Two polymorphisms in the promoter region (~3279T>G and TA repeat) and four additional SNPs that lead to reduced activity [211G>A (G71R), 247T>C (F83L), 686C>A (P229Q), and 1456T>G (Y486D)] were analyzed. All samples were successfully genotyped at all polymorphic sites. The genotyping results were identical to those obtained by direct sequencing. Fig. 1 shows representative sequencing patterns (pyromgrams) for the UGT1A1 TA repeat (TA)_9→(TA)_7 (Fig. 1A) and 211G>A (G71R; Fig. 1B). DNA samples derived from established cell lines or in vitro-mutated cDNA plasmids were also used as templates for the genotypes that were not detected, such as the homozygous (TA)_7 repeat, heterozygous and homozygous 24T>C (F83L), homozygous 686C>A (P229Q), and heterozygous and homozygous 1456T>G (Y486D). Correct genotyping results were successfully obtained from these control samples (data for the TA repeat are shown). This genotyping method was also applicable to the other TA repeat variants, (TA)_8 and (TA)_6, which have been found in African Americans, with the same primers and only a slight modification to the sequencing program (data not shown).

The allelic frequencies of these polymorphisms were 0.281 for ~3279T>G (4 homozygous G/G and 19 heterozygous T/G patients), 0.135 for (TA)_9→(TA)_7 (13 heterozygous 6/7 repeat patients) and 211G>A (G71R; 2 homozygous A/A and 9 heterozygous G/A patients), and 0.010 for 686C>A (P229Q; 1 heterozygous C/A patient), respectively. These frequencies were similar to those reported previously (4, 6–8). Two low-frequency SNPs, 247T>C (F83L) and 1456T>G (Y486D) (9, 11), were not detected in this study. We also did not find any Japanese patients with the (TA)_6 or (TA)_8 repeat.

In summary, we developed a pyrosequencing-based genotyping method for six functionally significant polymorphisms that are especially important in the Japanese. The pyrosequencing data were identical to those obtained from direct sequencing. Pyrosequencing thus can expedite studies on the association between genetic polymorphisms and pharmacokinetic or clinical data.

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References


Quantification of Human DNA in Feces as a Diagnostic Test for the Presence of Colorectal Cancer. Corné H.W. Klaassen,1* Marcel A.F. Jeunink,1 Clemens F.M. Prinsen,1 Theo J.M. Ruers,1 Adriaan C.I.T.L. Tan,2 Luc J.A. Strobbe,3 and Frederik B.J.M. Thunnissen1 (Departments of 1 Pathology, 2 Internal Medicine, and 3 Surgery, Canisius Wilhelmina Hospital, NL 6532 SZ Nijmegen, The Netherlands; 4 De- partment of Surgery, University Medical Center St. Radboud, NL 6500 HB Nijmegen, The Netherlands; address correspondence to this author at: Department of Pathology C66, Canisius Wilhelmina Hospital, Weg door Jonkerbos 100, NL 6532 SZ Nijmegen, The Netherlands; fax 31-24-365-8844, e-mail c.klaassen@cwz.nl)

Analysis of nuclear DNA extracted from stool specimens (1) is a recent addition to cancer diagnostics (2–4). Most studies have focused on the detection of sequence variations in tumor suppressor genes and oncogenes and on their correlation with clinical stage. In addition, however, the amount of human DNA in feces may be increased in individuals with colorectal cancer. Villa et al. (5) found that β-globin sequences were amplified by PCR more frequently in patients with either colorectal carcinoma or adenomas than in healthy individuals. Ahlquist et al. (6) demonstrated that large DNA fragments were amplified from DNA in stool samples from colorectal cancer patients more frequently than from healthy volunteers. In view of these results, we developed a real-time PCR assay for quantification of human DNA in stool samples.

Human stool samples were collected from 15 healthy adult volunteers (mean age, 46 years; range, 21–78 years)
measurements of individual samples was 20%. Real-time PCR was performed (1). Under these circumstances, solid stool samples can be stored at 4 °C for several days without significant degradation of the DNA. All stool samples from both groups were processed within 48 h after collection.

DNA was isolated from 200-gram fresh solid human stool samples with use of the QIAamp DNA Stool Mini Kit essentially according to the manufacturer’s recommendations for human DNA extraction. The purified DNA was eluted in 200 μL of elution buffer. The total yield of DNA was determined by ultraviolet absorbance at 260 nm on a DU 7600 spectrophotometer (Beckman); the A260/A280 ratio was ~1.8.

We targeted a 71-bp fragment within exon 3 of the human β-globin gene and used a combination of PCR primers and TaqMan probe to amplify and detect only human DNA sequences. We selected human DNA sequences that included several mismatches with respect to known β-globin gene sequences of edible species in the 3'-end region of both PCR primers and in the TaqMan probe. Primer and probe sequences were as follows: forward primer, 5'-GGGCAACGTCCTGTCTG-3'; reverse primer, 5'-AGGACACCTGCACCTGT-3'; TaqMan probe, 5'-FAM-CTGCCCATCATCTTTGGCCAAAGAA-TAMRA-3' (where FAM is 6-carboxyfluorescein, and TAMRA is 6-carboxytetramethylrhodamine). The specificity of the primers and probe was verified by BLAST analysis (7).

For PCR, a LightCycler (Roche) reaction mixture consisted of 1× FastStart Master Mix for Hybridization Probes (Roche), 250 nM each PCR primer, 100 nM TaqMan probe, 3 mM MgCl2 (total concentration), and up to 500 ng of total DNA in a reaction volume of 20 μL. PCR conditions were as follows: 10 min at 95 °C followed by 50 cycles of 0 s at 95 °C and 15 s at 60 °C, with maximum heating and cooling settings (20 °C/s). In each cycle, the fluorescent signal from the liberated FAM reporter group was measured in channel 1 at the end of the primer extension phase. The amounts of human DNA in the samples were extrapolated, using the "second derivative maximum" method, from a calibration curve constructed with fixed amounts of human placenta DNA. All quantification experiments were performed in triplicate. The technician performing the quantification experiments had no knowledge of the origin of the samples.

Table 1. Human DNA in stool specimens from colorectal cancer patients.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location of tumor</th>
<th>Patient gender</th>
<th>Patient age, years</th>
<th>Tumor size, cm</th>
<th>Cancer stage</th>
<th>DNA, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sigmoid</td>
<td>M</td>
<td>75</td>
<td>T3,N0</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>74</td>
<td>T4,N1,M1</td>
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<tr>
<td>3</td>
<td>Ascendens</td>
<td>F</td>
<td>77</td>
<td>T4,N1,M1</td>
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</tr>
<tr>
<td>4</td>
<td>Sigmoid</td>
<td>F</td>
<td>75</td>
<td>pT3,N0</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ascendens</td>
<td>F</td>
<td>81</td>
<td>T4,N1</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>Ascendens</td>
<td>F</td>
<td>72</td>
<td>pT3,N2,M1</td>
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<tr>
<td>7</td>
<td>Rectum</td>
<td>F</td>
<td>75</td>
<td>T3,N2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Ascendens</td>
<td>F</td>
<td>72</td>
<td>T3,N+</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Rectum</td>
<td>F</td>
<td>75</td>
<td>&lt;1 4γT1,N0</td>
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<td></td>
</tr>
<tr>
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<td>M</td>
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<td>T4,N2</td>
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<tr>
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<td>T3,N2</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>Rectum</td>
<td>F</td>
<td>56</td>
<td>T3,N1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Sigmoid</td>
<td>F</td>
<td>49</td>
<td>T2,N0</td>
<td>865</td>
<td></td>
</tr>
</tbody>
</table>

a DNA is expressed as mg of human DNA/g of total DNA in the samples.

b NR, not reported.

c After radiotherapy.
other hydrolyzing enzymes. Because the relative state of hydration decreases toward the more distal parts of the colon, a more favorable environment will be created for preservation of the DNA.

In this DNA, there may have been a confounding age difference between the patient group and controls. The mean age of the patient group was higher than that of the control group. However, within the patient group we demonstrated a difference between left-sided and right-sided colorectal tumors with no age difference being present. We therefore believe that this does not play a critical role in this study.

Usually, the amount of DNA in feces is calculated in absolute values (in mg of DNA/g dry weight). To compensate for differences in hydration states, the dry weight must be determined, for example, by freeze-drying. Although ideal for preservation of the DNA, freeze-drying is time-consuming and laborious and not available to every laboratory. Another complicating factor has been the need for quantitative extraction of human DNA from any amount of stool sample. We found, however, that all DNA (regardless whether it is of host, microbial, or food origin) that is liberated on lysis of the samples is further purified with equal efficiency (unpublished observations). We therefore quantified the amount of human DNA relative to the amount of total DNA. Thus, the amount of feces analyzed would be irrelevant, simplifying the entire procedure. Although stool samples are heterogeneous in composition, sampling errors introduced by analyzing small fractions may be circumvented by analyzing multiple fractions from each stool sample.

In conclusion, we describe a quantitative assay to determine the relative amounts of human DNA in feces. This approach allowed the detection of increased concentrations of human DNA in stool samples from patients with colorectal tumors, making this noninvasive assay a simple and potentially interesting approach to colorectal cancer screening.

Iodinated Contrast Media Interfere with Gel Barrier Formation in Plasma and Serum Separator Tubes, Tom Spiritus, Zahur Zaman, and Walter Desmet (1 Department of Laboratory Medicine–Clinical Chemistry and 2 Department of Cardiology, University Hospitals Leuven, Herestraat 49, B-3000 Leuven, Belgium; * author for correspondence: fax 32-16-34-79-31, e-mail Zahur.Zaman@uz.kuleuven.ac.be)

Vacutainer® serum separator tubes (SST® II) and plasma separator tubes (PST® II) manufactured by Becton Dickinson are widely used for blood collection. These tubes contain an inert, thixotropic polymer gel with a specific gravity of ~1.04 (1). Aspiration of blood into the tube and subsequent centrifugation displace the gel, and it forms a disk between the cells and the supernatant.

We recently encountered blockages of sample needles on our clinical chemistry Modular automated analyzer (Roche Diagnostics) with samples collected in PST II tubes. The tubes were placed directly on the analyzer after centrifugation for primary-tube sampling. The problematic samples had been collected from patients shortly after radiologic examinations requiring administration of iodinated radio-opaque contrast medium (Visipaque® or Omnipaque®). Instead of forming a separation layer between the sediment and the plasma after centrifugation, the gels had floated to the top and thus hindered analytical processing.

We investigated the influence of iodinated contrast media on gel barrier formation in PST II and SST II tubes. The most commonly used contrast media in our hospital are two nonionic contrast media, Omnipaque (iohexol; 350 g/L iodine; Nycomed) and Visipaque (iodixanol; 320 g/L iodine; Nycomed), and to a much lesser extent, two ionic contrast media, Telebrix® (sodium meglumine ioxithalamate; 350 g/L iodine; Guerbet) and Urografin® (sodium meglumine diatrizoate; 146 g/L iodine; Schering).

In one experiment we investigated the interference in whole blood samples. On three separate occasions, a total of 43 blood samples were collected from a healthy male volunteer (age, 26 years) into PST II (cat. no. 367376) and SST II (cat. no. 367955) tubes. Immediately after blood collection, we added different amounts of Omnipaque (0–44 mL/L; equivalent to 0–16 g/L iodine, final whole blood concentration), Visipaque (0–33 mL/L; equivalent to 0–11 g/L iodine), Telebrix (0–44 mL/L; equivalent to 0–16 g/L iodine), and Urografin (0–91 mL/L; equivalent to 0–13 g/L iodine). The tubes were mixed, and the serum specimens were allowed to clot for 30 min at room temperature before centrifugation. After centrifugation at 1700g for 10 min, the tubes were inspected for the position of the separating gel. Density was measured by weighing 1 mL of plasma or serum, and osmolality was measured with an Advanced Osmometer 3D3 (Advanced Instruments). The concentrations at which the gel failed to form a barrier under the plasma after centrifugation are shown in Table 1A.