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Detection of Hypermethylated DNA or Cyclooxygenase-2 Messenger RNA in Fecal Samples of Patients With Colorectal Cancer or Polyps

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BACKGROUND: Detection of fecal DNA is a promising approach to colorectal cancer screening. However, the sensitivity of current fecal DNA tests for colorectal polyps is low. We evaluated the feasibility of detecting aberrantly methylated DNA or cyclooxygenase-2 (COX-2) mRNA in feces of patients with colorectal cancer or polyps.

METHODS: Fecal samples were collected prior to colonoscopy from 20 patients with colorectal cancer, 30 patients with colorectal polyps, and 30 subjects with normal colonic examination. Presence of hypermethylated DNA in 7 tumor-related genes (*APC*, *ATM*, *hMLH1*, *sFRP2*, *HLTF*, *MGMT*, *and GSTP1*) in stool was analyzed by methylation-specific PCR. COX-2 mRNA in fecal samples was detected by RT-PCR.

RESULTS: With the use of this panel of methylation markers, the sensitivity of detecting colorectal cancer and adenoma was 75% (95% CI 50.9–91.3%) and 68% (95% CI 46.5–85.1%), respectively. Three normal subjects also had methylated DNA detected in stool, which gives a specificity of 90% (95% CI 73.5–97.9%). The mean number of genes methylated in DNA from the stool of patients with colorectal cancer and adenoma was 1.4 and 0.9, respectively. In contrast, COX-2 mRNA was detected in the stool samples of 10 (50%) cancer patients and one (4%) patient with advanced adenoma only. Two (6.7%) stool samples from normal subjects also had COX-2 mRNA detected.

CONCLUSION: Detection of aberrantly methylated DNA in fecal samples is more sensitive than COX-2 mRNA for detection of colorectal cancer and adenoma.

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INTRODUCTION

Colorectal cancer is the second most common cancer in the United States (1) as well as in Hong Kong (2). There are approximately 950,000 new cases being diagnosed in the world each year with 480,000 patients dying from this cancer at the same time (3). Because most colorectal cancers follow the adenoma-carcinoma sequence, early detection and removal of adenoma has been shown to reduce the incidence and mortality of colorectal cancer (4).

Among various screening tests for colorectal neoplasms, fecal occult blood testing (FOBT) is generally considered to be the best noninvasive screening test for colorectal cancer. It is the only screening test that is supported by long-term randomized controlled clinical trials to reduce cancer-related mortality (5). However, the sensitivity of FOBT is quite low (15–35%), which implies that a substantial proportion of col-

dase activity present in dietary components may lead to false-positive results, which mandates the need for special dietary restrictions prior to stool collection. With a better understanding of molecular changes associated with colorectal cancer development, there is intense interest in searching for tumor-derived DNA alterations in stool as a noninvasive molecular screening test for colorectal neoplasms. Studies that focus on the detection of mutated DNA in stool have shown encouraging results for the detection of colorectal cancer (7–11). However, the sensitivity in identifying colorectal adenoma remains suboptimal and the positive rate for patients with advanced adenoma is only 15% (11).

Epigenetic changes mediated by promoter hypermethylation of tumor-related genes are increasingly recognized to play an instrumental role in cancer development (12). With the use of multiple methylation markers, virtually all colorectal cancers harbor these epigenetic changes (13). Unlike some

we have shown that epigenetic changes are also frequently detected in colorectal adenoma and even nonadvanced adenoma (14). Recently, we and others have demonstrated the feasibility of detecting epigenetic changes in DNA from the stool of patients with colorectal cancer (15–18) and polyps (19).

Another novel approach for colorectal cancer screening is the detection of aberrantly expressed RNA in stool. A group of Japanese investigators has demonstrated the potential of detecting cyclooxygenase-2 (COX-2) mRNA, which is frequently overexpressed in colorectal cancer, in the fecal samples of patients with colorectal cancer (20). However, whether COX-2 mRNA is also detected in the stool samples of patients with colorectal polyps remains unknown. In this pilot study, we sought to explore the feasibility of screening for colorectal cancers and polyps in fecal samples using two different molecular approaches: detecting methylated DNA and detecting COX-2 mRNA.

METHODS

Patients and Fecal Samples

Fecal samples were collected from subjects who attended a colonoscopy screening program in Prince of Wales Hospital, Hong Kong (21). They were all asymptomatic subjects aged 50–70 yr. Subjects who had inflammatory bowel diseases, a family history of familial adenomatous polyposis or hereditary nonpolyposis colon cancer, or previous colonic surgery were excluded. All stool samples were collected one day prior to the initiation of bowel preparation for colonoscopy. The preendoscopy stool specimens were stored in patients' household freezers and patients were asked to return the stool samples on the day of endoscopy for long-term storage at −80°C in our laboratory. During colonoscopy, all polypoid lesions were removed or biopsied. The size of the lesion was determined by the opening of a biopsy forcep, and the location of the lesions was determined on withdrawal of the colonoscope. Histology of colonic polyps was according to the World Health Organization Classification (22). Advanced colonic adenoma was defined as size ≥1 cm, the presence of villous histology, or high-grade dysplasia (23). Carcinoma was not counted as advanced colonic adenoma in this study. In cases of patients with more than one lesion in the colon, the most advanced lesion was used in subsequent classification.

As a positive control, stool samples were collected from 20 colorectal cancer patients prior to surgical resection and more than 2 wk from the initial colonoscopy. All participants gave informed consent for obtaining stool specimens for the study. The study protocol was approved by the institutional review board of the Chinese University of Hong Kong.

DNA Isolation and Methylation-Specific PCR

All fecal samples were randomly coded before storage and processing. Staff who worked on these samples were unaware of the clinical diagnosis of the patients. DNA was isolated from each stool sample (250 mg) by the QIAamp DNA Stool amplification of the human β-globulin gene. With our storage protocol, human β-globulin DNA was successfully amplified in all stool samples.

The presence of methylated DNA in stool was detected by methylation-specific PCR (MSP). Briefly, 2 μg of DNA was chemically modified to convert all unmethylated cytosine to uracil by the EZ DNA methylation kit (Zymo Research, Orange, CA). A total of seven tumor-related genes (*APC*, *ATM*, *HLTF*, *MGMT*, *hMLH-1*, *SFRP2*, and *GSTP1)* were examined as descried previously (14–16). This selection was based on previous studies that found that the former six genes were frequently methylated in colorectal cancer (14, 15). The *GSTP1* gene was found to be unmethylated in colorectal cancer and was included as a negative control. CpGenome™ Universal methylated DNA (Chemicon International Inc., Temecula, CA) was used as a positive control, whereas template-free distilled water was included as a negative control for each amplification. All PCR reactions were duplicated to ensure consistency and reproducibility of the results.

RNA Isolation and RT-PCR

COX-2 expression in stool samples was detected by RT-PCR. Messenger RNA was extracted from stool samples by the QIAamp Viral Mini Spin Kit (Qiagen). All RNA samples were purified with DNase and stored at −80°C after extraction. Complementary DNA (cDNA) was synthesized using random primers and MMLV Reverse Transcriptase (Promega, Madison, WI). RNA integrity was checked by human β-actin mRNA expression. The primers used for β-actin detection were: 5′-CTT CAA CAC CCC AGC CAT GTA CG-3′ (forward) and 5′-CAT GAG GTA GTC AGT CAG GTC CCG G-3′ (reverse). β-actin mRNA was detected in 77 (96.3%) of the 80 stool samples in this study. The primer sequences for COX-2 were: 5′-TTC AAA TGA GAT TGT GGA AAA ATT GCT-3′ (forward) and 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′ (reverse). The cycling conditions were as follows: 95°C for 2 min, followed by 45 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The 195-bp β-actin and 305-bp COX-2 PCR products were identified by agarose gel electrophoresis. All samples were reverse-transcribed and amplified in duplicate to ensure reproducibility. Colon cancer tissue with high COX-2 expression was used as a positive control, whereas template-negative water was included in all amplifications as a negative control.

Statistical Analysis

The sensitivity and specificity (with 95% confidence interval) of the stool assay were computed. Categorical data were analyzed by Fisher's exact test and numerical values were compared by the student's *t*-test. A *P* value of less than 0.05 was considered to be statistical significant.

RESULTS

Patients

(mean age 70.5 yr), and 30 age-matched controls with a normal colonic examination. There was no significant difference in age among the three groups. Among the 30 patients with colonic polyps, 25 (83.3%) had adenoma and 5 (16.7%) had hyperplastic polyps. Eight (26.7%) patients had advanced colonic adenoma and 11 (36.7%) patients had more than one polyp in the colon. Sixteen (53.3%) patients had polyp distal to the splenic flexure.

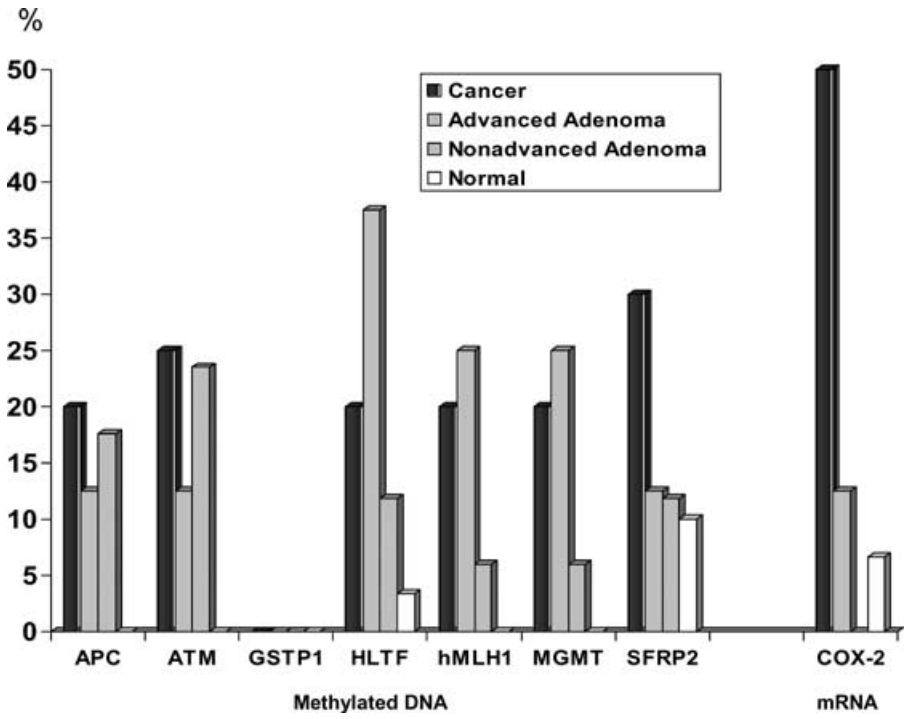
Methylation in Fecal DNA

The β-globulin gene was successfully amplified in all the 80 stool samples. With the exception of *GSTP1*, methylation was frequently detected in the other six genes in patients with colorectal cancer or adenoma Table 1. The frequency of methylation in DNA from the stool of patients (Table 1) with colorectal cancer, advanced adenoma, or nonadvanced adenoma and normal controls is shown in Figure 1. The most frequently methylated gene in the stool of cancer patients and adenoma patients was *sFRP2* (30%) and *HLTF* (20%), respectively. The overall frequency of methylation tended to be higher in the stool samples of cancer patients than of patients with adenoma: *sFRP2* (30% *vs* 12%), *ATM* (25% *vs* 16%), *APC* (20% *vs* 16%), *hMLH1* (20% *vs* 12%), and *MGMT* (20% *vs* 12%). The frequency of *HLTF* methylation was comparable in the stool samples of patients with colorectal cancer and adenoma (20% *vs* 20%). Among patients with colorectal adenoma, methylation was more frequently detected in the stool of patients with advanced adenoma than with nonadvanced adenoma in *HLTF*, *hMLH1*, and *MGMT* (Fig. 1). Notably, methylation was also detected in patients with hyperplastic polyps only. Methylation of *ATM*, *HLTF*, *hMLH1*, and *sFRP2* were each detected in the stool sample of one patient with a hyperplastic polyp.

With the use of six methylation markers (excluding *GSTP1*), 15 (75%) cancer patients, 17 (68%) patients with colonic adenoma, 2 (40%) patients with hyperplastic polyps, and 3 (10%) normal controls had methylation detected in DNA from their stool (Fig. 2). Five (62.5%) patients with advanced adenoma and 12 (70.6%) patients with nonadvanced adenoma had methylated DNA detected in their stool. The corresponding sensitivities for patients with colorectal cancer and adenoma with this panel of methylation markers were 75% (95% CI 50.9–91.3%) and 68% (95% CI 46.5–85.1%), respectively. The overall specificity of this panel of methylation markers was 90% (95% CI 73.5–97.9%).

The mean number of methylated genes in DNA from the stool of patients with colorectal cancer or adenoma and controls was 1.4, 0.9, and 0.1, respectively. Three (15%) cancer patients had methylation detected in $\geq$3 genes and 7 (35%) patients had methylation in 2 genes. Among the 25 patients with colonic adenoma, only 1 (4%) had methylation in $\geq$ 3 genes and 3 (12%) had methylation detected in 2 genes. Two (6.7%) normal controls had methylation in the *SFRP2* gene, whereas one (3.3%) had concurrent methylation in *HLTF* and *SFRP2*.

We further analyzed the characteristics of colorectal adenoma with detection of aberrant methylation in stool samples. The mean numbers of methylated genes in stool samples of patients with advanced adenoma tended to be higher (1.4) than those with nonadvanced adenoma (0.8). There was, however, no association between methylation in DNA from



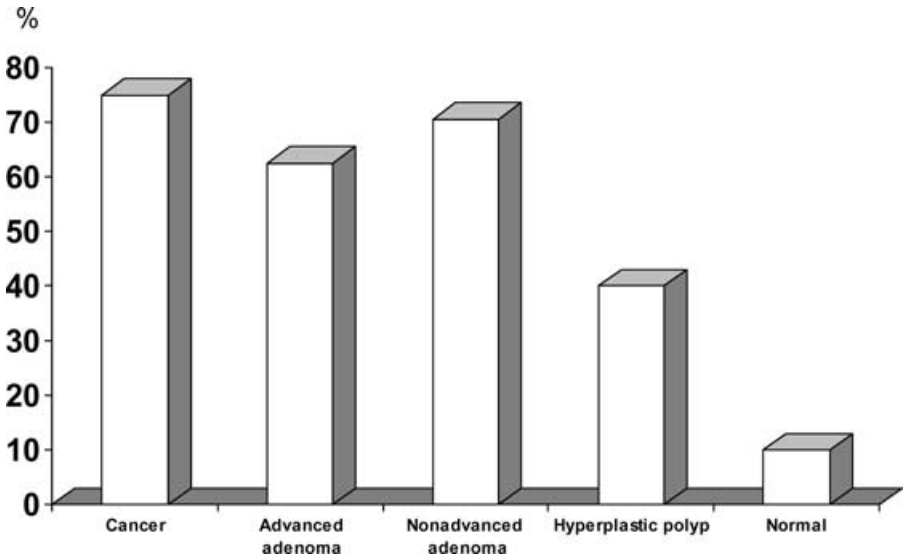


Figure 2. Overall frequency of detecting methylation changes in fecal DNA of patients with different colonic pathology.

stool samples and adenoma location or patient characteristics, including age.

COX-2 mRNA in Feces

We also detected the presence of COX-2 mRNA in these stool samples. β-actin mRNA was successfully amplified in the stool samples of 19 (95%) patients with colorectal cancer, 29 (96.7%) patients with colonic polyps, and 29 (96.7%) control subjects. COX-2 mRNA was detected in 10 (50%) cancer patients, 1 (4%) patient with colonic adenoma, and 2 (6.7%) normal controls. Hence, detection of COX-2 mRNA in stool has a sensitivity of 50% (95% CI 27.2–72.8%) for colorectal cancer and 4% (95% CI 0.1–20.4%) for colorectal adenoma. The specificity for colorectal neoplasm by COX-2 mRNA detection was 93% (95% CI 77.2–99.2%).

To verify the expression levels of COX-2 in tissue samples, we further tested the expression of COX-2 in the primary cancer tissues of the 20 cancer patients and the normal colonic biopsies of the 30 controls. COX-2 mRNA was detected in 14 (70%) cancer samples and 4 (13.3%) normal colonic tissues. Stool samples with COX-2 mRNA expression were all collected from patients with COX-2 mRNA in the corresponding primary tissues.

DISCUSSION

Detection of epigenetic alterations in various body fluids, including serum (24, 25), urine (26), and feces (15–19), is a novel approach for noninvasive cancer detection. We and others have recently demonstrated the feasibility of detecting epigenetic changes or aberrant mRNA expression in stool samples of patients with colorectal cancer (15–19). Herein, we expanded this application to the detection of colorectal polyps through analysis of stool DNA or RNA. Our results showed that methylated DNA was readily detected in the stool vanced and nonadvanced adenoma. With the use of this panel of six methylation markers, it was found that 75% of cancer and 68% of colonic adenoma patients had methylated DNA detected in their stool samples. The use of methylation markers appears to have a higher sensitivity than the commercially available fecal DNA panel in detecting colorectal adenoma (15.1%) and minor polyps (7.6%) (11). On the other hand, detecting COX-2 mRNA in stool may not be sensitive enough for identification of patients with colonic adenoma (4%).

Unlike previous studies that focus on identification of patients with advanced colonic adenoma (18), we found that methylation could be detected in the DNA from stool samples of patients with hyperplastic polyps (Fig. 2). In contrast, a previous study that used a single methylation marker (HIC1) failed to detect any methylation changes in hyperplastic polyps (18). Recently, Petko *et al*. also demonstrated the feasibility of detecting methylated DNA in the fecal DNA of patients with colorectal polyps (19). However, DNA was isolated from colon lavage effluent during colonoscopy instead of from patients' stool samples. In contrast to our study, a considerable proportion of the fecal DNA from normal subjects also had methylation detected. It remains elusive whether the low specificity is related to the method of sample collection or the choice of methylation markers. Further study is necessary to clarify this discrepancy.

Among the six methylation markers, the sFRP2 methylation marker was previously shown to be highly sensitive for colorectal cancer (15, 18). Although the positive rate of this marker in stool samples of cancer patients was highest among other methylation markers, the inclusion of the sFRP2 methylation marker may impose specificity concerns. In this study, three (10%) subjects with normal colonoscopy had sFRP2 methylation detected in their stool. In keeping with our finding, 23% of normal subjects were found to have sFRP2 methylation in their stool in a previous study (15). The

Table 1. DNA Methylation and COX-2 mRNA in Fecal Samples of Patients With Colorectal Cancer and Polyps

No.	Sex	Age	Location	Histology	Size(mm)	*APC*	*ATM*	*HLTF*	*hMLH1*	*MGMT*	*sFRP2*	COX2
1	F	71	Descending	CA		−	−	−	−	−	−	−
2	F	45	Splenic	CA		−	−	−	−	+	+	−
3	M	75	Rectum	CA		−	+	+	−	+	−	+
4	F	58	Sigmoid	CA		+	−	−	−	−	−	+
5	M	81	Splenic	CA		−	+	−	−	−	−	−
6	F	85	Descending	CA		+	−	+	−	−	−	+
7	F	84	Ascending	CA		−	+	+	−	−	−	+
8	M	73	Ascending	CA		−	−	−	−	−	−	NA
9	M	87	Rectum	CA		−	−	−	−	−	+	+
10	F	90	Hepatic	CA		−	−	−	+	+	−	−
11	M	66	Sigmoid	CA		−	−	+	+	−	+	−
12	F	54	Cecum	CA		−	+	−	−	−	−	+
13	F	78	Transverse	CA		−	+	+	+	−	−	+
14	F	53	Ascending	CA		+	−	−	−	−	−	−
15	F	55	Ascending	CA		+	−	−	−	−	+	+
16	F	81	Ascending	CA		−	−	−	+	−	+	−
17	M	71	Ascending	CA		−	−	−	−	−	−	−
18	F	69	Rectum	CA		−	−	−	−	+	+	+
19	F	51	Cecum	CA		−	−	−	−	−	−	−
20	M	48	Sigmoid	CA		−	−	−	−	−	−	+
22	F	86	Descending	TV	10	−	−	−	−	−	−	+
24	M	46	Sigmoid	TV	8	+	−	+	+	−	+	NA
25	M	61	Cecum	TV	10	−	−	+	−	−	−	−
30	M	67	Sigmoid	TV	10	−	−	−	−	−	−	−
34	M	80	Ascending	TV	14	−	+	−	−	+	−	−
42	F	81	Ascending	Ad	20	−	−	−	+	+	−	−
44	M	64	Rectum	Ad	20	−	−	−	−	−	−	−
48	F	79	Rectum	TV	15	−	−	+	−	−	−	−
23	F	69	Sigmoid	Ad	5	−	+	−	−	−	−	−
26	M	76	Ascending	Ad	2	−	−	−	−	−	+	−
27	M	76	Sigmoid	Ad	2	−	+	−	−	−	−	−
28	F	53	Transverse	Ad	3	−	−	−	−	−	−	−
31	F	64	Ascending	Ad	2	−	−	−	−	−	−	−
32	F	91	Ascending	Ad	5	−	−	−	−	−	−	−
33	F	68	Sigmoid	Ad	4	−	−	−	−	−	−	−
36	F	87	Ascending	Ad	3	−	−	−	−	−	−	−
37	M	68	Descending	Ad	3	+	−	−	−	−	−	−
38	F	64	Sigmoid	Ad	5	−	+	−	−	−	−	−
39	F	55	Cecum	Ad	2	+	−	−	−	−	−	−
40	M	65	Ascending	Ad	5	−	+	−	−	−	−	−
43	F	66	Transverse	Ad	4	−	−	+	−	−	−	−
45	F	58	Ascending	Ad	5	+	−	−	−	+	−	−
46	F	66	Sigmoid	Ad	2	−	−	+	−	−	−	−
47	F	77	Transverse	Ad	4	−	−	−	+	−	−	−
49	M	81	Rectum	Ad	3	−	−	−	−	−	+	−
21	M	60	Descending	HP	3	−	+	−	−	−	−	−
29	F	88	Transverse	HP	2	−	−	+	+	−	+	−
35	M	73	Rectum	HP	2	−	−	−	−	−	−	−
41	M	82	Sigmoid	HP	2	−	−	−	−	−	−	−
50	M	66	Rectum	HP	3	−	−	−	−	−	−	−

CA = carcinoma; Ad = adenoma; HP = hyperplastic polyp; TV = villous adenoma; NA = mRNA not detected.

sFRP2 methylation could also be detected in aberrant crypt foci, which is not easily detected by ordinary colonoscopy (27). Long-term follow-up of these subjects with methylated sFRP2 in stool samples is necessary to elucidate the true specificity of this methylation marker.

Although methylation is frequently detected in colorectal cancer, it has been demonstrated that methylation changes could also be detected in normal colonic tissues of cancer pa-

the exact origin of methylated DNA in stool remained uncertain in the absence of follow-up samples collected after surgery or polypectomy. However, because of the rapid turnover of cancer cells, there is a strong reason to believe that methylated DNA in stool is largely derived from neoplastic cells rather than normal colonic cells. In this regard, Klaassen *et al*. have demonstrated the increased concentrations of human DNA in stool samples from colorectal cancer

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