Use of DNA from Human Stools to Detect Aberrant CpG Island Methylation of Genes Implicated in Colorectal Cancer

Nigel J. Belshaw,[1] Giles O. Elliott,[1] Elizabeth A. Williams,[4] David M. Bradburn,[3] Sarah J. Mills,[3] John C. Mathers,[2] and Ian T. Johnson[1]

[1]Institute of Food Research, Colney, Norwich, United Kingdom; [2]Human Nutrition Research Centre, School of Clinical Medical Sciences, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, United Kingdom; [3]Wansbeck Hospital, Ashington, Northumberland, United Kingdom; and [4]Centre for Human Nutrition, University of Sheffield, Northern General Hospital, Sheffield, United Kingdom

Abstract

Hypermethylation of cytosine residues in the CpG islands of tumor suppressor genes is a key mechanism of colorectal carcinogenesis. Detection and quantification of CpG island methylation in human DNA isolated from stools might provide a novel strategy for the detection and investigation of colorectal neoplasia. To explore the feasibility of this approach, colorectal biopsies and fecal samples were obtained from 32 patients attending for colonoscopy or surgery, who were found to have adenomatous polyps, colorectal cancer, or no evidence of neoplasia. A further 18 fecal samples were obtained from healthy volunteers, with no bowel symptoms. Isolated DNA was modified with sodium bisulfite and analyzed by methylation-specific PCR and combined bisulfite restriction analysis for CpG island methylation of *ESR1*, *MGMT*, *HPP1*, *p16^{INK4a}*, *APC*, and *MLH1*. CpG island methylation was readily detectable in both mucosal and fecal DNA with methylation-specific PCR. Using combined bisulfite restriction analysis, it was established that, in volunteers from whom biopsies were available, the levels of methylation at two CpG sites within *ESR1* assayed using fecal DNA were significantly correlated with methylation in DNA from colorectal mucosa. Thus, noninvasive techniques can be used to obtain quantitative information about the level of CpG island methylation in human colorectal mucosa. The methods described here could be applied to a much expanded range of genes and may be valuable both for screening purposes and to provide greater insight into the functional consequences of epigenetic changes in the colorectal mucosa of free-living individuals. (Cancer Epidemiol Biomarkers Prev 2004;13(9):1495–1501)

Introduction

Colorectal carcinoma is the second most common cause of death from cancer in many Western countries. Most cases of sporadic colorectal cancer are thought to develop via the adenoma-carcinoma sequence (1). This process is, by definition, a localized phenomenon in which the emerging lesion grows by clonal expansion. At each stage, the abnormal epithelial cells acquire somatic mutations, or other genetic changes, leading to a progressive loss of differentiation and growth regulation (2). In addition to mutations, it is now recognized that the process is associated with *epigenetic mechanisms*, whereby somatic cells acquire changes in gene expression that are transmissible through mitosis but which do not involve any alterations to the DNA sequence (3-5). One of the principle epigenetic mechanisms known to be involved in the pathogenesis of colorectal cancer is the methylation of the cytosine residues of CpG-rich sequences (CpG islands) located within the promoter regions of genes regulating cell proliferation, apoptosis, and DNA repair (6). These CpG islands are normally unmethylated in expressed genes, but methylation interferes with the binding of transcription factors and is thought also to recruit protein complexes that modify chromatin structure and lead to suppression of gene transcription (7).

Aberrant silencing of methylated genes has been shown to make a major contribution to the genetic dysfunction associated with the emergence of colorectal and other neoplasias (3). For example, silencing of the DNA repair gene *MLH1* by promoter methylation is strongly associated with microsatellite instability in sporadic colorectal cancer (8). Methylation often affects multiple genes, and this has led to the emerging recognition of the CpG island methylator phenotype as an important mechanism of carcinogenesis (9, 10). Methylation of the CpG islands of genes, such as *MLH1* and *APC*, is known to occur in focal lesions of the adenoma-carcinoma sequence at both early and late stages of carcinogenesis (11, 12), but it also occurs as an age-related field effect in morphologically normal mucosa (13). This is of great potential interest because, unlike somatic mutation, it affects many epithelial cells simultaneously and may cause abnormalities such as hyperproliferation, failure of DNA repair, and suppression of apoptosis well before the appearance of localized lesions.

Apart from the novel insights into the pathogenesis of colorectal cancer provided by the study of CpG island methylation patterns, the phenomenon can be exploited

Received 11/17/03; revised 3/8/04; accepted 4/12/04.

Grant support: Food Standards Agency (United Kingdom).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ian T. Johnson, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom. Phone: 44-1603-255330; Fax: 44-1603-255167. E-mail: ian.johnson@bbsrc.ac.uk

as a strategy for the molecular characterization of tumors (14). Moreover, the detection of DNA derived from cells with aberrant methylation patterns might provide a valuable approach to screening for early lesions, particularly if it could be applied noninvasively. It has recently been shown that aberrant methylation of the promoter regions of the $p16^{INK4a}$ and *MGMT* genes can be detected in DNA from the sputum of patients with squamous cell lung carcinoma at least 3 years before clinical diagnosis (15). Previous studies on CpG methylation in colorectal epithelial cells have been conducted almost exclusively with mucosal or tumor samples obtained at endoscopy or surgery. The detection of hypermethylated fecal DNA has been briefly reported by others (16), but the quantification of CpG island methylation in fecal DNA has not. In the present study, we describe techniques for both detection and quantification of aberrant methylation patterns in human DNA residues obtained from stool samples.

Materials and Methods

Patients. Ethical approval for the project was received from the Northumberland Local Research Ethics Committee (project reference NLREC2/2001). Patients awaiting surgery for colorectal cancer, or flexible endoscopy for a range of gastrointestinal symptoms including change of bowel habit and rectal bleeding, were sent information about the study along with notification of their outpatient appointment. Those expressing interest were visited at home to obtain formal written consent and to provide collection apparatus and a container for fecal samples. Biopsies were collected at surgery or endoscopy as described below. Six to 8 weeks after the procedure, the medical notes for each of the volunteers were reviewed and final diagnosis was recorded based on the findings of the pathology report and the conclusions of the responsible consultant. A second series of stool samples was obtained from a group of 18 healthy volunteers (10 male, 8 female; average age 52 years) with no symptoms or history of gastrointestinal disease. One of these volunteers was subsequently diagnosed with cancer and was excluded from the healthy volunteer category in subsequent analyses.

Stool Samples. Volunteers were asked to provide a stool sample in advance of the hospital appointment, using a sealable pathology pot, and a modified bedpan that enabled the pot to be held in position over the toilet seat. Samples were collected from volunteers' homes and brought to the laboratory, typically within 2 hours of defecation. A subsample (~250 mg) was frozen and stored at −20°C.

Tissue Samples. Samples of flat mucosa and tumor tissue were collected from the freshly resected colon of patients undergoing surgery for colorectal cancer. All samples were collected under the supervision of the surgeon to ensure correct identification of tumor tissue and nonneoplastic tissue at least 10 cm from the tumor margin. Biopsies from macroscopically normal flat colorectal mucosa were obtained from patients undergoing endoscopy. All biopsies and tissue samples were snap frozen in liquid nitrogen immediately after collection, transferred to the laboratory, and stored at −80°C.

Control DNA and Cell Culture. Human placental DNA (4 μg, Sigma, Poole, United Kingdom) was artificially methylated by incubation with 24 units *Sss*I methylase (New England Biolabs, Hitchin, United Kingdom) and 160 μmol/L *S*-adenosylmethionine in 10 mmol/L Tris-HCl (pH 7.9), 120 mmol/L NaCl, 10 mmol/L EDTA, and 1 mmol/L DTT at 30°C for 2 hours. DNA was extracted from the colorectal carcinoma cell lines HT29 and SW48, which were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to the manufacturer's instructions.

DNA Extraction. DNA was purified from feces (~250 mg) using the QIAamp DNA stool mini kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's protocol, which is designed to preferentially release and purify DNA from the human colonocytes present in the feces. Genomic DNA was extracted from tissue samples (5–10 mg) or cultured cells ($\sim 1 \times 10^6$) using a Genelute mammalian genomic DNA extraction kit (Sigma) according to the manufacturer's instructions.

Analysis of CpG Island Methylation. Genomic DNA (2 μg) was treated with sodium bisulfite according to the method of Raizis et al. (17). Modified DNA was purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions and desulfonated in 0.3 mol/L NaOH at 37°C for 15 minutes. The alkali was neutralized with 10 mol/L ammonium acetate, and the DNA was precipitated with ethanol. Bisulfite-modified DNA was resuspended in 10 mmol/L Tris-HCl (pH 7.5) and 1 mmol/L EDTA, and a one-tenth volume was used as template in PCR reactions to amplify regions of the CpG islands of the genes *APC*, *ESR1*, *MLH1*, *MGMT*, *p16*, and *HPP1* using the primers listed in Table 1. PCR reactions (20 μL) containing 10 μL HotStarTaq master mix (Qiagen), 4 pmol of each forward and reverse primer, and supplemented with $MgCl_2$ to 0.5 mmol/L, were subjected to the following cycling conditions: 1 cycle of 95°C for 15 minutes, 35 cycles of 95°C for 30 seconds, annealing temperature (see Table 1) for 1 minute, 72°C for 1 minute followed by a 5-minute extension at 72°C. Products from the PCR reactions were either used as templates for methylation-specific PCR (MSP) reactions or analyzed directly by a modified combined bisulfite restriction analysis (COBRA) assay, as described below.

MSP reactions (18) were carried out with 1 μL of 50-fold diluted product from the primary PCR as template. Reactions (20 μL) were conducted as described above, except that 30-second annealing and extension steps were used. The primers and annealing temperatures used for the unmethylated and methylated MSP reactions for six genes are listed in Table 2. MSP products were analyzed on ethidium bromide–stained 3% agarose gels and electrophoresed in 1× TAE buffer. Random methylated MSP products for each of the six genes from fecal samples were excised from the gels, purified using a QIAquick gel extraction kit, and cloned into pCR4-TOPO (Invitrogen, Paisley, United Kingdom) prior to transformation into *Escherichia coli* TOP10 competent cells (Invitrogen). Random clones were selected and grown overnight in Luria-Bertani broth containing antibiotic selection, and the plasmid DNA was extracted and purified using a miniprep kit (Qiagen). Plasmid inserts were sequenced using a BigDye terminator cycle

Table 1. Primer sequences for the amplification of CpG islands from bisulfite-modified DNA by PCR together with their annealing temperatures

Gene	Genbank accession No.	Primer sequences	Annealing temperature (°C)	Restriction enzyme (Cuts at)
APC	U02509, D13980	GTTAGGGTTAGGTAGGTTGT CCATAATAACTCCAACACCTA	59.5	*Cla*I (166)
HPP1	AF264150	AGAGTTTTTTTTTTATGGTAGTAGT ACTCCCACAACACCATAACTA	56	*Taq*I (108)
MLH1	U83845	TTAGATTATTTTAGTAGAGGTATATAAG ATACCTTCAACCAATCACCTCAATA	53	*Afl*III (123)
ESR1	AL356311	ATGGTTTTATTGTATTAGATTTAAGGGAA AAACTCRTTCTCCAAATAATAAAACACCTA	58	*Sau*3AI (195), *Taq*I (132, 281)
MGMT	gi|10944181	GTTTYGGATATGTTGGGATAGTT CTACAAACCACTCRAAACTACCA	58	*Taq*I (48)
p16^{INK4a}	AF527803	GGTTTTTTTTAGAGGATTTGAGGGATA AAACAAACCCTCTACCCACCTAA	62	*Sau*3AI (100)

NOTE: Methylation at each locus was determined by COBRA assay using the indicated restriction enzyme.

sequencing kit (Applied Biosystems, Warrington, United Kingdom) and an 373 DNA sequencer (Applied Biosystems).

A modified COBRA assay (19), sometimes called bisulfite PCR, was used to determine the methylation status of CpGs within the CpG islands of the six genes. Products from the primary PCR were precipitated with ethanol prior to an overnight digestion with the chosen restriction enzyme (Table 1). The digested DNA was separated on 3% or 4% agarose gels in 1× TAE and stained with SYBR green I (Molecular Probes, Leiden, Netherlands). The DNA SYBR green fluorescence was captured using a CCD camera (ProXpress proteomic imager, Perkin-Elmer, Buckinghamshire, United Kingdom) fitted with ProFinder software (Perkin-Elmer) and band intensities were quantified using TotalLab 1D analysis software (Nonlinear Dynamics, Newcastle-upon-Tyne, United Kingdom). The proportion of methylated versus unmethylated DNA was determined from the relative intensities of cut and uncut PCR product.

Determination of Human DNA Content of Fecal DNA. Fecal DNA (5 or 12.5 ng) was added to a PCR containing 10 μL HotStarTaq master mix supplemented with $MgCl_2$ to 0.5 mmol/L and 4 pmol of each of the primers NB308 (5′-AGAGGGAATGGTCAGTGAT) and NB309 (5′-CAAGGAGAGGTCTGAGTAT) specifically designed to amplify a 548-bp fragment from the MYOD1 genomic locus. The PCRs were subjected to 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 59°C for 1 minute, and 72°C for 1 minute and finally a 5-minute extension at 72°C. The PCR products were electrophoresed in a 2% agarose gel alongside similar PCRs with placental DNA (0–2 ng) as template. DNA was stained with SYBR green I and quantified as above.

Statistical Analysis. Methylation data were obtained for each of the six genes using DNA derived from the normal mucosa, tumor tissue, and feces from the volunteers, and arithmetic means and SEM were calculated. The significance of differences between mean values

Table 2. Primer sequences and annealing temperatures for the analysis of methylation at the indicated CpG islands using MSP

Gene	MSP reaction	Primer sequences	Annealing temperature (°C)	Reference
APC	U-F	GTGTTTTATTGTGGAGTGTGGGGTT	66	Esteller et al. (11)
	U-R	CCAATCAACAAACTCCCAACAA		
	M-F	TATTGCGGAGTGCGGGTC	69	
	M-R	TCGACGAACTCCCGACGA		
HPP1	U-F	GTAGTTTTTTGTGTTTTTGGTGT	64	
	U-R	CAAAACATCCCACAAACAACAA		
	M-F	GTTTTTCGCGTTTTCGGCGT	72.5	
	M-R	ATCATCCCGCGAACGACGA		
MLH1	U-F	TTAATAGGAAGAGTGGATAGTG	57	Ueki et al. (28)
	U-R	TCTATAAATTACTAAATCTCTTCA		
	M-F	TTAATAGGAAGAGCGGATAGC	60.5	
	M-R	CTATAAATTACTAAATCTCTTCG		
MGMT	U-F	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	65	Esteller et al. (27)
	U-R	AACTCCACACTCTTCCAAAAACAAAACA		
	M-F	TTTCGACGTTCGTAGGTTTTCGC	70	
	M-R	GCACTCTTCCGAAAACGAAACG		
p16^{INK4a}	U-F	TTATTAGAGGGTGGGGTGGATTGT	64	Herman et al. (18)
	U-R	CAACCCCAAACCACAACCATAA		
	M-F	TTATTAGAGGGTGGGGCGGATCGC	77.5	
	M-R	GACCCCCGAACCGCGACCGTAA		

NOTE: Abbreviations: U, unmethylated; M, methylated; F, forward; R, reverse.

for the three sources of DNA were assessed by one-way ANOVA using transformed data (log 10) in which there was evidence of skewness. Mean levels of methylation for the six genes, determined using fecal DNA samples from cancer patients, patients with adenomatous polyps, and patients with no evidence of colorectal disease, were compared by one-way ANOVA. Pearson's correlation coefficients (r) were calculated to assess the significance of any associations between levels of methylation for different genes or for different DNA sources in the same patient. All statistical analyses were carried out using Minitab Release 13 (Minitab, Inc., State College, PA).

Results

Extraction of DNA from Fecal Samples. The yield of DNA obtained from ~250 mg of fecal material varied from 2 to 30 μg. The purity of human DNA obtained was assessed initially using quantitative PCR with TaqMan chemistry (Perkin-Elmer) to determine the numbers of copies of the *APC* gene present per microgram of DNA. However, we were unable to achieve any amplification with the fecal DNA, whereas, with human placental DNA, quantifiable amplification was achieved with the selected probe and primers (data not shown). Therefore, a simpler semiquantitative PCR approach was adopted. By comparing the relative intensities of a PCR product obtained with fecal DNA with those obtained for a range of concentrations of placental DNA, the proportion of fecal DNA derived from the human host was estimated to be <1% (data not shown).

CpG Island Methylation in Tissue and Fecal DNA by MSP. The presence of methylated alleles for *ESR1*, *APC*, *HPP1*, *MLH1*, *MGMT*, and $p16^{INK4a}$ in DNA from both tissue biopsies (normal and tumor) and corresponding fecal samples was determined using MSP after an initial PCR amplification step. This has been shown previously to substantially increase the sensitivity for the MSP (15). We were able to detect the presence of methylated alleles in all samples, for all genes studied, as indicated by the specific amplification of fragments in methylated MSP reactions (Fig. 1). The conversion of unmethylated cytosines by bisulfite was assessed by cloning and sequencing random PCR products from methylated MSP reactions with fecal DNA. For *APC*, *HPP1*, *MLH1*, *MGMT*, and $p16^{INK4a}$, a total of 34 PCR products were sequenced revealing only six unconverted non-CpG cytosines. From the genomic sequences, a total of 115 non-CpG cytosines are present in the regions to be amplified. Thus, assuming error-free PCR, >99.8% of unmethylated cytosines were converted. This level of conversion rules out the possibility that the positive methylated MSP reactions were artifacts generated from the incomplete conversion of cytosines by bisulfite. Interestingly, the sequencing results revealed gene-specific differences in the methylation profiles. For *APC*, *MGMT*, and $p16^{INK4a}$, CpGs in the interprimer region were uniformly methylated, indicating that complete methylation or hypermethylation occurs at these loci, whereas, for *MLH1* and *HPP1*, the methylation of the intervening CpGs was nonuniform and incomplete.

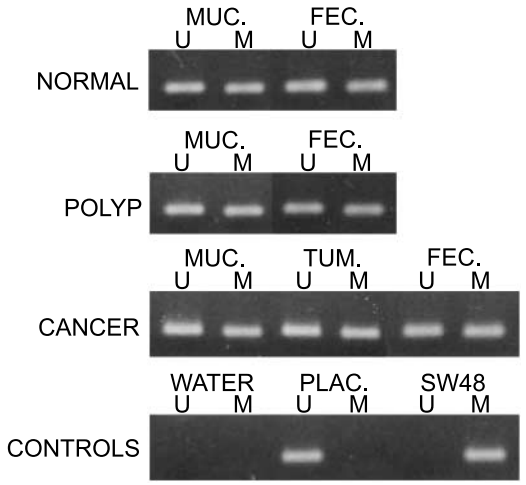

Figure 1. Methylation of *MGMT* by MSP in normal mucosa (*MUC.*), tumor tissue (*TUM.*), or fecal (*FEC.*) DNA from patients without disease (*NORMAL*), with polyps, or with cancer. PCR products from the unmethylated (*U*) reaction are alongside products from the methylated (*M*) reaction for each sample. The presence of a visible product in the *U lanes* indicates the presence of unmethylated CpG islands; the presence of product in *M lanes* indicates the presence of methylated CpG islands. DNA from the colorectal carcinoma cell line SW48 was used as a positive control for methylation (*SW48*), placental DNA was used as a negative control for methylation (*PLAC.*), and water was used as a negative PCR control (*WATER*).

CpG Island Methylation in Tissue and Fecal DNA by Modified COBRA. Because the results from MSP indicated the presence of aberrantly methylated CpG islands for each of the genes in all tissue and fecal samples analyzed, we used a modified COBRA assay to quantify the extent of this methylation. This assay determines the degree of methylation of a single CpG in genomic DNA by measuring the proportion of restrictable DNA in a pool of PCR-amplified fragments from bisulfite-modified DNA.

Methylation of Target Genes in the Study Population. Fecal samples were available for analysis from a total of 50 individuals, 22 of whom were endoscopy patients (10 male, 12 female; average age 59 years) who also provided samples of flat mucosa and 10 were surgical patients (8 male, 2 female; average age 67 years) who provided samples of both mucosa and tumor tissue. Of the 22 endoscopy patients, 4 showed no evidence of neoplasia, 10 had one or more adenomatous polyps at endoscopy, 2 were subsequently diagnosed as having cancer, and 6 had inflammatory conditions.

Using the modified COBRA assay with DNA from both tissue and fecal samples, we measured the degree of methylation at one CpG in the CpG islands of each of six genes *APC* (at −42 from the start of transcription), *MGMT* (at +270), *HPP1* (at −165 from the start of translation), *MLH1* (at −252), and $p16^{INK4a}$ (at −59) and at three CpGs for the CpG island of the *ESR1* gene (CpG 1 at +164, CpG 2 at +223, and CpG 3 at +313). Levels of methylation measured in biopsies from the

Table 3. Frequency of methylation at a single CpG in each of six selected genes in mucosal tissue, tumor tissue, and fecal DNA collected from patients undergoing surgery for colorectal carcinoma

Site	Gene locus							
	ESR1 (132)	*ESR1* (195)	*ESR1* (281)	*MGMT* (48)	*HPP1* (108)	*p16* (100)	*APC* (166)	*MLH1* (123)
Mucosa	38.7 ± 1.1	29.9 ± 0.8	33.8 ± 1.1	1.3 ± 0.3	0.51 ± 0.2	ND	ND	ND
Tumor	40.6 ± 1.0	34.0 ± 1.2*	35.2 ± 1.3	4.0 ± 2.2	8.4 ± 1.2†	0.06 ± 0.1	0.4 ± 0.4	ND
Feces	44.2 ± 1.3*	33.6 ± 0.9*	39.1 ± 1.4*	14.1 ± 2.5†	1.8 ± 1.0	2.1 ± 1.2	1.6 ± 0.5	0.7 ± 0.7

NOTE: Values are means and SEM of percentage values. ND, not determined.
*$P < 0.05$, fecal or tumor samples differed significantly from the normal mucosa.
†$P < 0.01$, fecal or tumor samples differed significantly from the normal mucosa.

flat mucosa, tumor tissue, and fecal samples obtained from the cancer patients are shown in Table 3. Methylation of the *ESR1* CpG island was quantifiable using COBRA in 100% of the mucosal, tumor, and fecal samples available from the cancer patients. For *MGMT*, methylation was quantified in 10 of 12 (83%) of mucosal samples, 8 of 9 (88%) of tumor samples, and 11 of 11 (100%) of available fecal samples. For *HPP1*, the corresponding figures were 5 of 12 (42%) for the mucosal samples, 9 of 9 (100%) for tumor samples, and 4 of 12 (33%) for fecal samples. Both *ESR1* and *MGMT* were significantly more methylated in DNA derived from feces than in the mucosa DNA ($P < 0.05$).

For *p16*, *APC*, and *MLH1*, the level of methylation detected in the normal mucosa by MSP was too low to be quantified by COBRA. Methylation of *MLH1* was not quantifiable in tumor tissue, but one tumor sample from a single patient (1 of 9, 11%) had quantifiable methylation of both *p16* and *APC*. The frequency of quantifiable methylation in fecal DNA by COBRA was 1 of 10 (10%) for *MLH1*, 6 of 11 (55%) for *p16*, and 9 of 11 (82%) for *APC*.

A comparison of CpG island methylation levels in fecal DNA for each of the six genes in normal volunteers ($n = 21$), patients shown to have polyps ($n = 10$), and cancer patients ($n = 12$) is given in Table 4. Across the population as a whole ($n = 50$), statistically significant correlations in the level of methylation were noted between *p16* and *MGMT* ($r = 0.45$; $P = 0.002$), *p16* and *APC* ($r = 0.44$; $P = 0.002$), and *APC* and *MGMT* ($r = 0.39$; $P = 0.009$).

For the 32 patients from whom mucosal tissue was available, there was a statistically significant correlation between age (mean 63 years, range 25–81 years) and level of methylation at CpG-132 ($r = 0.383$; $P = 0.033$) and CpG-281 ($r = 0.369$; $P = 0.041$). To confirm that the level of methylation detected in fecal DNA was a reflection of the methylation status of the same site in the host mucosa, we searched for correlations in the methylation levels of the three sites in *ESR1* using fecal and mucosal DNA samples from all 32 patients, regardless of diagnosis. Significant correlations were obtained for CpG-132 ($r = 0.459$; $P = 0.008$) and CpG-281 ($r = 0.517$; $P = 0.002$), but the relationship was weaker for CpG-195 ($r = 0.330$; $P = 0.069$).

Discussion

It is well established that aberrant promoter methylation is a fundamental mechanism of neoplasia, which causes inactivation of tumor suppressor genes in many types of cancer and which can be exploited as a biomarker for the early detection and characterization of tumors at a variety of sites (4). For example, aberrant *p16* promoter methylation has been detected in the sera of colorectal cancer patients (20) and in patients with esophageal squamous cell carcinoma (21). Aberrantly methylated *MLH1* promoter was detected in the serum of patients with microsatellite unstable colon cancer (22). Methylation of *p16* and *MGMT* in DNA derived from the sputum is a sensitive marker for lung cancer (15), and aberrant *APC* methylation has been detected in both serum and plasma from lung cancer patients (23). In the present study, we have established that it is feasible both to detect and to quantify methylation of CpG islands using DNA derived from colonocytes present in human feces. The methods described here provide, for the first time, a noninvasive approach to the quantification of colorectal CpG island methylation in free-living human populations.

Of the genes chosen for this study, *ESR1* was among the first to be shown to undergo a high degree of aberrant methylation, even in the morphologically normal human colonic mucosa. The phenomenon occurs in healthy subjects (13), increases progressively with age, and seems to be accelerated in patients with inflammatory bowel disease (24). In our study, the level of methylation at CpG-195 within the *ESR1* promoter region was 33.4% (±SD 2.5) in five subjects shown by endoscopy to be free of both neoplasia and inflammatory bowel disease. This

Table 4. Frequency of methylation at CpGs in each of six selected genes measured in fecal DNA from healthy volunteers, patients with current or previously removed polyps, and patients with colorectal carcinoma

Diagnosis	Gene locus							
	ESR1 (132)	*ESR1* (195)	*ESR1* (281)	*MGMT* (48)	*HPP1* (108)	*p16* (100)	*APC* (166)	*MLH1* (123)
Normal ($n = 21$)	42.8 ± 0.9	36.8 ± 0.4	38.5 ± 0.7	20.4 ± 1.8	1.5 ± 0.8	1.1 ± 0.4	1.7 ± 0.2	0.6 ± 0.4
Polyps ($n = 10$)	42.3 ± 0.9	34.4 ± 1.6	38.3 ± 1.1	14.6 ± 2.3	2.8 ± 2.3	1.0 ± 0.5	1.8 ± 0.3	ND
Cancer ($n = 12$)	44.2 ± 1.3	33.6 ± 0.9	39.1 ± 1.4	14.1 ± 2.5	1.8 ± 1.0	2.1 ± 1.2	1.6 ± 0.5	0.7 ± 0.7

NOTE: Values are means and SEM of percentage values. ND, not determined.

is comparable with the levels reported by Issa et al. (13) for this age group. A similar degree of methylation was observed in the macroscopically normal mucosa of 12 cancer patients. Interestingly, the level of methylation was significantly higher in fecal DNA than in the normal mucosa. The reasons for this are unclear at present, but the statistically significant correlations between fecal and mucosal DNA obtained for two sites of methylation within the *ESR1* gene confirm the feasibility of using fecal DNA to obtain quantitative information about the level of methylation in the host mucosa.

The recently identified hyperplastic polyposis protein gene (*HPP1*), which encodes a transmembrane protein predicted to contain follistatin and epidermal growth factor–like domains, contains a CpG island in its promoter region that was shown to be aberrantly hypermethylated in colorectal cancers, including those associated with ulcerative colitis, and in hyperplastic polyps and adenomas (25, 26). We were able to quantify methylation of *HPP1* in 9 of 9 colorectal tumor samples, but levels were detectable by COBRA in only 5 of 12 mucosal samples and 4 of 12 fecal samples from surgical patients. Methylation of the remaining genes *MLH1*, *p16*, and *APC* was detectable by MSP, but not quantifiable by COBRA, in the macroscopically normal mucosa of all patients. The significant positive correlation between the levels of methylation determined in fecal DNA for the *p16*, *APC*, and *MGMT* loci suggests that these genes may become methylated simultaneously, but at different rates, with *MGMT* methylated more frequently than *p16* and *APC*.

The DNA mismatch repair gene *MLH1* is of particular interest in the present context because it contains a CpG island in the promoter region that is aberrantly methylated in a variety of human cancers (14, 27, 28), including colorectal cancer, and inflammatory bowel disease–associated neoplastic lesions (29). Nakagawa et al. (30) concluded that hypermethylation of the *MLH1* promoter is an age-related phenomenon, detectable in the normal mucosa of ~50% of colorectal patients, a high proportion of whom have microsatellite instability. In the present study, methylation of *MLH1* was detectable by MSP in the normal mucosa, tumor tissue, and feces from all cancer patients, but it was generally not quantifiable using the less sensitive COBRA assay. The two-stage MSP assay used by us has been shown to detect 1 methylated allele in 50,000 unmethylated alleles. This is an ~50-fold increase in sensitivity compared with the more widely used one-stage MSP assay, whereas the COBRA assay has a limit of detection in this study of ~1 methylated allele in 200 unmethylated alleles. Using MSP, we were able to detect aberrant methylation of *MLH1* in all samples. Interestingly, the donor of one of the fecal samples who had no previous history of bowel disease, and at the time of fecal collection lacked any abnormal bowel symptoms and was presumed to be healthy, had a level of *MLH1* methylation, which was quantifiable by COBRA (6%), and was subsequently diagnosed as having colorectal carcinoma. Abnormal expression of *MLH1* is firmly implicated in the development of colorectal neoplasia. Although *MLH1* may indeed be subject to age-related methylation in the normal mucosa (30), the use of sensitive techniques to quantify the degree of methylation will enable us to explore its potential as a risk marker for colorectal neoplasia, and this issue deserves further investigation.

The exfoliated epithelial cells from the distal part of the colon and the rectum seem more likely to survive intact in the stool than cells from the proximal colon, but we assume that fecal DNA is derived from a relatively large colorectal field. All three CpGs in the *ESR1* gene and the single CpG studied in *MGMT* were significantly more heavily methylated in the fecal samples than in the mucosal tissues. One possible explanation is that DNA derived from exfoliated cells in the feces is prone to increased CpG island methylation perhaps as a side effect of apoptosis. There seems to be no obvious mechanism for increased methylation as a direct consequence of apoptosis, but there is published evidence to suggest that DNA degradation during apoptosis occurs preferentially at unmethylated C-G sequences (31). Selective cleavage of unmethylated CpGs might spare methylated CpGs in the fecal DNA pool, leading to an apparent enrichment of methylated sites. However, it is also possible that the phenomenon simply reflects the microanatomy of the colorectal mucosa and the mechanisms of epithelial cell replacement and differentiation. Nakagawa et al. (30) used MSP *in situ* hybridization for the *in situ* detection of methylated alleles of *MLH1* and showed that, in the normal colonic epithelium, higher levels of methylation were detected in cells near the mucosal surface compared with cells at the base of the crypts. In that case, the level of methylation in fecal DNA would provide an accurate reflection of that in mature colonocytes, whereas the level in biopsy material would be diluted by the presence of colonocytes at earlier stages of maturation.

The techniques for the detection and quantification of CpG island methylation in human stool described here have two potential applications. The first is the study of the origins and significance of colorectal DNA methylation in human populations. The original proposal by Issa et al. (13) that age-related CpG island methylation is a field defect that may play a crucial role in human colorectal carcinogenesis deserves to be studied in depth, as does the involvement of diet and other environmental factors as potential modulators of DNA methylation. The methods described here provide the means to achieve this.

The second obvious application, namely, the use of this approach as a clinical screening technique to detect tumors in individual patients, is more problematic. None of the genes chosen for investigation in the present study seem to be suitable as unambiguous biomarkers for the presence of cancer, because the level of methylation in the tumor has been proven to be irresolvable against the background methylation derived from the mucosa. Indeed, in the cancer patients, the level of fecal methylation for several CpG sites exceeded that of the tumor. This might simply reflect the relatively small number of subjects used in the present study. For example, Herman et al. (32) reported that, whereas hypermethylation of *hMLH1* is found in the majority of tumors with microsatellite instability, it is much less common in tumors without this abnormality. Random selection of cancer patients would therefore tend to dilute the apparent methylation ''signal'' obtained from fecal analysis. However, by applying the methods to genes for which

methylation of CpG islands is confined only to neoplastic lesions, it should be possible to use CpG island methylation-based biomarkers for tumor detection. The identification of such genes should therefore be given a high priority. To provide even greater precision, or perhaps to provide a means of identifying particular tumor subgroups, fecal methylation analysis could be combined with other noninvasive methods such as detection of fecal DNA mutations (33, 34), fecal immunocytochemical analysis (35) or blood-based loss-of-imprinting assays (36).

In conclusion, we believe that the methods for analysis of CpG island methylation in the colorectal mucosa described here are robust, relatively inexpensive, and readily applicable to many subjects. The use of these techniques therefore has the potential to provide much greater insight into the functional consequences of these epigenetic phenomena in free-living populations.

Acknowledgments

We thank Wendy Bal, Julie Coaker, and Catherine Lamb for technical support and the volunteers for cooperation.

References

1. Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg 2002;89:845–60.
2. Bodmer WF. 1998 Runme Shaw Memorial Lecture: somatic evolution of cancer. Ann Acad Med Singapore 1999;28:323–9.
3. Issa JP. The epigenetics of colorectal cancer. Ann NY Acad Sci 2000;910:140–53; discussion 153–5.
4. Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumors. J Pathol 2002;196:1–7.
5. Feinberg AP. Cancer epigenetics takes center stage. Proc Natl Acad Sci U S A 2001;98:392–4.
6. Jubb AM, Bell SM, Quirke P. Methylation and colorectal cancer. J Pathol 2001;195:111–34.
7. Bestor TH. Gene silencing. Methylation meets acetylation. Nature 1998;393:311–2.
8. Furukawa T, Konishi F, Masubuchi S, Shitoh K, Nagai H, Tsukamoto T. Densely methylated MLH1 promoter correlates with decreased mRNA expression in sporadic colorectal cancers. Genes Chromosomes Cancer 2002;35:1–10.
9. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.
10. Chan AO, Issa JP, Morris JS, Hamilton SR, Rashid A. Concordant CpG island methylation in hyperplastic polyposis. Am J Pathol 2002;160:529–36.
11. Esteller M, Sparks A, Toyota M, et al. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 2000;60:4366–71.
12. Chan AO, Broaddus RR, Houlihan PS, Issa JP, Hamilton SR, Rashid A. CpG island methylation in aberrant crypt foci of the colorectum. Am J Pathol 2002;160:1823–30.
13. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the estrogen receptor CpG island links aging and neoplasia in human colon. Nat Genet 1994;7:536–40.
14. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225–9.
15. Palmisano WA, Divine KK, Saccomanno G, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res 2000;60:5954–8.
16. Ahlquist DA, Klatt KK, Harrington JJ, Cunningham JM, Han J, Shuber AP. Novel use of hypermethylated DNA markers in stool for detection of colorectal cancer: a feasibility study. Gastroenterology 2002;122:A40; 354.
17. Raizis AM, Schmitt F, Jost JP. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. Anal Biochem 1995;226:161–6.
18. 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 U S A 1996;93:9821–6.
19. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25:2532–4.
20. Nakayama H, Hibi K, Taguchi M, et al. Molecular detection of p16 promoter methylation in the serum of colorectal cancer patients. Cancer Lett 2002;188:115–9.
21. Hibi K, Taguchi M, Nakayama H, et al. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res 2001;7:3135–8.
22. Grady WM, Rajput A, Lutterbaugh JD, Markowitz SD. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Cancer Res 2001;61:900–2.
23. Usadel H, Brabender J, Danenberg KD, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. Cancer Res 2002;62:371–5.
24. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573–7.
25. Young J, Biden KG, Simms LA, et al. HPP1: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. Proc Natl Acad Sci U S A 2001;98:265–70.
26. Sato F, Shibata D, Harpaz N, et al. Aberrant methylation of the HPP1 gene in ulcerative colitis-associated colorectal carcinoma. Cancer Res 2002;62:6820–2.
27. Esteller M, Catasus L, Matias-Guiu X, et al. hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 1999;155:1767–72.
28. Ueki T, Toyota M, Sohn T, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000;60:1835–9.
29. Fleisher AS, Esteller M, Harpaz N, et al. Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1. Cancer Res 2000;60:4864–8.
30. Nakagawa H, Nuovo GJ, Zervos EE, et al. Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res 2001;61:6991–5.
31. Chen DL, Swe M, Sit KH. G-band expression and megabase fragmentations in apoptosis. Exp Cell Res 1998;240:293–304.
32. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A 1998;95:6870–5.
33. Traverso G, Shuber A, Olsson L, et al. Detection of proximal colorectal cancers through analysis of fecal DNA. Lancet 2002;359:403–4.
34. Traverso G, Shuber A, Levin B, et al. Detection of APC mutations in fecal DNA from patients with colorectal tumors. N Engl J Med 2002;346:311–20.
35. Davies RJ, Freeman A, Morris LS, et al. Analysis of minichromosome maintenance proteins as a novel method for detection of colorectal cancer in stool. Lancet 2002;359:1917–9.
36. Cui H, Cruz-Correa M, Giardiello FM, et al. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. Science 2003;299:1753–5.

Use of DNA from Human Stools to Detect Aberrant CpG Island Methylation of Genes Implicated in Colorectal Cancer

Nigel J. Belshaw, Giles O. Elliott, Elizabeth A. Williams, et al.

Cancer Epidemiol Biomarkers Prev 2004;13:1495-1501.