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Detection of Epigenetic Changes in Fecal DNA as a Molecular Screening Test for Colorectal Cancer: A Feasibility Study, Wai K. Leung,1 Ka-Fai To,2 Ellen P.S. Man,1 Michael W.Y. Chan,1 Alfa H.C. Bai,1 Aric J. Hui,1 Francis K.L. Chan,1 Janet F.Y. Lee,3 and Joseph J. Y. Sung1 (Departments of 1 Medicine & Therapeutics, 2 Anatomical and Cellular Pathology, and 3 Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong) * address correspondence to this author at: Department of Medicine & Therapeutics, Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, Hong Kong; fax 852-2637-3852, e-mail wkleung@cuhk.edu.hk)

Colorectal cancer is the fourth most common cancer worldwide (1). There has been intense interest in the search for potential tumor markers that can be used in the screening of colorectal cancer. Because of the continuous shedding of intestinal cells into the lumen, genetic alterations found in tumors can also be detected in stool, which offers a golden opportunity for the noninvasive screening of colorectal cancer. Previous studies have demonstrated the feasibility of detecting altered DNA, including BAT26, APC, K-ras, and p53 mutations, in the feces of colorectal cancer patients (2–5).

Epigenetic gene silencing by promoter hypermethylation is increasingly recognized to play a crucial role in carcinogenesis (6). In colorectal cancer, several tumor-related genes have been found to have promoter hypermethylation in the CpG islands (7–10). These epigenetic changes are detected in the early phase of colorectal cancer development before the development of K-ras mutations (11). We tested the feasibility of detecting promoter hypermethylation of multiple tumor-related genes in fecal DNA of patients with colorectal cancer.

We recruited 20 colorectal cancer patients (mean age, 69 years; range, 45–90 years; 7 males). Patients who had familial adenomatous polyposis or hereditary nonpolyposis colon cancer, inflammatory bowel diseases, or previous colon surgery were excluded. Colon tumor biopsies were obtained during colonoscopy. Stool samples were collected before initiation of bowel preparation. The stool specimens were stored in a household freezer and then transferred for long-term storage at −80°C. Twenty age-matched controls were randomly selected from individuals who participated in a colonoscopy screening program during the same period and had normal colonoscopy results (12). Stool samples were collected before bowel preparation as described for cancer patients, and healthy colon tissues were collected during colonoscopy from these controls. All patients and controls gave informed consent for the collection of tissues and stool specimens for the study. The study protocol was approved by the Institutional Review Board of the Chinese University of Hong Kong.

Samples obtained from cancer patients and controls were randomly coded before processing. DNA was isolated from colonic tissues by use of the QIAamp DNA Mini Kit (Qiagen) and from stool samples (250 mg) by use of the QIAamp DNA Stool Mini Kit (Qiagen). The quality of human DNA recovered from stool was verified by PCR amplification of the human β-globulin gene. We treated 2 μg of DNA with the EZ DNA Methylation Kit (Zymo Research) and resuspended it in Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.5). The presence of methylated DNA in colonic tissues and stool was detected by methylation-specific PCR (MSP; Fig. 1) (13). We examined six tumor-related genes: APC, ATM, HLF, MGMT, hMLH-1, and GSTP1 (14–19). This selection was based on our previous study, which found that the former five genes were frequently methylated in colorectal cancer (11). GSTP1 was previously found to be unmethylated in colorectal cancer and was included as a negative control. CpGenome Universal Methylated DNA (Chemicon International Inc.) was used as the positive control, and template-free distilled water was included as a negative control for amplification. Moreover, tumor DNAs with

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methylated DNA and controls for colorectal cancer, and the bottom panel shows the corresponding MSP results for stool samples. Positive control, in vitro-methylated DNA; negative control, water (H2O).

Table 1. Clinicopathologic characteristics of colorectal cancer and promoter hypermethylation in tumor DNA.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ATM</th>
<th>APC</th>
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<tr>
<td>Methylation</td>
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<tr>
<td>Unmethylated</td>
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<tr>
<td>Methylated</td>
<td>9</td>
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<td>10</td>
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ATM: 25%, APC: 20%, MGMT: 20%, hMLH1: 20%, and hMTF: 25%. Methylation in GSTP1 was again detected in any stool samples. With the combination of all five markers, 14 cancer patients had methylated fecal DNA detected, giving a total of 14 positive cases.

We successfully amplified the beta-globulin gene in stool samples from all 20 cancer patients. In contrast, 3 (15%) non-cancer controls had no detectable human DNA in their stool, suggesting that the amount of human fecal DNA is lower in individuals without cancer (22). The corresponding frequency of detectable methylation in fecal DNA of cancer patients was as follows: ATM (25%), APC (20%), MGMT (20%), hMLH1 (20%), and HLF (25%). Methylation in GSTP1 was again detected in any stool samples. With the combination of all five markers, 14 cancer patients had methylated fecal DNA detected, giving a total of 14 positive cases.

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ing a sensitivity of 70% (95% confidence interval, 46–88%). Methylation was not detected in the stool samples of colonoscopy-negative individuals (100% specificity). To ensure that the negative results for fecal DNA from non-cancer controls was not attributable to a low human DNA concentration, we successfully amplified unmethylated promoter sequences in all stool samples with detectable β-globulin gene. The corresponding positive and negative predictive values were 100% and 73.9%, respectively.

The mean number of methylated genes in the fecal DNA of cancer patients was 1.1 (range, 0–3). Concordant methylation patterns were detected in the tumor and the paired fecal DNA for all patients. There was a significant association between the numbers of methylated markers in stool and in primary tumors (1.1) and distal colon cancer (1.1; difference in the mean number of genes methylated in stool samples from six patients with significant colorectal neoplasia). We examined stool samples from 144 PCR amplifications for a single sample in stool and in primary tumors (1.1). The presence of methylation in fecal DNA was not associated with any clinicopathologic characteristics of the cancer.

To further test the feasibility of detecting methylated DNA in stool as a screening test for colorectal cancer, we examined stool samples from six patients with significant colonic adenoma. The human β-globulin gene was successfully amplified in all samples, and methylated DNA was detected in four (67%) samples.

In this study, we explored the feasibility of detecting methylated fecal DNA as a possible screening test for colorectal cancer. The feasibility of detecting aberrant methylation in the serum and urine of patients with gastrointestinal and bladder cancers has been demonstrated (17–19, 23, 24). To overcome the low sensitivity of single methylation markers, we used a combination of five markers. The sensitivity (70%) was superior to that reported for detection of APC mutation in stool (57%), which involved 144 PCR amplifications for a single sample (4). It is estimated that the percentage of mutant APC genes present in stool could be as low as 0.4% of all APC genes (4). To detect this minute amount of mutated DNA within a larger pool of wild-type DNA, highly sophisticated techniques that are capable of distinguishing 1 mutant molecule in more than 250 wild-type molecules are necessary.

An inherent advantage of MSP is the detection of a positive signal rather than the identification of signal loss and the low detection limit of 0.1% methylated DNA in a background of wild-type DNA (13). For that reason, the requirement for stool processing and storage was also less stringent in our study than in previous reports (2–5). Stool samples were collected by patients, and DNA isolation was carried out with commercially available reagents. We used only 250 mg of stool sample for DNA isolation, whereas up to 4 g of stool sample was used in previous studies focusing on genetic alterations (2–5). Furthermore, unlike detection of microsatellite instability markers in fecal DNA, which is limited to proximal colon cancers (5), methylation was detected in the fecal DNA of patients with both proximal and distal colorectal cancer. Recently, Müller et al. (25) reported the use of assays for methylation changes in fecal DNA. Using a very different panel of markers, they found that three genes (SFRP2, SFRP5, and PGR) were differentially methylated in cancer patients. With the use of a single methylation marker, SFRP2, they were able to detect 10 of 13 patients with colorectal cancer. On the other hand, three apparently healthy individuals were found to have methylated SFRP2 in their stool (77% specificity). The discrepancy in performance of our study and that of Müller et al. (25) is likely attributable to the selection of different methylation markers, but the use of a more sensitive methylation assay, MethyLight, may also account for the higher sensitivity and lower specificity. Moreover, it remains to be determined whether the methylation patterns of cancers differ in diverse ethnic groups.

The results from our preliminary work demonstrate the feasibility of using a panel of epigenetic markers in screening for colorectal cancer. With the selection and refinement of methylation markers, epigenetic testing of fecal DNA may be a simple and promising screening test for colorectal cancer.

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References
Rapid Detection of \textit{ITPA} \textit{94C}>A and IVS2 + 21A>C Gene Mutations by Real-Time Fluorescence PCR and in Vitro Demonstration of Effect of \textit{ITPA} IVS2 + 21A>C Polymorphism on Splicing Efficiency, Tanja Heller, Michael Oellerich, Victor William Armstrong, and Nicolas von Ahsen \textsuperscript{1} (Department of Clinical Chemistry, George-August-University, Robert-Koch-Strasse 40, 37099 Göttingen, Germany; *author for correspondence: fax 49-551-39-8551, e-mail nahsen@gwdg.de)

Inosine triphosphatase (ITPA; EC 3.6.1.19) catalyzes the hydrolysis of ITP to inosine monophosphate, thereby recycling purines that might otherwise be trapped in the form of ITP (1, 2). Two single-nucleotide polymorphisms associated with ITPA deficiency have been identified in the \textit{ITPA} gene. Individuals who are homozygous for a 94C>A (P32T) mutation have a total deficiency of enzyme activity and accumulate ITP intracellularly, whereas 94C>A heterozygotes have decreased ITPA activity that is 22.5% of the control mean value (2). A second mutation, IVS2 + 21A>C, was detected in ITPA-deficient families. This intrinsic mutation has a more subtle effect on ITPA activity, and heterozygotes have activities that are, on average, ~60% of the control mean. It was presumed that the IVS2 + 21A>C mutation alters the relatively conserved adenine of a putative splicing branch site, leading to abnormal mRNA splicing (2).

Although ITPA deficiency is not related to any defined pathology in humans, it was recently demonstrated that polymorphisms in the \textit{ITPA} gene associated with ITPA deficiency have pharmacogenomic implications for patients treated with thiopurines (3). In a retrospective study involving patients with inflammatory bowel disease receiving azathioprine, Marinaki et al. (3) observed that the 94C>A deficient allele was significantly related to the adverse drug reactions (ADRs) flu-like symptoms, rash, and pancreatitis.

The purine analog 6-mercaptopurine and its prodrug azathioprine (AZA) are widely used in the treatment of leukemia and autoimmune disease, and in transplantation. ADRs to these drugs have been related to a genetic deficiency of thiopurine S-methyltransferase (TPMT; EC 2.1.1.67), which is a key enzyme of thiopurine drug catabolism (4). TPMT deficiency leads to life-threatening myelosuppression by accumulation of active thiopurine metabolites (5). Most ADRs to thiopurines, however, cannot be explained by TPMT deficiency. Thiopurines are more frequently discontinued because of non-dose-dependent ADRs (fever, pancreatitis, nausea) than because of dose-dependent side effects (recurrent infections, thrombocytopenia, leukopenia) (6).

In the light of the findings of Marinaki et al. (3), reliable methods are required for screening for the functional polymorphisms in the \textit{ITPA} gene. We present a procedure for genotyping the \textit{ITPA} 94C>A and IVS2 + 21A>C point mutations by rapid cycle real-time PCR on the LightCycler\textsuperscript{TM} (Roche Molecular Biochemicals). We also investigated the molecular basis for the reduced ITPA activity observed with the IVS2 + 21A>C genotype, using a dual-reporter vector system to characterize the splicing efficiencies of the different genotypes.

Primers for amplification of the region of interest in the \textit{ITPA} gene were located in intron 1 (forward primer; 5'-CTT TAG GAG ATG GGC AGC AG-3') and intron 2 (reverse primer; 5'-CAC AGA AAC TCA GGT CAG AGG-3'). Accumulation of specific PCR product was monitored by use of adjacent hybridization probes designed to bind on one amplicon strand. The 3' end of one probe was labeled with fluorescein (FLU), whereas the 5' end of the adjacent anchor probe was labeled with either Cy5.5 (94C>A) or Bodipy630/650 (IVS2 + 21A>C). Anchor probes were 3'-phosphorylated to prevent probe elongation by the \textit{Taq} polymerase. All oligonucleotides were synthesized by MWG Biotech. Fluorescence resonance energy transfer occurs when both probes hybridize in close proximity and is detected by the LightCycler. Increasing the temperature during fluorescence reading yields a temperature/fluorescence curve from which the melting point of the probe can be derived. When the appropriate conditions are selected, the mismatch under the detection probe caused by a single-nucleotide polymorphism leads to a substantial decrease in the melting point. Probe and anchor pairs were designed with use of the MeltCalc software (\text{http://www.meltcalc.com}), to

\textbf{References:}