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DNA Integrity as a Potential Marker for Stool-based Detection of Colorectal Cancer

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Background: **Molecular genetic analysis of DNA in patient stools has been proposed for screening of colorectal cancer (CRC). Because nonapoptotic cells shed from tumors may contain DNA that is less degraded than DNA fragments from healthy colonic mucosa, our aim was to show that DNA fragments isolated from stools of patients with CRC had higher integrity than DNA isolated from stools of patients with healthy colonic mucosa.**

Methods: **We purified DNA from the stools of a colonoscopy-negative control group and patients with CRC and examined the relationship between long DNA fragments and clinical status by determining stool DNA integrity, using oligonucleotide-based hybrid captures with specific target sequences in increasingly long PCR reactions (200 bp, 400 bp, 800 bp, 1.3 kb, 1.8 kb, 24 kb). DNA fragments obtained from CRC patients were compared with fragments obtained from colonoscopy-negative individuals for length and/or integrity.**

Results: **DNA fragments isolated from CRC patients were of higher molecular weight (>18 bands detected of a total of 24 possible bands) than fragments isolated from fecal DNA of the colonoscopy-negative control group.**

Conclusions: **The presence of long DNA fragments in stool is associated with CRC and may be related to disease-associated differences in the regulation of proliferation and apoptosis. An assay of fecal DNA integrity may be a useful biomarker for the detection of CRC.**
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Colorectal cancer (CRC)[4] accounts for 10% of all cancer deaths and is second only to lung cancer as a leading cause of cancer-related mortality in industrialized nations *(1)*. An extensive body of work has been published demonstrating CRC progression along a series of predictable neoplastic pathways *(2)*. This sequential progression allows early diagnosis of CRC at a time when treatment has a high probability of being curative and may facilitate preventive intervention *(3, 4)*.

Stool testing methods are noninvasive, do not require cathartic preparation of the colon, and can be performed on stools at a centralized testing facility. However, the promise of screening tools such as the fecal occult blood test has not been fully realized because of major performance limitations and low compliance rates. Although the fecal occult blood test has been available for some years and may have lowered CRC mortality to some degree *(5)*, early-stage CRC is only poorly diagnosed by this method *(6, 7)*.

Molecular genetic screening assays that test stool DNA examine the genetic composition of the colonic mucosa, which is exfoliated into the colon *(8)*. Many molecular markers associated with colorectal carcinogenesis have been well characterized *(9)* and have been shown to be represented in the DNA isolated from the stools of cancer patients *(10–12)*. Previous work demonstrated that the use of multiple markers to screen patients for CRC is useful in detecting the heterogeneity of genetic lesions characteristic of CRC *(8)*. A multitarget assay was developed to evaluate DNA isolated from human stools for 15 mutational hot spots in the K-*ras*, *APC*, and *p53* genes, as well as for microsatellite instability in the *BAT-26* locus *(10)*. During the course of this study, it was observed that stool DNA from a colonoscopy-negative control group and CRC patients exhibited various efficiencies in PCR amplification, with DNA prepared from stools of CRC

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[4] Nonstandard abbreviations: CRC, colorectal cancer; GE, genome-equivalent(s); FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; and DMSO, dimethyl sulfoxide.

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patients consistently amplifying more efficiently. It was postulated that differences in PCR efficiency may be related to the presence of a greater concentration of tumor-associated human DNA in the stools of CRC patients shed from lesions. Studies have demonstrated greater shedding of nonapoptotic colonocytes exhibiting cell surface tumor markers from CRC tumors into the mucocellular layer compared with shedding from healthy mucosa *(13)*.

We have observed an association between high-efficiency PCR amplification and the presence of high-molecular-weight DNA fragments in the stool. Additionally, we observed that the presence of high-molecular-weight DNA fragments in the stool is associated with the presence of cancer. These observations suggest that DNA integrity may be a valuable CRC biomarker.

Materials and Methods

MATERIALS

All capture probes and PCR primers were synthesized by Midland Certified Reagent Company. LA Taq® DNA polymerase was obtained from PanVera Corporation. Nucleotides were purchased from Promega Corporation.

STUDIES ON CLINICAL SPECIMENS

Stool collection. We consecutively recruited 25 patients with CRC and 77 patients with negative colonoscopic findings at the Mayo Clinic (Rochester, MN). All stools were collected before colonoscopy or purgative bowel cleansing. To prevent artifacts from toilet water, stools were collected in toto by use of a plastic bucket-type container mounted to the toilet seat and were then delivered to an onsite processing laboratory within 12 h of defecation. On receipt, stools were immediately aliquoted into 30-g portions, frozen at −60 °C, and later sent (at least 30 g per participant) on dry ice by 24-h express mail to EXACT Sciences laboratories (Maynard, MA) for analysis.

Stool homogenization. All stool samples were thawed at room temperature and homogenized in an excess volume (7 mL of buffer per gram of stool) of EXACT buffer A (EXACT Sciences) using an Exactor™ stool shaker (EXACT Sciences). After stools were homogenized, we centrifuged a 4-g stool equivalent of each sample to remove all particulate matter and incubated the supernatants at 37 °C after addition of proteinase K (0.5 g/L) and sodium dodecyl sulfate (5 g/L). The supernatants were subsequently extracted with Tris-saturated phenol (Invitrogen), phenol–chloroform–isoamyl alcohol (25:24:1 by volume), and chloroform. Total nucleic acids were then precipitated (1/10 volume of 3 mol/L sodium acetate and an equal volume of isopropanol), removed from solution by centrifugation, and resuspended in Tris-EDTA [0.01 mol/L Tris (pH 7.4), 0.001 mol/L EDTA] buffer containing RNase A (2.5 mg/L), incubated for 15 min at 37 °C, and then stored at −20 °C. For each group of samples prepared, we included process positive-control samples as well as component negative controls.

Sequence-specific hybridization and selective capture. Sequence-specific DNA fragments were purified from total nucleic acid preparations by oligonucleotide-based hybrid captures as described previously *(10)*. In this method, DNA was combined in an equal volume of a solution of 6 mol/L guanidinium isothiocyanate with biotinylated oligonucleotide capture probe sequences (33 pmol; Midland Certified Reagent Company) that were complementary to the regions of the DNA fragments that were outside of the regions that were amplified in subsequent PCR amplification reactions. The mixture was heated to 95 °C for 5 min, incubated on ice for 5 min, and then incubated at room temperature overnight to allow for hybridization of complementary sequences. After incubation, the mixture with hybridized sequences was diluted in sterile H_2O and added to paramagnetic polystyrene beads coated with streptavidin (Dynabeads® M-280 Streptavidin; Dynal ASA), which had been freshly washed according to the manufacturer's instructions. The sample-bead suspension was rotated gently and continuously for 1 h at room temperature, after which beads with bound biotin-oligonucleotide-DNA complexes were isolated with a magnetic separator and washed with capture binding and washing buffer [1 mol/L NaCl, 10 mmol/L Tris (pH 7.2), 1 mmol/L EDTA, and 1 mL/L Tween® 20]. The captured sequences were then eluted from the bead complexes by the addition of 40 μL of a low-salt buffer [0.1× Tris-EDTA; 1 mmol/L Tris (pH 7.4), 100 mmol/L EDTA] and then heating the bead complex to 85 °C for 4 min. After removal of the beads, eluted sequences were stored at −20 °C.

PCR amplification of stool DNA samples. Amplification reactions consisted of captured human stool DNA or human genomic DNA (Boehringer Mannheim Biochemicals) mixed with 1× PCR buffer with 1.5 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphate (dATP, dGTP, dTTP, and dCTP), 300 nM PCR primers, and 5 U of LA Taq polymerase. We used 5 μL of captured DNA in the PCR reactions. Although DNA concentrations varied, TaqMan® analysis indicated that, on average, each sample contained 114 genome-equivalents (GE); thus, each reaction contained ~800 pg of DNA. DNA fragments for integrity analysis were amplified from four different loci: *APC*, *p53*, *BRCA1*, and *BRCA2* (see Fig. 1 and the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol49/issue7/). Independent reactions were performed for the detection of the 400- and 800-bp and 1.3-, 1.8-, and 2.4-kb fragments as follows: thermal cycling began with an initial denaturation step (92 °C for 2 min) followed by 10 cycles of sequential DNA denaturation (92 °C for 30 s), primer annealing (58 °C for 1 min), and primer extension

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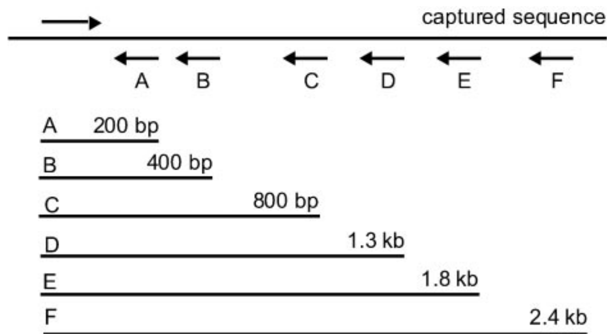

Fig. 1. DNA integrity analysis PCR amplification scheme.

One forward primer and six reverse primers (*A–F*) were used to amplify six different fragment lengths (200 bp, 400 bp, 800 bp, 1.3 kb, 1.8 kb, and 2.4 kb) from four different loci (*APC*, *p53*, *BRCA1*, and *BRCA2*; also see the online Data Supplement).

(68 °C for 3 min), and 30 additional cycles of 92 °C for 30 s, 58 °C for 1 min, and 68 °C for 2 min. These reactions were completed with a final primer extension step (68 °C for 7 min). Reactions performed on stool DNA had a PCR profile different from that for tissue DNA. Because considerably more DNA is available from tissue than from stool, fewer cycles were required. PCR products were stagger-loaded on an agarose gel (3% SeaKem agarose; BioWhittaker Molecular Applications) and electrophoresed, and the ethidium bromide-stained PCR products were visually inspected. The PCR reactions for the 200-bp fragment were performed for 40 cycles with the same conditions as above, and products were visually inspected. These reactions were performed to determine whether the capture of target DNA was successful. Although independent PCR reactions were performed in this study, we are currently developing a multiplex PCR format.

TaqMan analysis. TaqMan analysis was performed on an ABI 7700 thermal cycler (Applied Biosystems) with primers against a 200-bp region of the *APC* gene. A probe labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) was used to detect PCR product. Primer and probe sequences are given in the online Data Supplement.

Amplification reactions consisted of captured human stool DNA mixed with TaqMan PCR Universal Master mixture (Applied Biosystems), 1× PCR primers (5 μM), and 1× TaqMan probe (2 μM; Applied Biosystems). We used 5 μL of captured DNA in the PCR reactions. TaqMan reactions were performed as follows: thermal cycling began with a primer annealing step (50 °C for 2 min), followed by 1 cycle of DNA denaturation (95 °C for 10 min) and 40 cycles of sequential DNA denaturation (95 °C for 1 min) and primer annealing (60 °C for 1 min). The ABI 7700 unit detected amplification products with the FAM/TAMRA probe, and data used in the calculation of GE per reaction were provided.

MODEL SYSTEM

Tissue culture and apoptosis induction. An established in vitro apoptosis induction cell culture system was used to determine whether this methodology was in fact examining DNA integrity. Genistein (Indoline Chemical Co.), a promoter of apoptosis, was prepared in dimethyl sulfoxide (DMSO) to a stock solution (10 000 mg/L) from which working concentrations of 10, 30, 50 and 70 mg/L were made. Human prostate carcinoma DU145 cells (American Type Culture Collection) were seeded at a density of 2.5×10^5 cells per plate in DMEM supplemented with 50 mL/L fetal calf serum, 15 mmol/L HEPES, 4.5 g/kg glucose, 65 mL/L horse serum, 100 kilounits/L penicillin, and 100 g/L streptomycin. Twenty-four hours post-seeding, cells were exposed to a range of genistein concentrations and maintained for 48 h in the presence of this agent. Controls received no DMSO or genistein. DMSO controls received equal volumes of DMSO without genistein. At the end of treatment, attached cells were harvested by scraping, and cells in suspension were pelleted out of the medium to yield three distinct sample fractions: monolayer cells, detached cells, and medium containing cell-free DNA fragments. DNA was prepared from all fractions with the miniprep Qiagen blood DNA extraction reagent set. All samples were eluted in 200 μL of elution buffer, and 5-μL volumes per reaction were used in DNA integrity analysis.

PCR amplification for model system. The amplification reactions consisted of DNA from each fraction mixed with 1× PCR buffer with 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 300 nM PCR primers, and 5 U of LA Taq polymerase. The mixtures underwent thermal cycling that began with an initial denaturation step (92 °C for 2 min) followed by 10 cycles of sequential DNA denaturation (92 °C for 30 s), primer annealing (58 °C for 1 min), and primer extension (68 °C for 3 min); 16 (for lanes A1–B6 in the gel in Fig. 4) or 26 (for lanes C1–C6 in the gel in Fig. 4) additional cycles of 92 °C for 30 s, 58 °C for 1 min, and 68 °C for 2 min; and a final primer extension step (68 °C for 7 min). PCR products were stagger-loaded on an agarose gel and electrophoresed, and ethidium bromide-stained PCR products were visually inspected.

Results

DIFFERENTIAL PCR EFFICIENCIES IN A CLINICAL POPULATION

Differences in the efficiencies of PCR reactions for stool DNA from CRC patients and disease-free individuals had been observed previously by our group *(10)*. Differential amplification was seen when a 200-bp PCR product was amplified from K-*ras* target sequences captured from the stools of six patients. Band intensities for PCR products amplified from DNA captured from stools of CRC patients were higher than the intensities for PCR products obtained from colonoscopy-negative individuals. This in-

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creased band intensity suggested increased PCR reaction efficiency, and we hypothesized that it may be attributable to a greater amount of template available in the reaction.

ASSOCIATION OF HIGH-MOLECULAR-WEIGHT STOOL DNA WITH THE PRESENCE OF CRC

To further characterize the nature of the DNA fragments being obtained from these individuals, we determined fragment sizes. We determined the presence of high-molecular-weight template DNA by amplifying 200-bp and 1.8-kb target sequences within the *APC* gene, using stool DNA from cancer and colonoscopy-negative patients. Although we observed differential amplification, cancer and colonoscopy-negative samples both contained the 200-bp fragment. However, cancer-positive samples (Fig. 2, lanes 1, 4, 9, and 14) had substantially greater amounts of the 1.8-kb fragment than samples from colonoscopy-negative patients (Fig. 2, lanes 2, 3, 5–8, 10–13, and 15). Lane 9 represents PCR products amplified from stool DNA of a patient with a malignant ileal carcinoid tumor.

With the correlation between the presence of the 1.8-kb fragment and cancer incidence, we wanted to further characterize DNA integrity in stools from colonoscopy-negative and cancer patients. We therefore developed a multiple-band high-molecular-weight DNA integrity detection scheme (200 bp, 400 bp, 800 bp, 1.3 kb, 1.8 kb, and 2.4 kb). As seen in Fig. 3, patients with CRC (lanes 9–15) contained high-molecular-weight human DNA sequences in their stool. This high-molecular-weight DNA supported the amplification of longer PCR products compared with those amplified from individuals who were disease-free (Fig. 3, lanes 1–8).

In addition, as observed with the 200-bp fragment in Fig. 3, samples from colonoscopy-negative patients tended to have lower DNA concentrations compared with CRC samples. However, we have observed samples from cancer-free individuals with high DNA concentrations but low DNA integrity and have observed samples from CRC patients with low DNA concentrations and high integrity (see the online Data Supplement). These observations suggest that there is no direct association between total DNA concentration and integrity.

A DNA integrity scoring algorithm was developed based on the comparison of relative intensities of PCR products found in colonoscopy-negative individuals and CRC patients. Because six PCR products from four separate loci were amplified from captured DNA, a total of 24 bands could have potentially been detected (DNA integrity score, 0–24). Analysis of PCR products amplified from a 77-member colonoscopy-negative control group indicated that the majority of stool DNA from disease-free patients had scores between 0 and 18. We then analyzed the integrity of DNA from the stools of 27 patients with CRC. Using the previously developed algorithm, we found that 15 of 27 CRC patients had >18 bands, whereas only 2 of the 77 in the colonoscopy-negative control group had >18 bands (see the online Data Supplement). We compared total GE, as determined by TaqMan analysis using a 200-bp sequence, and DNA integrity scores. The left-hand column of Table 2 of the Data Supplement (http://www.clinchem.org/content/vol49/issue7/) has samples sorted by GE, whereas the right-hand column has the same samples sorted by DNA integrity score (total bands). When this data set was sorted by GE score and the specificity was set at 97%, sensitivity was 7%. When the sensitivity was set at 50%, data sorted by GE score yielded

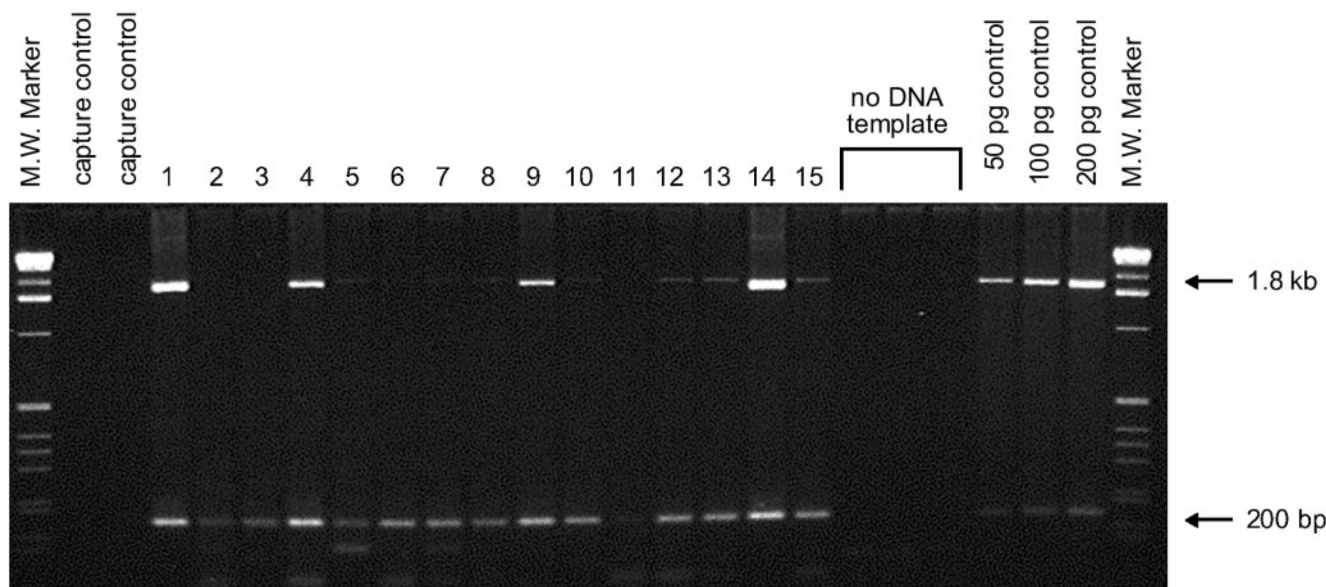

Fig. 2. DNA integrity analysis is more efficient than PCR efficiency alone for differentiating CRC.

Detection of a high-molecular-weight (1.8-kb) band, using a different reverse primer than the 200-bp reaction (see Fig. 1), was specific to those patients with cancer. *Patients 1*, *4*, and *14* have CRC, whereas *patients 2*, *3*, *5–8*, *10–13*, and *15* do not. *Patient 9* has a carcinoid tumor of the ileum. *M.W.*, molecular weight.

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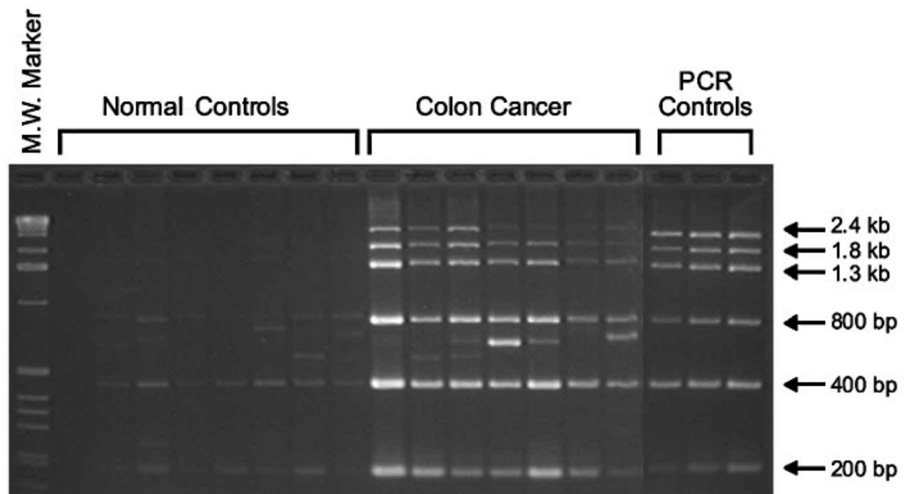

Fig. 3. Application of the DNA integrity assay to a clinical population.

Detection of multiple high-intensity high-molecular-weight bands in CRC patients. Examples of stool DNA profiles are shown from colonoscopy-negative patients (*Normal Controls*; *lanes 1–8*) and from CRC patients (*lanes 9–15*). Multiple reverse primers were used to detect 2.4-kb, 1.8-kb, 1.3-kb, 800-bp, 400-bp, and 200-bp fragments of the *APC* gene in these samples. See Fig. 1 for primer scheme and Data Supplement for sequence-specific PCR primer sequences. *M.W.*, molecular weight.

77% specificity. When these same data were sorted by DNA integrity score and the specificity was set at 97%, sensitivity increased to 57%. When the sensitivity was set at 50%, data sorted by DNA integrity score yielded a 97% specificity. When samples were sorted by the number of bands (DNA integrity), there was a highly significant association between cancer and the presence of high-molecular-weight bands ($P < 0.001$). When the same samples were sorted for GE, although there was a significant association between higher DNA concentration (200-bp band) and the incidence of cancer ($P < 0.01$), the association between DNA integrity and cancer was stronger than the association between total human DNA concentration in a patient sample and cancer.

DNA integrity analysis in a model system

To better understand the origin of the high-molecular-weight DNA fragments associated with the presence of CRC, we used our DNA integrity analysis method to examine the integrity of DNA generated from a tissue culture cell model exposed to the apoptosis promoter genistein. Subconfluent human prostate carcinoma DU145 cells *(14)* were exposed to 10, 30, 50, and 70 μg of genistein over a 48-h period. The 200-bp, 400-bp, 800-bp, 1.3-kb, 1.8-kb, and 2.4-kb DNA fragments were amplified from DNA isolated from adherent cells, detached floating cells in the culture medium, and cell-free DNA in the medium. As seen in Fig. 4, neither the adherent monolayer cells (section C, lanes C3–C6) nor the cells in suspension (section B, lanes B3–B6) revealed a genistein dose-dependent response. High-molecular-weight DNA fragments were found in the monolayer cell fraction and may represent uncleaved DNA isolated from a largely nonapoptotic induced cell population. DNA from cells in suspension contained both high- and low-molecular-weight DNA fragments. This fraction may be a mixture of pyknotic fragments as well as nonapoptotic cells that have lost adhesion with the culture substrate. Conversely, the cell-free DNA amplified from the medium contained predominantly low-molecular-weight DNA fragments (Fig. 4, section A, lanes A3–A6) that increased in concentration as genistein concentration increased. Thus, DNA integrity analysis monitored changes in high-molecular-weight DNA concentration as apoptosis was induced in tissue culture cells.

Discussion

The colonic mucosa maintains surface crypt architecture integrity through a balance of programmed cell death and cell proliferation *(15)*. The disruption of this balance by a shift away from regulated apoptosis to steadily decreasing degrees of apoptosis and increasing cell proliferation has been implicated as a factor leading to changes in colonic mucosa undergoing neoplastic transformation *(16)*. Furthermore, *p53 (17)*, *APC (18)*, and K-*ras (19)* mutations in CRC may be involved in the deregulation of apoptosis as well as causing an increase in proliferation. We previously observed a correlation between the presence of disease and the band intensities of PCR products obtained from stools of CRC patient and hypothesized that this indicated the presence of high-molecular-weight DNA fragments that may originate from nondegraded sources in the colon *(10)*. A method that provides quantitative analysis of DNA integrity in patient samples may allow both noninvasive diagnosis and monitoring over time.

Adenomatous and neoplastic tissues along with healthy mucosa shed cells and/or cell fragments into the lumen of the colon *(13)*. DNA isolated from stool samples is representative of the genetic composition of the colonic mucosa at the time of stool sample collection. Whereas the majority of mucosal tissues lose cells to apoptosis, there is evidence that transformed colonic mucosa cells have dysfunctional apoptotic mechanisms *(16, 20)* and thus may shed cells that have not undergone apoptosis.

The role of apoptosis is critical for the dynamic response of the healthy mucosa to both acute and chronic environmental injuries. Bile acids, necessary for the emul-

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