Highly Methylated Genes in Colorectal Neoplasia: Implications for Screening

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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

Received 6/7/07; revised 8/29/07; accepted 9/17/07.

Grant support: Charles Oswald Foundation.

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doi:10.1158/1055-9965.EPI-07-0518

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.

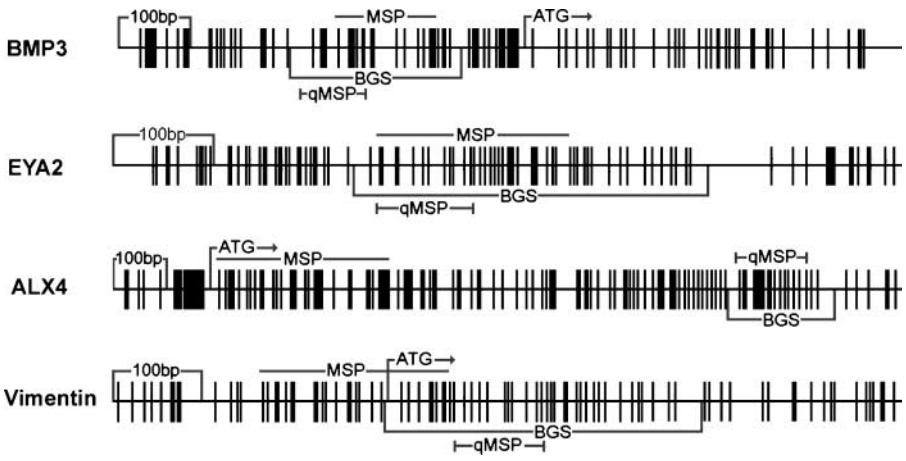

Figure 1. Schematic graph of the 5′ regions of *BMP3, EYA2, ALX4*, and *vimentin* genes. *Vertical bars,* CpG sites. Regions analyzed by MSP, quantitative MSP (*qMSP*), and bisulfite genomic sequencing, and the start codons were indicated.

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).

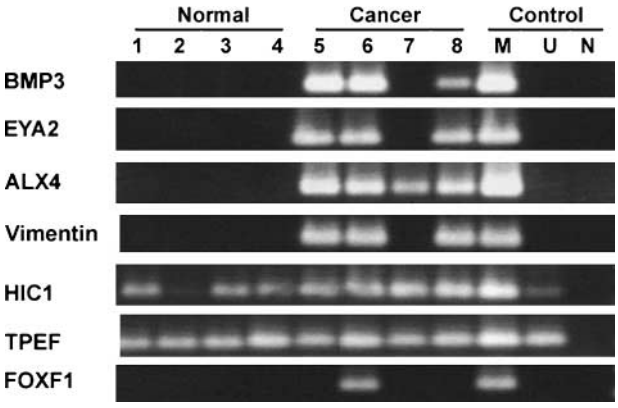

Figure 2. Tumor-specific methylated gene markers selected for study. Among 41 candidate genes, *BMP3, EYA2, ALX4*, and *vimentin* were methylated in at least three of four of the colorectal cancers, but in none of four normal colon tissues screened. HIC1, TPEF, and FOXF1 as representative of less specific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

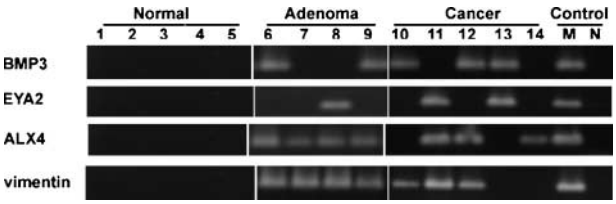

Figure 3. Neoplasm-specific methylation of *BMP3, EYA2, ALX4*, and *vimentin* genes. Methylation status was determined by conventional MSP using methylation-specific primers. Representative tissues from normal colon epithelia, adenomas, and cancers. Universally methylated DNA and water were amplified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

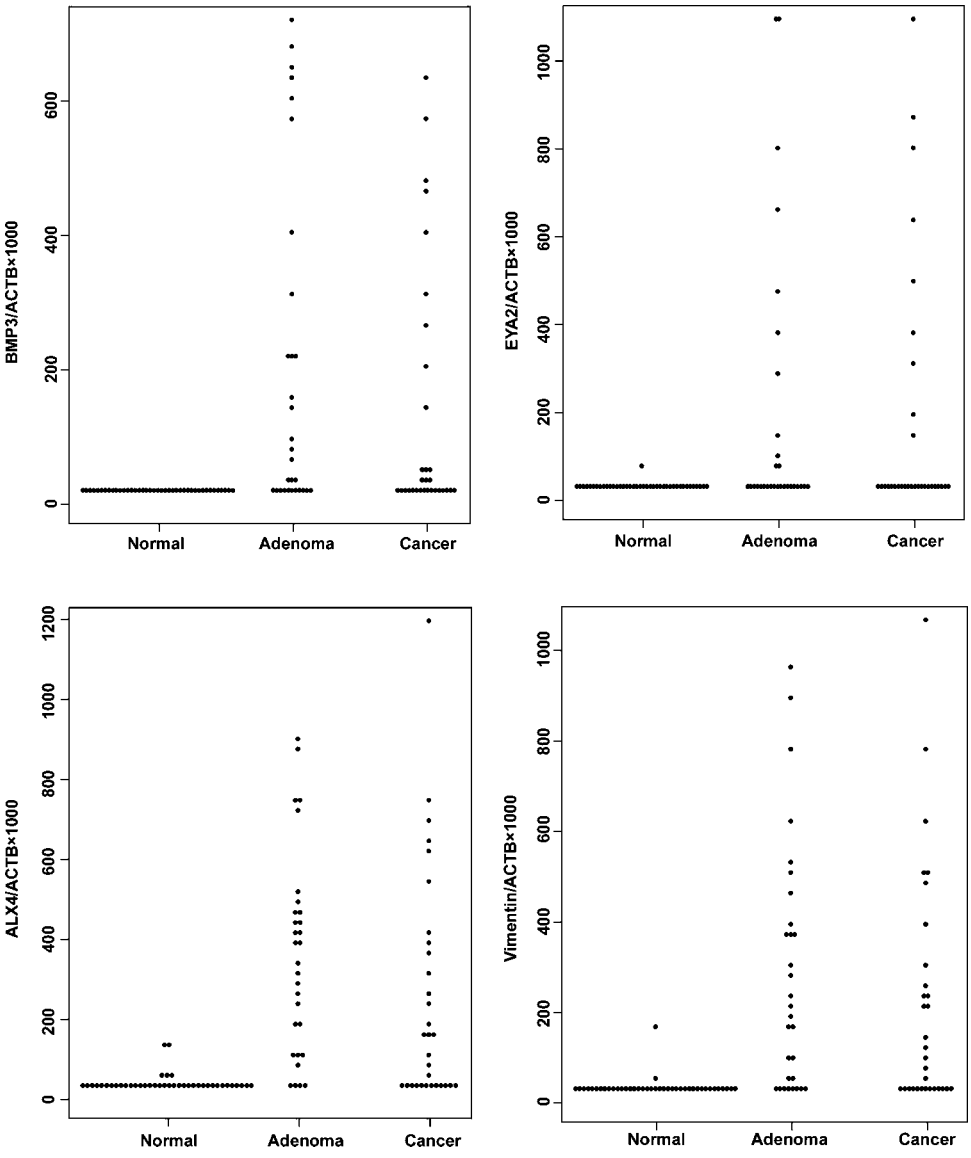

Figure 4. Methylation levels of *BMP3, EYA2, ALX4*, and *vimentin* measured by quantitative real-time MSP in colorectal cancer, adenoma, and normal epithelia. Each dot represents a sample.

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