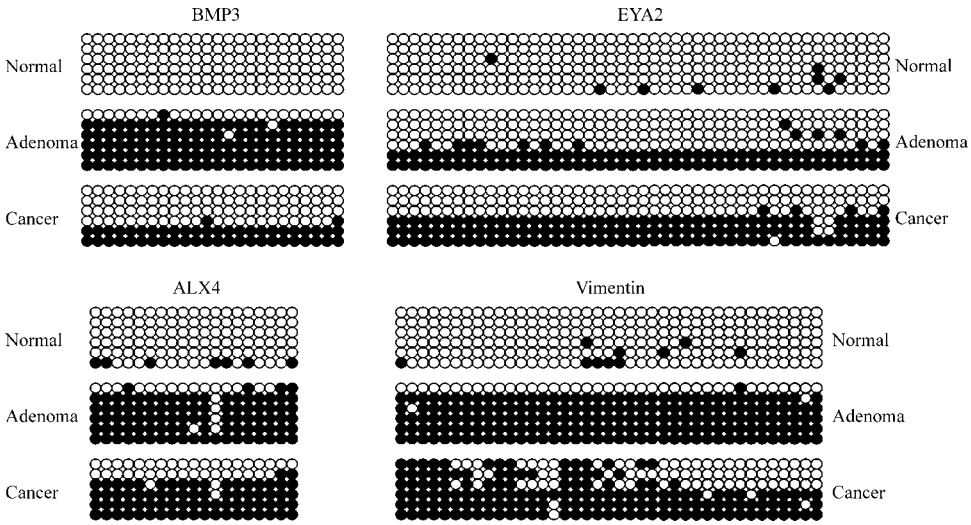

Figure 6. Methylation status of representative colon tissues confirmed by bisulfite genomic sequencing. The analyzed regions of the four CpG islands evaluated with the methylation status of each. Six clones were sequenced for each sample. *Closed circles,* methylated CpGs; *open circles*, unmethylated CpGs.

frequencies with clinical characteristics of tumor patients in study I. Wilcoxon rank-sum test was used to compare the methylation levels between each of the three different tissue groups and evaluate the association of methylation levels with tumor location, gender, Dukes stage, and differentiation grade in study II. Correlation of methylation levels with tumor size and patient age in study II was calculated with logistic procedure. Receiver operating curve was constructed to compare methylation level in cancers or adenomas versus normal subjects for each of the four markers and their combinations in study II, and area under the curve (AUC) value was also calculated for each curve. The association of gene comethylation with clinical characteristics of tumor patients and *K-ras* or *BRAF* mutations were calculated with χ^2 test. Statistical analysis was conducted with SAS software (SAS Institute).

Results

Methylation of *BMP3, EYA2, ALX4*, and *Vimentin* Genes in Colorectal Tumors. From a total of 41 candidates, four genes (*BMP3, EYA2, ALX4*, and *vimentin*) were found to be methylated in at least three of four colon cancers but in none of four normal colon epithelia on prestudy triage. Methylation of these four selected genes was evaluated more comprehensively in this investigation in two tissue studies.

In tissue study I, using conventional MSP, methylation of *BMP3, EYA2, ALX4*, and *vimentin* was detected in 60%, 51%, 74%, and 77% of 43 cancers; 72%, 44%, 91%, and 91% of 32 adenomas; and 7%, 3%, 17%, and 17% of 29 normal mucosa samples, respectively. Methylation was more frequently detected in cancer or adenoma than in normal epithelia for each of the four genes ($P < 0.01$; Fig. 3). Methylation was significantly more frequent in cancer from proximal colon than from distal colon for *BMP3, EYA2, ALX4*, and *vimentin* ($P < 0.05$; Table 3), but not associated with age, sex, tumor size, Dukes stage, or grade for any of the four genes ($P > 0.05$). Methylation in adenomas was not associated with age, sex, tumor size, degree of dysplasia, or villous component ($P > 0.05$).

In tissue study II, methylation levels were quantified using quantitative MSP. Mean methylation levels in 31 cancers, 30 adenomas, and 41 normal colon epithelia were observed respectively as follows (Fig. 4): 116 (0-628), 189 (0-712), and 0.3 (0-8.2) for *BMP3*; 158 (0-1082), 167 (0-1066), and 1.5 (0-51) for *EYA2*; 230 (0-1182), 335 (0-868), and 10.1 (0-113) for *ALX4*; and 193 (0-1055), 258 (0-955), and 5.0 (0-144) for *vimentin*. Methylation levels were significantly higher in cancer or adenoma than in normal epithelium for each of the four genes ($P < 0.01$ for each gene) but were comparable between cancer and adenoma for each gene after stratification by tumor location ($P > 0.05$ for each gene). Methylation levels were significantly higher in cancers from the proximal colon than from the distal colon for all four genes ($P < 0.05$; Table 3) but higher in adenomas from proximal colon than from distal colon for *ALX4* only ($P = 0.02$). Methylation levels in cancers correlated with larger size for *ALX4* only ($P = 0.004$) but were not associated with age, sex, Dukes stage, and grade for any of the four genes ($P > 0.05$). Methylation levels of adenomas correlated with larger size for *BMP3* only ($P = 0.04$) and with older age for *vimentin* only ($P = 0.04$) but not with other clinical characteristics, including sex, degree of dysplasia, and villous component, for any of the four genes ($P > 0.05$).

For quantitative data obtained in study II, receiver operating curves were constructed for each of the four genes (Fig. 5). Comparing cancer to normal epithelia, AUC values were 0.85, 0.9, 0.89, and 0.88 for *BMP3, EYA2, ALX4*, and *vimentin*, respectively (Fig. 5A); comparing adenoma to normal epithelia, AUC values were 0.87, 0.79, 0.93, and 0.89 for *BMP3, EYA2, ALX4*, and *vimentin*, respectively (Fig. 5A). AUC value was not significantly improved by combining any or all markers compared with the best single marker ($P > 0.05$; Fig. 5B). At a specificity of 93%, methylation of *BMP3, EYA2, ALX4*, and *vimentin* detected 74%, 87%, 58%, and 65% of 31 cancers and 77%, 53%, 93%, and 77% of 30 adenomas.

Combining studies I and II, methylation of *BMP3, EYA2, ALX4*, and *vimentin* was detected in 66%, 66%, 68%, and 72% of 74 cancers; 74%, 48%, 89%, and 84% of 62 adenomas; and 7%, 5%, 11%, and 11% of 70 normal

epithelia, respectively ($P < 0.01$, cancer or adenoma versus normal for each gene).

Using representative samples, bisulfite genomic sequencing confirmed that these four genes are densely methylated in cancer and adenoma but rarely or not methylated in normal colon mucosa (Fig. 6).

Comethylation in Colorectal Tumors. *BMP3, EYA2, ALX4*, and *vimentin* genes were commonly comethylated in colorectal neoplasms, and the subset of subjects with neoplasms showing comethylation shared certain characteristics. Methylation levels in study II were dichotomized to simplify panel assembly and to allow easier translation of quantitative to qualitative panels as obtained in study I (27). The dichotomization threshold at a methylation level of 10 was chosen as a point sufficiently above background levels measured with quantitative MSP but well below the much higher levels for the four markers in both colorectal cancers and adenomas (27). Methylation of one or more of four (at least one), two or more of four, three or more of four, or four of four genes was noted in 88%, 72%, 53%, and 41% of 74 cancers (Fig. 7; Table 4) and 98%, 84%, 60%, and 39% of 62 adenomas (Fig. 7; Table 5) compared with 24%, 7%, 3%, and 0% of 70 normal epithelia, respectively. Thus, comethylation is much more common in neoplasia than in normal epithelia, and comethylation is associated, progressively so with increasing specificity but decreasing sensitivity for colorectal neoplasia. Comethylation of two or more of four and three or more of four genes in cancer was significantly associated with older age ($P < 0.05$) and proximal colon location ($P \leq 0.001$) but not with other clinical characteristics (Table 4); comethylation of four of four genes in cancer was associated with proximal location only ($P = 0.0004$; Table 4). Comethylation of two or more of four and three or more of four genes in adenoma was significantly associated with proximal location ($P < 0.01$; Table 5), and comethylation of four of four genes in adenoma was associated with older age ($P = 0.008$; Table 5).

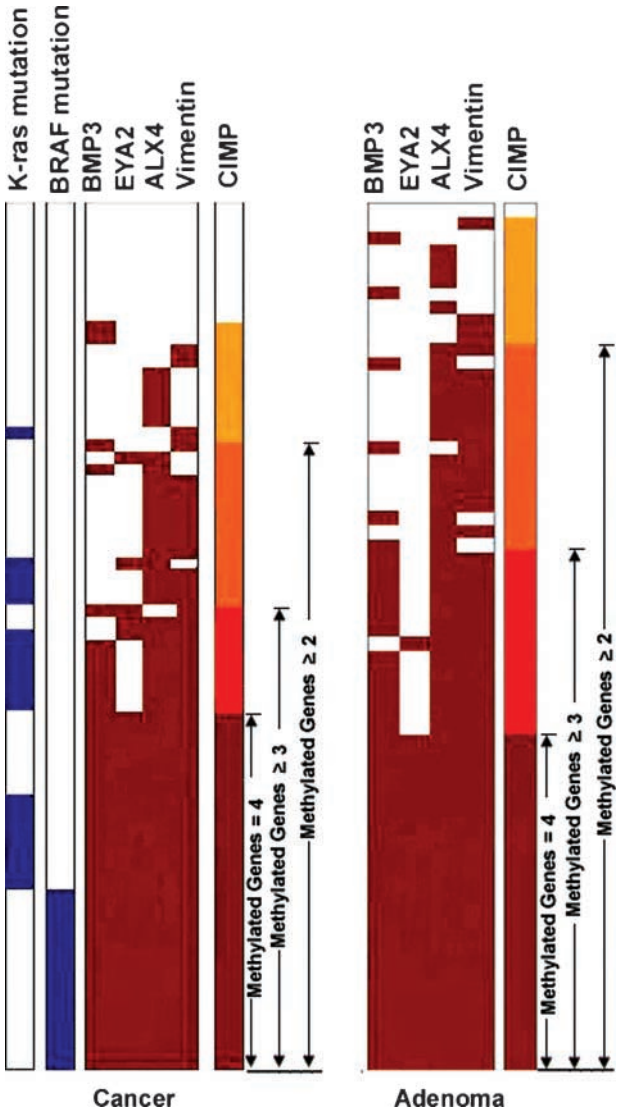

Figure 7. Heat maps demonstrating relationship of specific gene methylation, K-ras and *BRAF* mutations, and categorization as CIMP in colorectal cancer and adenoma. *Red bars,* methylated samples for the corresponding gene. The methylation levels across all cancer or adenoma samples are indicated from low to high using a long bar with increasing depth of red color. *Blue bars, BRAF* and *K-ras* mutations.

Association of BRAF and K-ras Mutations with Tumor Methylation. *BRAF* V600E and *K-ras* codons 12 and 13 mutations were found in 20% (15 of 74) and 27% (20 of 74) of cancers and were mutually exclusive. All BRAF and K-ras mutations occurred in tumors exhibiting methylation in at least one of the four study genes, and addition of neither BRAF nor K-ras mutations improved sensitivity over the most informative methylation marker alone. *BRAF* was strongly associated with gene comethylation; each of the 15 cancers with BRAF mutations also showed methylation in all four study genes (odds ratio, ∞; $P = 9 \times 10^{-8}$; Fig. 7; Table 4). Most cancers (19 of 20) with mutant *K-ras* also showed methylation of two or more genes (odds ratio, 11.2; $P = 0.007$; Fig. 7; Table 4), but this association was not apparent when tumors were dichotomized into those with all four genes methylated and those with less than four genes (Fig. 7; Table 4).

Re-expression of Methylated Genes in Colon Cancer Cell Lines by Demethylation. In SNUC4, HCT15, and WIDR cell lines, all four genes were found to be methylated; in the SW620 cell line, methylation was found in *BMP3* and *ALX4* genes; and in the SW480 cell line, only the *ALX4* gene was methylated (Fig. 8). Suppression of mRNA expression in these genes was generally observed in the methylated cell lines without 5-aza-deoxycytidine treatment. With the 5-aza-deoxycytidine treatment, *BMP3* mRNA was re-expressed from an undetectable level in HCT15 and increased by 22-fold, 26-fold, or 3225-fold in SNUC4, SW620, and WIDR cell lines, respectively. No changes in mRNA expression of *BMP3* were observed in the unmethylated cell SW480. *EYA2* mRNA was increased or re-expressed by 5-aza-deoxycytidine in methylated cell lines SNUC4 and WIDR but also in an unmethylated cell line SW620. *ALX4* mRNA was re-expressed from an undetectable level in four of five methylated cancer cells, SNUC4, HCT15,

Table 4. The association of gene comethylation with clinical variables and gene mutations in cancer subjects

		Comethylated genes ≥2			Comethylated genes ≥3			Comethylated genes = 4		
		+	−	*P*	+	−	*P*	+	−	*P*
Total		53	21		39	35		30	44	
Age	≤60 y	12	11	0.01	8	15	0.04	6	17	0.09
	>60 y	41	10		31	20		24	27	
Sex	Male	25	12	0.4	17	20	0.2	12	25	0.2
	Female	28	9		22	15		18	19	
Location	Proximal	31	2	0.0002	28	5	6×10^{-7}	21	12	0.0004
	Distal	22	19		11	30		9	32	
Dukes stage	A/B	27	11	0.9	16	22	0.06	11	27	0.04
	C/D	26	10		23	13		19	17	
Grade	1/2	12	3	0.5	8	7	1.0	5	10	0.5
	3/4	41	18		31	28		25	34	
BRAF	Mutant	15	0	0.006	15	0	1×10^{-5}	15	0	9×10^{-8}
	Wild-type	38	21		24	35		15	44	
K-ras	Mutant	19	1	0.007	15	5	0.02	8	12	1.0
	Wild-type	34	20		24	30		22	32	

SW620, and WIDR; *vimentin* mRNA expression was increased by 8-fold, 147-fold, and 346-fold, respectively, in the methylated cells SNUC4, HCT15, and WIDR, but only slightly changed in the unmethylated cells SW480 and SW620 (Table 6).

Discussion

Methylated genes have been detected in the blood and stool of patients with colorectal cancer and proposed as candidate screening markers (8, 9, 11, 14-20). In this study, we found four genes, *BMP3, EYA2, ALX4*, and *vimentin*, to be methylated in the majority of both colorectal cancers and premalignant adenomas. As these methylated gene markers were rarely found in normal epithelia, their methylation seems to be neoplasm specific or cancer related (type C; ref. 51). Each of these candidate markers can be considered for further evaluation in screening or diagnostic applications for colorectal neoplasia because of their broad coverage and early onset in the tumorigenesis of colorectal cancer.

Of note, the four methylation markers evaluated in the current study were found in the same subset of neoplasms and were associated with certain clinical features and genetic alterations. Comethylation of these markers was particularly associated with *BRAF* mutations, proximal colon location, and older age, which is consistent with the previous reports of the so-called CIMP (22, 26-28, 52). *K-ras* gene mutation occurred almost exclusively (95%) in cancers with methylation of two or more genes, but the relationship was lost when comparing tumor subsets with all four genes methylated against those with fewer than four methylated genes; and this observation suggests the possibility of a mutant *K-ras*–related CIMP-low group (28).

From a clinical standpoint, this phenomenon of marker comethylation has potential relevance to the performance of methylation markers in colorectal cancer screening. Because comethylation disproportionately affects neoplastic tissue, comethylation could be incorporated into a stringent definition of test positivity to improve specificity if a panel of markers was assayed. For example, when the definition of test positivity in the present study is changed from ''methylation of any of the four target genes'' to ''comethylation of at least two genes,'' the false-positive rate drops by 71% (24% rate to 7% rate, respectively) but the true-positive rate for cancer decreases by only 18% (88% rate to 72% rate, respectively).

Due to the biology underlying CIMP, methylation events may be insufficient to provide a panel of completely informative tumor markers. Although methylation markers identified in this study covered most colorectal tumors, no methylation markers were found in an important minority subset. It is not known if the addition of any other tumor-specific methylation markers would have improved lesion detection in the

Table 5. The association of gene comethylation with clinical variables in adenoma subjects

		Comethylated genes ≥2			Comethylated genes ≥3			Comethylated genes = 4		
		+	−	*P*	+	−	*P*	+	−	*P*
Total		52	10		37	25		24	38	
Age	≤60 y	18	5	0.4	12	11	0.4	4	19	0.008
	>60 y	34	5		25	14		20	19	
Sex	Male	27	5	0.9	22	10	0.1	11	21	0.5
	Female	25	5		15	15		13	17	
Location	Proximal	35	2	0.005	27	10	0.01	14	23	0.9
	Distal	17	8		10	15		10	15	
Dysplasia	Low	37	9	0.2	25	21	0.1	14	23	0.9
	High	15	1			4		10	15	

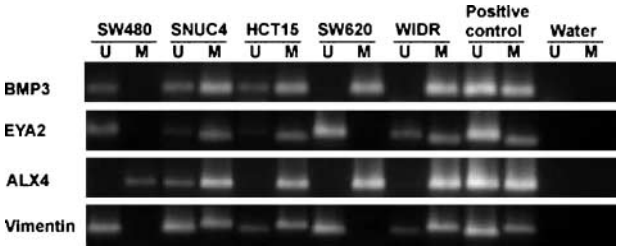

Figure 8. Methylation status of colon cancer cell lines checked with MSP. PCR products in lanes U or M indicates the presence of unmethylated or methylated genes, respectively. Universally methylated DNA and human genomic DNA were used as positive controls for methylation and unmethylation. Water was used as negative control.

methylation-negative subset because the panel of markers we evaluated was not exhaustive. Some have reported that methylated genes could yield near 100% coverage, such as with the combination of *ER, MYOD1*, and *SFRP1* (32, 41, 53); however, as such genes are also frequently methylated in normal mucosa of older individuals and may be more age-related (type A; ref. 51), nonspecificity rates could be unacceptably high. Thus, to accurately detect both CIMP-positive and CIMP-negative tumors, it would seem logical to consider a screening panel that combines tumor-specific methylated markers with genetic markers mutant in CIMP-negative lesions, as others have suggested (15, 21, 30). In the present study, neither BRAF nor K-ras mutations proved to be complementary to the most informative single methylation marker for tumor detection sensitivity.

Recently, *vimentin, ALX4*, and *BMP3* have been found to be methylated in colorectal cancer (9, 15, 19, 54), and the present study extends and corroborates these findings. Vimentin has been evaluated as a candidate stool marker, alone (9) and in combination with other markers (14), and has proved to be informative for colorectal neoplasia. This is the first report that *EYA2* is frequently methylated and epigenetically silenced in cancer. Despite their frequent methylation in colorectal neoplasia, the carcinogenic roles of these genes are not well understood. As inactivation of tumor suppressor genes by aberrant promoter methylation is a mechanism of oncogenesis (55, 56), it is possible that these genes function as tumor suppressors. Evidence from the current study to support this hypothesis includes the findings that methylation is associated with markedly reduced or absent gene expression in colon cancer cell lines and that *in vitro* demethylation with 5-aza-deoxycytidine re-expressed these genes. Further basic investigation will be helpful to elucidate their cellular function and mechanisms of action.

In summary, *BMP3, EYA2, ALX4*, and *vimentin* genes are commonly methylated in colorectal cancers and adenomas but rarely in normal epithelia. Application studies on stool, serum, or other biological samples are indicated to explore the value of these methylated genes as markers for screening colorectal cancer. The comethylation of these four genes across a majority of colorectal cancers supports the existence of a subset that may be broader than the conventionally described CIMP. Such a broad panel of methylation markers has special relevance to neoplasm detection but may need to be distinguished from methylation markers that are mechanistically linked to CIMP-high associated with BRAF mutation and the serrated pathway to carcinogenesis (27, 29). An important minority of colorectal neoplasms does not seem to exhibit gene methylation and may be missed by tests that target methylated genes only. Complementary use of markers that detect nonmethylated or rarely methylated neoplasia is a biologically rational approach to optimize screening sensitivity.

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Table 6. The effect of 5-aza-deoxycytidine treatment on the mRNA expression levels of genes in colon cancer cells

Cell	Fold change after 5-aza-deoxycytidine treatment			
	BMP3	*EYA2*	*ALX4*	*Vimentin*
SW480	−0.1	−0.1	+0.5	+0.3
SNUC4	+22	+3	Re-expressed*	+8
HCT15	Re-expressed*	0	Re-expressed*	+147
SW620	+26	Re-expressed*	Re-expressed*	+0.5
WIDR	+3225	Re-expressed*	Re-expressed*	+346

*mRNA expression was only detected in these cells with 5-aza-deoxycytidine treatment and was not detectable without 5-aza-deoxycytidine treatment.

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