Detection of Mutations Associated With Colorectal Cancer in DNA From Whole-Gut Lavage Fluid

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The overall 5-year survival rate for colorectal cancer is 30%–40% despite surgical intervention (1). Early diagnosis is associated with better survival (2), and the benefits of developing tests that improve early diagnosis are clear. The colorectal cancer mortality reduction from fecal occult blood testing in the general population has been well demonstrated (3); however, the poor specificity of such testing suggests the need for better strategies (4). While colonoscopy remains the most sensitive method of detecting colorectal cancer, it is costly and has associated morbidity (4). The detection of tumor-specific genetic mutations holds promise as a tool for early diagnosis and screening for colorectal cancer. Ki-ras (i.e., Kirsten-RAS) gene mutations associated with colorectal cancer have been detected in the stool of Ki-ras mutation-positive case patients (5–11), as have p53 (also known as TP53) gene mutations (12,13). The potential for the detection of premalignant lesions by use of genetic tests on stool specimens has also been recognized (10). Ki-ras mutations occur in 40%–50% of cases of colorectal cancer (14); p53 mutations occur in 50%–75% of cases (15,16). Therefore, examination of a range of mutation sites is required to achieve an acceptable level of sensitivity for genetically based diagnostic tests (17). To our knowledge, no systematic study of an array of genes in stool or colonic washings has been reported. In several studies (5,9,11,12), persistent fecal contamination of the DNA template frequently inhibited the polymerase chain reaction (PCR), which was being used for genetic analysis, so that many samples were unsuitable for examination. Colonic washings may be more suitable for PCR analysis (5,9,11). Whole-gut lavage fluid (WGLF), the clear effluent obtained via the rectum following bowel preparation (for colonoscopy), with solutions containing polyethylene glycol (PEG) and balanced electrolytes, is suitable for protein analysis in inflammatory bowel disease (18), and well-preserved inflammatory cells can be isolated (19). Unlike inflammatory cells, colonic epithelial cells are seldom found in WGLF (19), but neoplastic cells from colorectal cancer have been identified in such samples (20–22). Since WGLF might permit noninvasive assessment of material from the entire colonic lumen, we evaluated its suitability for coordinate screening of cancer-associated mutations in the Ki-ras (codon 12) gene, the p53 (codon 248) gene, the transforming growth factor-β receptor II (TGF-β RII) gene, and the mutation cluster region of the adenomatous polyposis coli (APC) gene.

WGLF (100 mL) was collected from 40 patients with confirmed colorectal cancer and from 14 control subjects. Written informed consent was obtained from the study subjects, and the study received ethical approval. DNA extracted from the WGLF was compared with corresponding tumor DNA and normal DNA, which were extracted from the same paraffin sections of colectomy specimens obtained from the patients (23). The presence of nonbacterial DNA in the WGLF samples was confirmed by analyzing mitochondrial DNA fragments that encoded cytochrome b and cytochrome oxidase—present in high-copy numbers in human and other eukaryotic cells (9).

WGLF mutations at codon 12 of the Ki-ras gene were detected using an enriched PCR strategy of proven high sensitivity that is capable of detecting one mutant allele in 10^5 wild-type alleles (24). Detected mutations were confirmed by direct DNA sequencing. Screening for mutations at the codon 248 hot spot of p53 was performed by use of an enriched PCR method (24,25) (Fig. 1, A). Single-strand conformational polymorphism (SSCP) analysis was used to assess the mutation cluster region of APC (26) (Fig. 1, B) and exon 3 of TGF-β RII (27). For each mutant locus, WGLF and DNA recovered from paraffin sections of the resected tumor were examined by similar methods, except for Ki-ras codon 12, where tumor mutations were detected by direct sequencing of PCR products. WGLF samples were analyzed for those samples in which mutations were detected in the tumors.

Tumor analysis detected 26 different mutations in 18 (45%) of the cancer patients (Table 1). Mitochondrial DNA analysis confirmed the presence of nonbacterial DNA in 37 (93%) of the 40 WGLF samples from these patients. The mean total amount of DNA per 100 mL WGLF sample was 2.35 μg (standard deviation, 3.75 μg). The single-copy Ki-ras gene was amplifiable in 19 (51%) of the 37 eukaryotic DNA-containing WGLF samples. The mean total DNA for these 19 samples was 3.42 μg (standard deviation, 4.92 μg), not significantly different from the mean total DNA concentration for the whole population of samples (standard error of the difference of the means, 1.63; P = .12).

In the WGLF samples from patients with tumors that had mutations in the examined genes, two of seven Ki-ras mutations, two of two p53 mutations, none of 15 APC mutations, and none of two TGF-β RII mutations were identified (Table 1) (Fig. 1). The Ki-ras gene was amplifiable in eight (57%) of the 14 WGLF samples from individuals with normal colonoscopies (i.e., the control subjects), and no mutations were detected.

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See "Notes" following "References."
Several methods of DNA extraction from samples of whole-gut lavage fluid (WGLF) were evaluated, and the best method (determined by the ability to amplify mitochondrial cytochrome b DNA sequences by the polymerase chain reaction [PCR]) was adapted from Smith-Ravin et al. (9). Samples were treated with 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 0.5% sodium dodecyl sulfate (final concentrations) at room temperature for 30 minutes. Samples were clarified in a centrifuge (room temperature; 5 minutes; 1000g), and the supernatants were extracted with equal volumes of TE (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA)-saturated phenol. DNA was precipitated from the aqueous phases with equal volumes of ice-cold isopropanol and then pelleted and dried. The dried DNA was resuspended in aliquots of 500 μL–1 mL TE before proceeding as described (9). DNA concentrations were measured by use of standard techniques, and total DNA yields were calculated. A) Enriched PCR analysis of p53 codon-248 mutations. DNA samples (50-ng) from WGLF were amplified in initial 100-μL reactions containing each primer (primers: forward, 5'-TAG GTT GGC TCT GAC TGT-3'; reverse, 5'-TGG CAA GTG GCT CCT GAC-3') and 1.5 mM Mg²⁺ for 15 cycles, using an annealing temperature of 58 °C. Ten-microliter aliquots of the first PCR reactions were each digested with 2 U of the restriction endonuclease Msp I in 20-μL volumes at 37 °C for 1 hour. A 1-μL aliquot of each digest was then used as the template for a second PCR reaction, using 150 ng of the same primers in a 50-μL reaction for 30 cycles. A second Msp I digest was performed, using 10 μL of the second PCR product. To be certain of complete restriction enzyme digestion, positive, negative, and blank controls were used at each stage of the assay. The final digestion products were then resolved in a 15% nondenaturing polyacrylamide gel and visualized by ethidium bromide staining. Mutant alleles remained undigested and appeared as single 143-base-pair (bp) bands, whereas wild-type alleles were cleaved into 85- and 58-bp products. All detected mutations were confirmed by direct DNA sequencing of gel-purified products using a 5' -nested primer (5'-TAG GTT GGC TCT GAC TGT-3'). Lanes: (M) Marker DNA (1-kilobase ladder; Life Technologies Inc., [GIBCO BRL], Gaithersburg, MD); (A) uncut PCR product, (B) Msp I digested positive control, (C) mutant-enriched positive control, (D) Msp I digested tumor DNA from paraffin sections, and (G and I) mutant-enriched PCR of corresponding WGLF samples showing mutations. B) Single-strand conformational polymorphism (SSCP) analysis of the mutation cluster region of the adenomatous polyposis coli gene. Normal and tumor DNA were amplified by means of PCR (26), and the resulting products were end-labeled, using γ-³²P-adenosine triphosphate (ICN Pharmaceuticals, Inc., Costa Mesa, CA) and T4 polynucleotide kinase, and resolved in 50% MDE gels (FMC Corp., Rockland, ME) that contained 10% glycerol. Electrophoresis was performed overnight at room temperature at 10 W constant power. Gels were then dried and subjected to autoradiography with intensifying screens. Mutations were identified by band shifts in the products of the tumor DNA specimens. When a mutation was detected, 5 μL of the corresponding WGLF DNA sample (i.e., 5–200 ng DNA) was amplified and compared by SSCP with the amplification products of normal and tumor DNA samples. With control DNA, we were able to detect only one mutant allele in 100 wild-type alleles for the APC gene and one allele in 50 alleles for the transforming growth factor-β receptor II gene (data not shown). Tumor (T) DNAs from paraffin sections show mutant band shifts. Neither of the corresponding WGLF samples (W) show a mutant band shift. N = corresponding normal tissue DNA.

According to the analysis of the four mutation sites described above, 18 (45%) of the 40 patients with colorectal cancer had the potential for a genetic diagnosis of their disease. This proportion is lower than expected from a review of the literature, even allowing for the inefficiencies of SSCP, and is explained by the fact that only one point-mutation site each in Ki-ras and p53 was analyzed, in addition to only the mutation cluster region of APC. A genetic diagnosis by WGLF examination was possible in only four (10%) of the 40 patients. Although high-copy-number DNA suitable for amplification was identified in 93% of the WGLF samples, single-copy genes could be amplified by PCR in only half of them. There was no significant difference in the amount of DNA in the samples that amplified and those that did not. This result suggests that PCR inhibition is not a significant problem in WGLF samples when amplifying genes of high-copy number, but it may be problematic when studying single-copy genes, as experienced in other studies (5–13). Human DNA is estimated to account for 10% of the total DNA extracted from stool specimens (6). It is probable that extreme dilution of single-copy genes in PEG-containing solutions accounts for the substantial proportion of samples in which PCR amplification was not achieved.

When PCR was successful, mutations in WGLF samples were only detected by selective enrichment (Table 1). All previous studies (5–12) examining Ki-ras mutations in stool have used an enrichment strategy. The identification of a Ki-ras mutation in one of our WGLF samples but not in the tumor (data not shown) may represent the detection of neoplastic cells not present in the paraffin section. A similar result has been obtained in another study (7) and underscores the potential of premalignant lesion detection by genetic tests. Yet another study (13) had limited success using SSCP to identify p53 mutations in the stool of patients with confirmed colorectal cancer; however, all of the patients had advanced cancers. Fecal occult blood testing on the same patients was four times more accurate (13). We found SSCP to be insensitive when examining WGLF samples. Since colonic epithelial cells are an infrequent finding in WGLF (19), it is presumed the number of cancer cells in our samples was below the limit of sensitivity for SSCP.

In conclusion, these findings show that WGLF is suitable for PCR analysis, but its usefulness as a screening resource is still limited because, in many samples, further examination of single-copy genes is not possible. For successful identification of mutations commonly associated with colorectal cancer in the stool (or similar specimens) of patients, methods that selectively enrich for the mutations must be used. At pre-
sent, detection is limited to point mutations at known, fixed sites (e.g., K-ras codons 12 and 13 and p53 codons 175 and 248). By use of increasingly sensitive techniques—such as radiolabeled-probe hybridization, fluorescence PCR (28), or generation of smaller PCR products—and by improving sample collection to reduce PCR inhibition, the detection of point mutations could approach 100%. However, successful genetic analysis at these four point mutation sites would still have the potential to detect only 60% of colorectal cancers (14–16), which is not better than fecal occult blood testing. Thus, the likelihood of the success of this method in early colorectal cancer diagnosis is constrained. Given current technology and knowledge of the mutations associated with colorectal cancer, it seems improbable that genetic techniques offer a significant advantage over existing screening methods. For such tests to be usefully employed in screening, either a new point mutation that occurs with high frequency in colorectal cancer must be identified, or other methods that enrich for unknown mutations must be developed.

### References


### Table 1. Mutations detected in tumor DNA and whole-gut lavage fluid (WGLF) of 40 patients with colorectal cancer**†

<table>
<thead>
<tr>
<th>Mutation site</th>
<th>No. of mutations according to sample type</th>
<th>Detection method</th>
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<tbody>
<tr>
<td>Ki-ras codon 12</td>
<td>Tumor 7 WGLF 2</td>
<td>Enriched PCR‡</td>
</tr>
<tr>
<td>p53 codon 248</td>
<td>Tumor 2 WGLF 2</td>
<td>Enriched PCR‡</td>
</tr>
<tr>
<td>APC MCR</td>
<td>Tumor 15 WGLF 0</td>
<td>SSCP§</td>
</tr>
<tr>
<td>TGF-β RII exon 3</td>
<td>Tumor 2 WGLF 0</td>
<td>SSCP§</td>
</tr>
<tr>
<td>Total</td>
<td>Tumor 26 WGLF 4</td>
<td></td>
</tr>
</tbody>
</table>

*Ki-ras = Kirsten-RAS gene; PCR = polymerase chain reaction; APC MCR = mutation cluster region of the adenomatous polyposis coli gene; SSCP = single-strand conformational polymorphism analysis; and TGF-β RII = transforming growth factor-β receptor II gene.

†Median age of the study population was 71 years (range, 39–90 years), with a male:female ratio of 24:16. There were seven Dukes’ A carcinomas, 14 Dukes’ B carcinomas, 17 Dukes’ C carcinomas, and 2 metastatic lesions. [See (29) for information on Dukes’ staging.] There were seven right-sided tumors, 16 left-sided tumors, and 17 rectal tumors. For Ki-ras and p53, failure to detect mutations was because the WGLF sample did not PCR amplify. In the samples that did amplify, identical mutations were found in both tumor and WGLF samples by means of enriched PCR; mutations were confirmed by direct DNA sequencing. In one WGLF sample, a Ki-ras mutation was identified that was not detected in the primary tumor, even when re-examined. For the APC and TGF-β RII genes, it was not possible to identify any mutations by means of SSCP, even when PCR amplification was successful.

‡See (24,25) for details concerning enriched PCR methods.

§See (26,27) for details concerning SSCP methods.


Notes

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