# Detection in Fecal DNA of Colon Cancer–Specific Methylation of the Nonexpressed Vimentin Gene

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***Background:*** **Increased DNA methylation is an epigenetic alteration that is common in human cancers and is often associated with transcriptional silencing. Aberrantly methylated DNA has also been proposed as a potential tumor marker. However, genes such as vimentin, which are transcriptionally silent in normal epithelium, have not until now been considered as targets for cancer-associated aberrant methylation and for use as cancer markers.** ***Methods:*** **We applied methylation-specific polymerase chain reaction to the vimentin gene, which is transcriptionally silent in normal colonocytes, and compared methylation of vimentin exon 1 in cancer tissues and in fecal DNA from colon cancer patients versus control samples from healthy subjects.** ***Results:*** **Vimentin exon-1 sequences were unmethylated in 45 of 46 normal colon tissues. In contrast, vimentin exon-1 sequences were methylated in 83% (38 of 46) and 53% (57 of 107) of tumors from two independently collected groups of colon cancer patients. When evaluated as a marker for colon cancer detection in fecal DNA from another set of colon cancer patients, aberrant vimentin methylation was detected in fecal DNA from 43 of 94 patients, for a sensitivity of 46% (95% confidence interval [CI] = 35% to 56%). The sensitivity for detecting stage I and II cancers was 43% (26 of 60 case patients) (95% CI = 31% to 57%). Only 10% (20 of 198 case patients) of control fecal DNA samples from cancer-free individuals tested positive for vimentin methylation, for a specificity of 90% (95% CI = 85% to 94%).** ***Conclusions*****: Aberrant methylation of exon-1 sequences within the nontranscribed vimentin gene is a novel molecular biomarker of colon cancer and can be successfully detected in fecal DNA to identify nearly half of individuals with colon cancer. [J Natl Cancer Inst 2005;97:1124–32]**

Aberrant (i.e., increased) methylation of CpG-rich sequences (CpG islands) is an epigenetic change that is common in human cancers *(1–4)*. Such CpG islands are most frequently located in the promoter regions or in untranslated first exons of human genes *(1–4)*. Most commonly, increased CpG methylation of gene promoters or first exons is associated with loss of gene transcription *(1–4)*. In human colon cancers, several genes have been identified that are commonly unmethylated and expressed in normal colon mucosa but are methylated and silenced in colon cancer *(1–7)*. There has been substantial interest in attempting to adapt such cancer-associated aberrant gene methylation for use as a marker for potential early detection of colon and other cancers *(3,8–12)*.

Colon cancer is the second-leading cause of cancer death in adults in the United States *(13)*. When these cancers are detected in early clinical stages, i.e., stages I and II, when the tumors are still confined to the bowel wall, surgical cure rates

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*See* "Notes" following "References."

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are 90% and 75%, respectively *(14)*. In contrast, chances for cure drop rapidly once colon tumors have spread beyond the confines of the bowel. Initial reports have confirmed the potential for early detection of colon cancer–derived aberrantly methylated DNA in both patient blood and feces, but the sensitivity and specificity of currently identified markers are not optimal *(8,10)*.

To expand the population of genomic DNA sequences that might potentially be useful as methylated DNA markers of colon cancer, we have investigated whether cancer-associated aberrant DNA methylation might target CpG-rich sequences within a gene that is not expressed by normal colonic epithelium and for which gene silencing would therefore not result from an aberrant methylation event. We chose for this approach the vimentin gene, which encodes a protein constituent of intermediate filaments and whose expression is considered a classic marker of mesenchymal cells, such as fibroblasts *(15)*, and which hence should not be expressed by normal colonic epithelium. We describe here the analysis of aberrant methylation of the human vimentin gene and then the assay of vimentin gene methylation as a potential marker of colon cancer in patient tumors and in fecal DNA.

## Materials and Methods

### Tissues, Cell Lines, and Nucleic Acid Isolation

Normal and malignant colon tissue samples were obtained from discarded tissue specimens from the department of surgical pathology at University Hospitals of Cleveland using a tissue procurement protocol approved by the University Hospitals of Cleveland internal review board. These samples included 12 samples of histologically normal colonic mucosa from individuals having resections for noncancer diagnoses (designated normal group 1) and 46 samples of histologically normal colonic mucosa from colon cancer resections (designated normal group 2), along with matching colon cancer tissue from these 46 patients (designated group A). An additional and independent set of 107 colon cancer tumor tissues (designated group B) were collected from consenting patients at the Lahey Clinic (Burlington, MA) and sent to Exact Sciences, which provided these samples for study at Case Western Reserve University. Colon cancer tumors included those arising in the proximal colon (cecum, ascending, and transverse colon), distal colon (descending and sigmoid colon), and rectum. VACO series colon cancer cell lines were established and maintained as described previously *(16)*. For initial screening of vimentin gene methylation the 11 cell lines studied were Vaco5, Vaco6, Vaco9m, Vaco10m, Vaco206, Vaco241, Vaco364, Vaco394, Vaco400, Vaco425, Vaco441, and Vaco576. Additional studies also employed Vaco6. RNA and DNA were prepared from colon tissues and cell lines after lysis in guanidine isothiocyanate and fractionation through cesium chloride as previously described *(17)*.

### Immunohistochemistry

Vimentin protein expression in paraffin-embedded normal colon tissue and colon tumors were evaluated using a mouse anti-vimentin monoclonal antibody, V9 (DAKO Cytomation, Carpinteria, CA). Briefly, 5-μm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated through graded alcohols to water. Antigen unmasking was performed by heat treatment (10 m*M* citrate, pH 6.0, in an 800-W microwave oven for two 5-minute cycles). Slides were incubated with the V9 anti-vimentin primary antibody at 1 : 100 dilution for 10 minutes and developed using the LSAB2 visualization system (DAKO) with 3,3′ diaminobenzidine tetrahydrochloride substrate, followed by hematoxylin counterstaining. In every analysis, longitudinally cut sections of peripheral nerve were included as a positive control and staining with preimmune mouse serum was performed as a negative control.

### Preparation of Colonic Mucosa and Colonic Crypts

Colonic mucosa was prepared by blunt dissection from normal portions of colectomy resections, with tissue maintained at 4 °C throughout. To further prepare colonic crypts, which are epithelial cell-enriched, mucosal samples were cut into 2- to 3-mm strips, incubated with approximately 5 mL of Cell Recovery Solution reagent (Becton Dickinson, Franklin Lakes, NJ) per square centimeter of tissue at 4 °C with gentle rocking for 1 hour, and then passed through a large-bore pipette. Released colonic crypts were collected by low-speed centrifugation at 350*g* for 5 minutes at 4 °C.

### Real-time Reverse Transcription–Polymerase Chain Reaction

The vimentin transcript was amplified from the isolated RNA of normal colon and colon cancer tissues and colon cancer–derived cell lines in an iCycler instrument (BioRad Laboratories, Hercules, CA) using 400 n*M* of forward primer, 5′-CACGAAGAGGAAATCCGGAGC-3′, and reverse primer, 5′-CAGGGCGTCATTGTTCCG-3′, to yield a 215-bp product. Each PCR was carried out in a 25-μL volume using SybrGreen Mastermix (BioRad) for 8 minutes, 30 seconds at 95 °C, followed by 50 cycles of 95 °C for 20 seconds, 60 °C for 20 seconds, and 72 °C for 20 seconds. To directly compare vimentin expression in crypt cell preparations and in whole-colonic mucosa, vimentin transcript expression was normalized in both crypt and whole-mucosal preparations to the transcript levels of Muc2, a marker of colonocyte epithelial cell mass. Muc2 transcript was amplified using forward primer 5′-TGAAGAAGACAGAGACCCCCT-3′ and reverse primer 5′-CAGGCAGTCCTCATTGTTCTGAC-3′, spanning exons 14 and 15. The RT-PCR conditions were 50 cycles of 94 °C for 20 seconds, 60 °C for 20 seconds, and 72 °C for 20 seconds. The level of vimentin expression was determined as the ratio of vimentin to Muc2 = $2^{CT_{vimentin} - CT_{Muc2}}$, where $CT_{vimentin}$ is the cycle number for crossing the iCycler detection threshold in real-time PCR amplification of vimentin, and $CT_{Muc2}$ is the cycle number for crossing the iCycler detection threshold in real-time PCR amplification of Muc2.

### Bisulfite Conversion of Genomic DNA and MS-PCR

Bisulfite conversion of DNA was performed as described previously *(6,18)* to create a template for methylation-specific PCR (MS-PCR). Briefly, 500 ng to 2 μg of genomic DNA from each sample in a volume of 50 μL was denatured by NaOH (freshly made, final concentration, 0.2 *M*) at 37 °C for 15 minutes. Next, 30 μL of 10 m*M* fresh hydroquinone and 520 μL of

were added, and the mixture was incubated at 55 °C for 16 hours. Bisulfite-modified DNA was purified using the Wizard DNA Cleanup kit (Promega, Madison, WI). The DNA was desulfonated by incubation with NaOH at a final concentration of 0.3 *M* at room temperature for 15 min and neutralized by adding ammonium acetate, pH 7.0, to a final concentration of 3 *M*. DNA was precipitated with ethanol and resuspended in distilled water to a final concentration of 5 ng/μL.

Bisulfite-treated DNA was then used as the template for MS-PCR, which was performed as described previously *(6,18)*. Briefly, 5 μL of bisulfite-converted genomic DNA served as the PCR template. The amplification was in a reaction of 25 μL containing 0.19 m*M* each dNTP, 1.5 m*M* MgCl2, 400 n*M* of forward and reverse primers, and 1.25 U of AmpliTaq Gold in the recommended buffer. Amplification primers and reaction conditions are provided (Supplementary Table, available at http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97issue15). Vimentin MS-PCR reaction #29 employed forward amplification primer 5′-TCGTTTCGAGGTTTTCGCGTTAGAGAC-3′ and reverse amplification primer 5′-CGACTAAAACTCGACCGAC TCGCGA-3′. PCR cycling parameters were as follows: hot start at 95 °C for 9 minutes, followed by 45 cycles of 95 °C (45 seconds), 70 °C (45 seconds), and 72 °C (45 seconds), then 72 °C for 10 minutes, and 10 °C to cool. For amplifications from fecal DNA, both forward and reverse MS-PCR primers were additionally extended by addition of a 5′ tag sequence 5′-GCGGTCCC-3′, which is not derived from the vimentin sequence but which provided, on the second and subsequent cycles of PCR, for more robust amplification of templates that had incorporated the PCR primers. For sequencing of bisulfite-converted DNA, products were amplified with methylation-indifferent primers and cloned into pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA); 10–15 individual clones per sample were then sequenced using an automated sequencer (Applied Biosystems, Foster City, CA).

### Preparation of Fecal DNA

Stools were collected from a population (n = 198) of average-risk individuals with no prior history of colon cancers or polyps and from a population (n = 94) of colorectal cancer patients, all of whom provided written informed consent, and who represented four different medical care organizations, of which one group contributed half of the total samples studied, with the remaining three groups contributing the balance. Stool samples were frozen within 72 hours after collection and stored at −80 °C. For recovery of human DNA, whole samples were thawed at room temperature and homogenized in excess volume (1 : 7) of EXACT buffer A (EXACT Sciences, Marlborough, MA). Homogenized samples were then archived at −80 °C for an average of 12 months (range = 6–18 months). No effect of the time of sample storage on ultimate sensitivity of the MS-PCR assay was found. To reduce the risk of sample degradation, homogenates were thawed only once, at the time of processing and analysis. At that time, a 4-g stool sample equivalent of each homogenate was centrifuged to remove all particulate matter, and the supernatants were treated with 20 μL of RNase A (2.5 mg/mL) (Roche, Indianapolis, IN) and incubated at 37 °C for 1 hour. Total DNA was then precipitated (by adding 1/10 volume of 3 *M* NaAc and [illegible] in 4 mL of 1× TE buffer (0.01 *M* Tris, pH 7.4, 0.001 *M* EDTA) (Pierce, Rockford, IL). Target human vimentin DNA fragments were purified from total DNA preparations by acrylamide gel–based affinity capture as previously described *(19)*. Total DNA yields from normal patients (median = 936 genome equivalents, range = 33–18 560 genome equivalents) and cancer patients (median = 1014 genome equivalents, range = 32–3700 genome equivalents) were similar. Total captured DNA from each sample was then subjected to bisulfite-modification and MS-PCR, and the results were analyzed in a manner blinded to patient's disease status.

### Statistical Analysis

Exact 95% confidence intervals (CIs) were calculated for all estimated proportions. Clinical variables were adjusted using a logistic regression model, and two-sided *P* values were calculated for the log-odds ratios using a Wald-type test *(20)*. Comparisons were determined to be statistically significant if $P<.05$. MS-PCR reactions were run independently in quadruplicate on all cell line samples and in duplicate on all patient tissue samples. Due to limitations of sample amount, assays on aberrant crypt foci and on fecal DNAs were single determinations.

## Results

### Expression and Methylation of the Vimentin Gene in Colon Cancer Cell Lines, Colon Cancers, and Normal Colonocytes

Immunohistochemical assay of vimentin expression in the human colon showed the absence of protein expression in the colonic epithelial cells in both normal colonic crypts and in colon cancers and positive vimentin expression in stromal cells and lymphocytes within both normal colonic crypts and colon cancers (Fig. 1, A). To confirm that the vimentin gene is transcriptionally silent in colon epithelial cells, we used real-time RT-PCR to analyze vimentin transcript levels in bluntly dissected normal colonic mucosa, which contains epithelial and stromal cells and in a purified preparation of normal colonic crypts that are highly enriched for colonic epithelial cells (Fig. 1, B). On average, colonic crypts retained only 3% (95% CI = 2.9% to 3.1%) of the vimentin transcript level present in the full mucosal tissue (Fig. 1, C), strongly suggesting that vimentin transcripts in the normal mucosal tissue are derived essentially completely from the non-epithelial cell population.

The structure of the vimentin gene demonstrates a dense region of CpG dinucleotides starting upstream of the first exon and continuing across the first two-thirds of this exon (Fig. 2, A). To assay this region for potential cytosine methylation, we designed a series of eight MS-PCR primer pairs that defined overlapping fragments spanning the region. These primers were initially used to assay the nonexpressed vimentin gene for DNA methylation in normal colonic mucosal samples from 12 control individuals who did not have colon cancer and in 11 colon cancer cell lines. Although the vimentin gene is transcriptionally silent in colonic mucosal epithelial cells, a 5′ portion of vimentin exon 1, defined by six overlapping MS-PCR primers, was free of detectable [illegible]

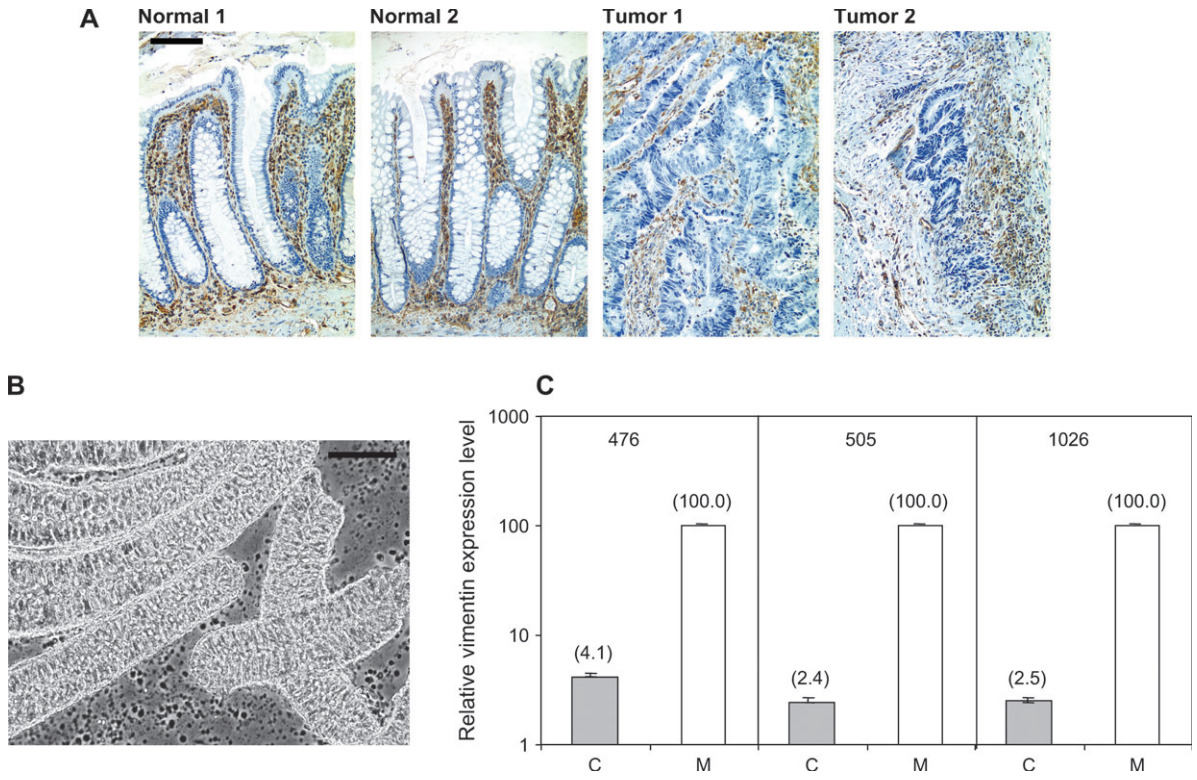


**Fig. 1.** Localization of vimentin expression. **A**) Immunohistochemical detection of vimentin expression (**brown**) using the monoclonal V9 anti-vimentin antibody (DAKO Cytomation, Carpinteria, CA) in tissue sections from two normal colonic mucosa specimens and two colon cancers. Comparison with a hematoxylin counterstain shows clear vimentin staining in stromal cells and lymphocytes but not in epithelial cells. **Bar** = 100 μm. **B**) Phase-contrast microscopy of colonic crypt preparations showing the enrichment of epithelial cells compared with the colonic mucosa specimens. **Bar** = 100 μm. **C**) Levels of vimentin transcript expression measured by real-time PCR in colonic crypt preparations (**solid bars**, labeled C) and in matched colonic mucosal tissue samples (**open bars**, labeled M) for three different individuals (case patients 476, 505, and 1026). For each patient, levels of vimentin expression in the mucosal tissue were set to equal 100%. Vimentin expression in mucosa and in crypt preparations was normalized to the epithelial cell mass of the tissue as assessed by the level of expression of the colonic epithelial cell marker Muc2. Graphed values are means and 95% confidence intervals of triplicate determinations. Where 95% confidence intervals are not visible, they represent a range of less than 1%.

the 12 normal colonic mucosa samples assayed. In contrast, the 11 colon cancer cell lines demonstrated clear acquisition of aberrant methylation across vimentin exon 1, with different MS-PCR primer pairs detecting methylation in from eight to 10 of the 11 cell lines assayed (Fig. 2, C and Table 1).

To quantify the extent of vimentin exon 1 methylation in the 11 cancer cell lines, we prepared bisulfite-converted DNA from three of these cell lines (Vaco5, Vaco6, Vaco400). For each cell line, we sequenced vimentin exon 1 from multiple individual PCR-amplified clones and assessed whether the antecedent cytosine at each CpG site was methylated or unmethylated. In this analysis, every CpG cytosine within the target vimentin exon 1 sequence was methylated in every clone sequenced, demonstrating that in all of these cell lines this region had become essentially 100% methylated (data not shown).

### Acquired Increased Vimentin Methylation in Tissues From Primary Colonic Neoplasms

MS-PCR assays for vimentin gene methylation were next used to characterize vimentin gene methylation in matched pairs of normal colonic mucosa and colon cancer tissues obtained from 46 colon cancer patients not mentioned above. In this second set of 46 normal mucosal tissue samples, MS-PCR primer sets 3 and 29 again defined a 216-bp region of vimentin exon 1 that was

(Fig. 2, D; Table 1). In contrast, 83% (38 of 46) of the colon cancers from the same 46 patients had acquired increased methylation in this 216-bp region, particularly when assayed by MS-PCR primer set 29 (Fig. 2, E, Table 1). Among these 46 colon cancers, acquired increased vimentin methylation was detected in 92% of cancers arising in the proximal colon (cecum, ascending and transverse colon), 67% of cancers arising in the distal colon (descending and sigmoid colon), and in 80% of cancers of the rectum (Table 2, group A).

To confirm these results, we used primer set 29 to assay a second independent collection of 107 colon cancer samples. Again, a majority, 53% (57 of 107 case patients), of this second set of colon cancer case patients demonstrated aberrant vimentin methylation. In this second patient series, 72% of cancers of the proximal colon assayed positive for aberrant vimentin gene methylation, and 45% of cancers of the distal colon were methylated (Table 2, group B). The smaller proportion of proximal colon cancers in the second patient cohort than the first likely accounts for this series having a somewhat lower overall frequency of vimentin methylation (Table 2). Also, in both series of tumors, early stage I and stage II cancers that have not spread beyond the wall of the colon showed rates of vimentin gene methylation at least equal to those of later stage III and stage IV cancers (Table 2). Detection of vimentin gene methylation was technically robust, and all normal samples that tested

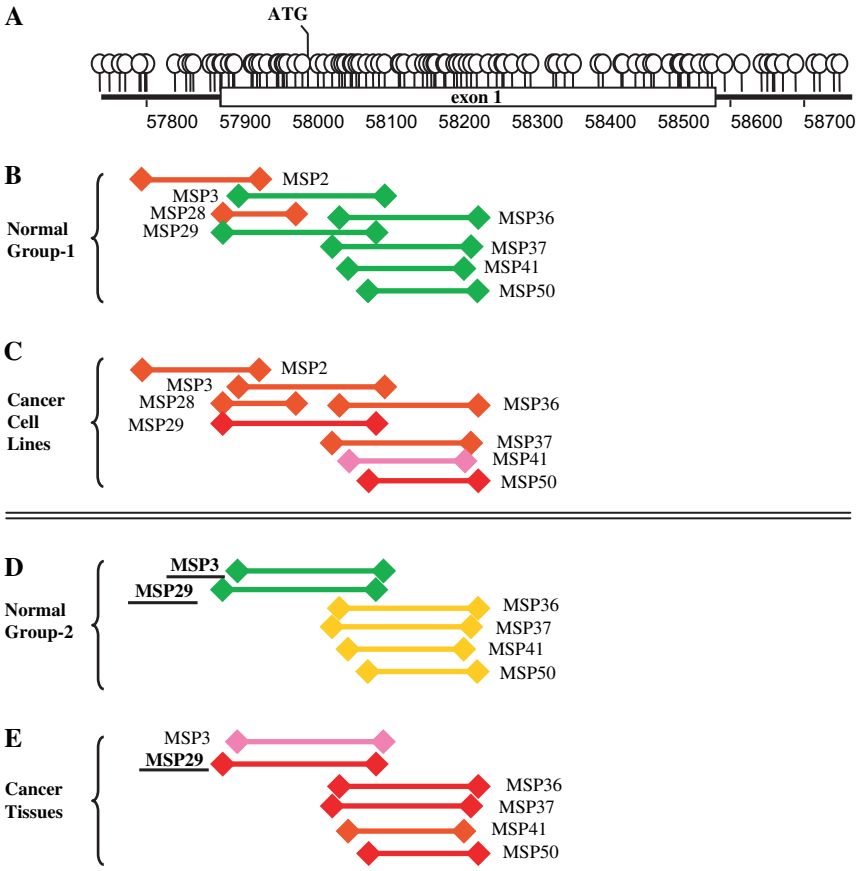


**Fig. 2.** Distribution of cytosine methylation across the vimentin gene. **A**) Balloons designate the distribution of CpG dinucleotides across the promoter and first exon of the vimentin gene. **B**) Different vimentin gene domains that were amplified by a panel of methylation specific polymerase chain reactions (MS-PCRs) that were applied to normal colon mucosa samples from 12 control noncancer patients (Normal Group 1). **C**) Using the same convention as in panel B, the results of MS-PCR assays for vimentin gene methylation in 11 colon cancer cell lines are shown. **D**) The results of MS-PCR testing for vimentin gene methylation in normal colonic mucosa samples from 46 colon cancer patients. **E**) The results of MS-PCR testing for vimentin gene methylation in colon tumor samples from the same 46 patients represented in panel D. The color code of the line denoting each MS-PCR indicates the percentage of case patients testing positive for methylation (**green** = <10% of samples methylated, **yellow** = 10%–49%, **pink** = 50%–80%, and **red** = >80%.

constitutively methylated control (MS-PCR assay F1-19M) (Fig. 3, A).

To determine the timing during colon carcinogenesis of acquisition of vimentin gene methylation, we next used MS-PCR primer set 29 to test a set of 10 colonic adenomas of 1 cm or greater in size. Of these 10 adenoma lesions, seven were positive for vimentin gene methylation (Fig. 3, B). We therefore used MS-PCR primer set 29 to assay DNA extracted from aberrant crypt foci, the microscopic lesions that are recognized as the earliest morphologic abnormality of the colonic mucosa *(21)*. Of nine aberrant crypt foci obtained from colons of six different individuals, seven aberrant crypts from five different individuals were positive for vimentin gene methylation (Fig. 3, C). In contrast, only one of 14 microdissected regions of histologically normal colon from these individuals tested positive for aberrant vimentin methylation (Fig. 3, C).

## Sensitivity of Detecting Aberrant Vimentin Methylation

To evaluate the potential use of increased vimentin gene methylation as a cancer biomarker, we tested the technical limits to the sensitivity of detecting DNA methylation by primer set 29. This primer set robustly detected vimentin methylation in colon cancer cell lines but not in normal colonic mucosa obtained from control noncancer colon resections (Fig. 3, D). Indeed, DNA from normal colonic mucosa remained negative in this assay, even after subjecting an aliquot of the MS-PCR to a second round of PCR amplification (i.e., 90 cycles total) (Fig. 3, D). Moreover,

**Table 1.** Vimentin exon-1 methylation in normal and cancer tissues and cancer cell lines*

| Primer set | Normal group 1† (n = 12) | Cancer cell lines (n = 11) | Normal group 2‡ (n = 46) | Cancer tissues (n = 46) |
|---|---|---|---|---|
| MSP2 | 83 (52 to 98) | 91 (59 to 99) | — | — |
| MSP3 | 0 (0 to 22) | 82 (48 to 98) | 0 (0 to 6) | 63 (48 to 77) |
| MSP28 | 83 (52 to 98) | 91 (59 to 99) | — | — |
| MSP29 | 0 (0 to 22) | 82 (48 to 98) | 2 (01 to 12) | 83 (69 to 92) |
| MSP36 | 8 (0 to 39) | 82 (48 to 98) | 22 (11 to 36) | 87 (74 to 95) |
| MSP37 | 0 (0 to 22) | 82 (48 to 98) | 24 (13 to 39) | 89 (76 to 96) |
| MSP41 | 0 (0 to 22) | 73 (39 to 94) | 46 (21 to 61) | 89 (76 to 96) |
| MSP50 | 8 (0 to 39) | 82 (48 to 98) | 24 (13 to 39) | 87 (74 to 95) |

*The percentage of subjects demonstrating vimentin gene methylation from methylation-specific polymerase chain reaction assays using the primer sets shown.
†Normal group 1 = normal colon mucosal tissues from non–colon cancer patients.
‡Normal group 2 = matched normal colonic mucosa from colon cancer patients whose cancer tissues were assayed; — = Normal group 2 and cancer tissues groups

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