Quantitation of DNA from Exfoliated Colonocytes Isolated from Human Stool Surface as a Novel Noninvasive Screening Test for Colorectal Cancer

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INTRODUCTION

Colorectal cancer is the second most important cause of cancer death in Western countries (1, 2). Reduction in colorectal cancer mortality can be achieved by the earliest possible detection and treatment of these tumors (2). Screening approaches to detection of colorectal tumors are presently based on the use of: (a) FOBT,4 (b) flexible sigmoidoscopy; (c) double-contrast barium enema; and (d) colonoscopy (2). Among these screening tests, only FOBT first proposed by Gregor (3) in 1967 and based on relatively high probability of bleeding from colorectal tumors is noninvasive, simple, and of low cost. It has been repeatedly shown that annual FOBT screening, followed by sigmoidoscopy or colonoscopy, can reduce colorectal cancer mortality by 15–30% (2, 4, 5). However, frequent false-positive and false-negative results of the test considerably limit its sensitivity and specificity (2, 6, 7). Moreover, precancerous adenomatous polyps and early cancers bleed rarely compared with advanced tumors, and the presence or absence of blood in stool is not due only to neoplastic growth and thus can be regarded biologically as a nonspecific marker. Thus, despite the wide use of FOBT, there is a need for a more cancer-specific, noninvasive test directly assessing changes in the gut epithelium.

It is generally accepted that physiological renewal of colonocyte populations in the gut results in exfoliation of millions of epithelial cells daily, and attempts to use these cells obtained by colonic lavage for diagnostic cytological examination were made over 40 years ago (8, 9). It had been assumed that exfoliated cells, prior to excretion in feces, inevitably lose their structure and turn into completely degraded waste products. This notion has been reversed by Nair and his colleagues (10, 11), who developed a technique of isolation of exfoliated cells from human stools by gradient fractionation of dispersed fecal material (8–10-g aliquots taken from stools were used). Nevertheless, this interesting approach has not thus far been developed into any diagnostically useful test.

Colorectal carcinogenesis is presently believed to result from a combination of mutational events involving several genes controlling cell proliferation, differentiation, and apoptosis (12, 13). There are many reports on the detection of mutant K-ras gene in DNA extracted from stools of colorectal cancer patients (14–17) and from animal feces during experimental induction of colorectal tumors (18). The diagnostic value of such approaches is, however, limited because neither mutant K-ras nor any other molecular marker that can be easily detected in a majority of colorectal cancers has hitherto been found. The most frequently mutated gene in these tumors is the...

Received 6/13/97; revised 10/15/97; accepted 10/27/97.
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The abbreviations used are: FOBT, fecal occult blood test; APC, adenomatous polyposis coli; SDNAI, stool DNA index.


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APC gene (12, 13), which has a complex structure and displays such a variable mutation spectrum that analysis of all these mutations in stool DNA at a proper level of sensitivity is presently beyond reasonable limits of both effort and cost efficiency.

We have recently developed a novel technique of isolating colonic exfoliated cells from the stool surface (19). Initially, we planned to apply the technique to assessment of gene mutation-associated biomarkers of colorectal carcinogenesis, but we discovered a simple quantitative characteristic that appears to be a powerful indicator of colorectal neoplasia in humans.

MATERIALS AND METHODS

Subjects. Newly diagnosed patients with colorectal cancer and polyps in the Cambridge area were recruited from those attending out-patient clinics prior to operation. Seventy-two and polyps in the Cambridge area were recruited from those associated biomarkers of colorectal carcinogenesis, but we discovered a simple quantitative characteristic that appears to be a powerful indicator of colorectal neoplasia in humans.

MATERIALS AND METHODS

Subjects. Newly diagnosed patients with colorectal cancer and polyps in the Cambridge area were recruited from those attending out-patient clinics prior to operation. Seventy-two people took part in the study. Among them, 30 (ages, 24–79 years) were healthy volunteers, 20 (ages, 51–80 years) were colorectal cancer patients, and 22 (ages, 19–77 years) were individuals with suspected polyps undergoing colonoscopy. The study had received the approval of the Ethical Committees of the Dunn Clinical Nutrition Center and Addenbrooke’s Hospital.

Exfoliated Colonocyte Isolation Procedure. The detailed procedure of exfoliated cell isolation is described in the patent application by O’Neill and Loktionov (19). Briefly, the stool samples were collected into plastic bags, cooled, and processed within 4 h. Ice-cold aqueous suspending solution was added to the stool in a proportion of 1 ml per 1 g of stool weight. The solution contained Modified Eagle’s Medium (pH 7.4), 500 mg/l streptomycin sulfate, 50 mg/l gentamicin, 500 units/l penciillin K, and 1 mg/l amphotericin B (11), to which freshly prepared 0.5 m N-acetylcysteine was added before use. The washings were sieved and placed in 50-ml polypropylene tubes.

Magnetic beads coated with epithelium-specific antibodies were supplied by Dynal AS (Oslo, Norway). A suspension of the magnetic beads (70 μl = 2.8 × 10^7 beads) was added to the stool washings and incubated with rotation for 20 min at room temperature. The bead-cell complexes were magnetically attracted to the side of the tube(s) with Dynal MPC-1 separators. Supernatants were discarded. The bead-cell complexes were resuspended and washed with the suspending solution. The procedure was repeated until the supernatant was clear and then repeated twice more. At the end of the washing procedure, a drop of suspension was taken for preparation of smears, which were fixed with acetone and stained with hematoxylin. Finally, the bead-cell complexes were put in a tube, and the pellet volume was measured.

DNA Extraction and Quantitation. A 10-fold volume of cell lysis buffer [400 mm Tris-HCl (pH 8.0), 60 mm EDTA, 150 mm NaCl, and 1% SDS] was added, and the suspension was stored at −80°C. Later, the DNA was extracted with a commercial kit (Qiagen, Ltd., Dorking, United Kingdom). The extraction procedure was modified (19) by initial addition of 5% hexadecyltrimethylammonium bromide (final concentration, 2%) to the defrosted sample. DNA concentrations were determined by absorbance measurement at 260 and 280 nm using a CE 2041S spectrophotometer (Cecil Institute, Cambridge, United Kingdom). Results were calculated for overall amount of DNA per stool sample and expressed as SDNAI or amount of DNA (ng) isolated per gram of stool. PCR amplification of a 113-bp fragment of K-ras gene exon 1 region was performed using sense primer 5′-GGCCCTGCTGAAAATGACTGAA-3′ and antisense primer 5′-GGATCATATFCGTCCACAAAA-3′ as described previously (18) to confirm quality of extracted DNA. All results were expressed as the mean and SD or SE as indicated in the text. Pooled t tests were conducted using Data Desk4 (Data Description, Inc., Ithaca, NY) statistical package.

RESULTS

Exfoliated cells were successfully isolated from all stools except five samples (cancer patients C05, C10, and C20 and healthy controls H51 and H61), which had to be excluded due to technical problems (material loss) during the isolation procedure. The presence of exfoliated epithelial cells and cell clusters in the samples was confirmed by microscopical analysis of the stained smears. The extracted DNA quality was confirmed by its concentration measurements at 260 and 280 nm (ratio within the range 1.6–1.9) and by successful K-ras PCR amplification in all cases (Fig. 1).

Stool samples were collected from all participants in the study except one cancer patient (C02), who failed to provide a sample before preoperative clearance of the intestine. One patient (C17) had to be excluded from the cancer group because after operation, it was revealed that diverticular disease with inflammatory changes was clinically simulating tumor. In the

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**Fig. 1** Amplification of the 113-bp fragment of the K-ras gene using DNA extracted from exfoliated epithelial cells isolated from stools of healthy people (Lanes 1–5), colorectal polyp patients (Lanes 6 and 7), and colorectal cancer patients (Lanes 8–13). Lane M, molecular weight marker (100-bp ladder).
group of 22 patients with suspected polyps, 10 individuals were polyp free or had polyps in combination with inflammatory bowel disease and had to be excluded from the study. They could not be used as healthy controls due to confounding gastrointestinal conditions (inflammatory bowel disease, diverticulosis) or polyps in the past. In one polyp case (C31), cancer was diagnosed during colonoscopy, and the patient was transferred to the cancer group, leaving 11 polyp patients for analysis. Thus, 16 colorectal cancer patients and 11 polyp patients successfully yielded cells.

The results of the SDNAI determination are shown in Fig. 2 and Tables 1 and 2. Healthy people participating in the study were divided into two groups according to their age. SDNAI increased with the age of healthy subjects, and its lowest values of 227 ± 41 (mean ± SE, 33.8 ± 2.3). The difference in the mean indexes between this group and older healthy people (>50 years; mean age ± SE, 68.3 ± 1.9; mean SDNAI ± SE, 469 ± 65) was statistically significant \((P = 0.002)\). Multiple stools (two to seven samples) were collected from eight healthy volunteers, and intraindividual variation of SDNAI (up to 5-fold difference between extreme values) was common, although SDNAI never exceeded 500. For these subjects, average SDNAI values are shown in Fig. 2.

Only older healthy people were used as controls for cancer patients because ages in the latter group varied between 51 and 80 years (mean age ± SE, 69.1 ± 1.9). Mean SDNAI was 4.5-fold higher in cancer patients compared with healthy subjects of the same age group \((P = 0.005)\). Substantial interindividual variation of SDNAI was observed in both groups (Fig. 2), but among the 16 controls in the older age group, SDNAI was within the range of 100–600 in 13 cases. The remaining three samples with higher SDNAI values belonged to persons in their seventies (ages: 72, 74, and 75), who did not present any complaints but were not subjected to a detailed physical examination or colonoscopy. In two of these cases, the SDNAI values overlapped, with the lowest two SDNAI values in the cancer group.

If 700 ng of DNA/g stool is taken as a cutoff SDNAI value in discriminating between healthy people over 50 years and cancer patients, both specificity and sensitivity (20) are very high: 1.00 for sensitivity and 0.81 for specificity.

Substantial heterogeneity was found in the group of colorectal polyp patients, who had a very wide age distribution (range, 19–76 years, with mean age of 55.2 ± 5.9). It seemed to be more appropriate to compare this group with all controls taken together (ages 24–78 years with mean, 53.5 ± 3.6). Although a statistically significant difference \((P = 0.018)\) was found between healthy people and polyp patients, a wide variation in SDNAI in the polyp group (Fig. 2) suggests that the test is much more efficient in detecting colorectal cancers than polyps.

Individual results of colorectal cancer cases are presented in Table 2. All tumors had histological features of adenocarcinomas of different degrees of differentiation, and most were classified as Dukes' stage B. There was also variability in tumor localization, but it did not seem to affect SDNAI because proximal tumors (C09, C12, C13, C14, and C15) displayed clearly elevated values of the index. It was also difficult to see any relationship of tumor size or Dukes' stage with SDNAI.

**DISCUSSION**

These results indicate that quantitation of DNA from exfoliated epithelial cells isolated from the human stool surface (19) provides a novel noninvasive screening test for colorectal cancer. We have been able to show an apparent progression of the SDNAI values along the sequence: young healthy people—older healthy people—colorectal polyp patients—colorectal cancer patients. Although further validation of the test is needed, it is likely to be highly specific and sensitive because DNA is extracted from epithelial cells from tissue giving rise to colorectal tumors.

Other authors have observed previously that dispersed or homogenized stool samples obtained from colorectal cancer patients may contain more exfoliated cells (21) or DNA (17) than material taken from healthy people. However, no diagnostic use of these observations has been proposed because the differences were not large enough. One problem with the use of homogenized or dispersed fecal material is the presence of various interfering/contaminating factors inside the anaerobic fecal mass (bacteria, food debris, elements of dead host cells, mucus, and others). Isolation of cells from the stool surface, which may be partially oxygenated due to close contact with colonic mucosa, provides a dramatically increased yield of relatively undamaged cells free of most interfering materials. The use of magnetic beads with epithelium-specific antibodies facilitates further purification of epithelial cells, which may then be used for different types of analysis.

Although the clinical importance of an SDNAI-based test
Table 1  SDNAI values in the study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. of subjects</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
<th>P for comparison with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy, all ages</td>
<td>28</td>
<td>365 (245)</td>
<td>247</td>
<td>50-967</td>
<td>0.0073</td>
</tr>
<tr>
<td>Healthy, &lt;50 years</td>
<td>12</td>
<td>227 (140)</td>
<td>196</td>
<td>50-597</td>
<td>0.0007</td>
</tr>
<tr>
<td>Healthy, &gt;50 years</td>
<td>16</td>
<td>469 (259)</td>
<td>469</td>
<td>130-967</td>
<td>0.0005</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>7</td>
<td>2177 (1721)</td>
<td>1696</td>
<td>780-7975</td>
<td>0.0005</td>
</tr>
<tr>
<td>Colorectal polyps</td>
<td>11</td>
<td>1215 (1817)</td>
<td>566</td>
<td>123-6341</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 2  Details of the 20 consecutive colorectal cancer cases and individual values of DNA UV absorbance ratio (260 nm:280 nm) and SDNAI before surgical removal of the tumors*

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor localization</th>
<th>Tumor size and histological type</th>
<th>Tumor Dukes' stage</th>
<th>DNA 260:280 ratio</th>
<th>SDNAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C01</td>
<td>Desc. colon</td>
<td>4 × 4 × 4 cm; AdCa</td>
<td>B</td>
<td>1.83</td>
<td>2010</td>
</tr>
<tr>
<td>C02</td>
<td>Trans. colon</td>
<td>4 cm-long, annular; AdCa</td>
<td>B</td>
<td></td>
<td>NA²</td>
</tr>
<tr>
<td>C03</td>
<td>Rectum</td>
<td>3 × 2 × 0.8 cm; AdCa</td>
<td>A-B</td>
<td>1.60</td>
<td>1565</td>
</tr>
<tr>
<td>C04</td>
<td>Rectum</td>
<td>4 × 3 × 1 cm; AdCa</td>
<td>C</td>
<td>1.71</td>
<td>3953</td>
</tr>
<tr>
<td>C05</td>
<td>Rectum</td>
<td>5.5 × 5.5 × 3 cm; AdCa¹</td>
<td>B</td>
<td>1.80</td>
<td>915</td>
</tr>
<tr>
<td>C06</td>
<td>Rectum</td>
<td>3 × 2.5 × 2 cm; AdCa and (2 tumors)</td>
<td>4.5 × 4.5 × 1 cm; AdCa¹</td>
<td>1.68</td>
<td>2831</td>
</tr>
<tr>
<td>C07</td>
<td>Rectosigmoid</td>
<td>4.5 × 4.5 × 3 cm; AdCa¹</td>
<td>C</td>
<td></td>
<td>NA²</td>
</tr>
<tr>
<td>C08</td>
<td>Sigm. colon</td>
<td>9 × 3.5 × 3 cm; AdCa</td>
<td>B</td>
<td>1.76</td>
<td>1740</td>
</tr>
<tr>
<td>C09</td>
<td>Trans. colon</td>
<td>4 cm-long, annular; AdCa</td>
<td>B</td>
<td></td>
<td>1480</td>
</tr>
<tr>
<td>C10</td>
<td>Cecum</td>
<td>12 × 10 × 8 cm; AdCa</td>
<td>C-D</td>
<td></td>
<td>excl³</td>
</tr>
<tr>
<td>C11</td>
<td>Rectosigmoid</td>
<td>3 × 2.5 × 1 cm; AdCa</td>
<td>B</td>
<td>1.73</td>
<td>1722</td>
</tr>
<tr>
<td>C12</td>
<td>Cecum</td>
<td>8 × 5 × 3 cm; AdCa</td>
<td>B-C</td>
<td>1.67</td>
<td>1671</td>
</tr>
<tr>
<td>C13</td>
<td>Trans. colon</td>
<td>5.5 cm-long, annular; AdC</td>
<td>B-C</td>
<td>1.70</td>
<td>1582</td>
</tr>
<tr>
<td>C14</td>
<td>Cecum</td>
<td>7 × 7 × 4 cm; AdCa</td>
<td>C</td>
<td>1.75</td>
<td>780</td>
</tr>
<tr>
<td>C15</td>
<td>Asc. colon</td>
<td>3 × 3 × 3 cm; AdCa</td>
<td>A-B</td>
<td>1.71</td>
<td>2133</td>
</tr>
<tr>
<td>C16</td>
<td>Rectum</td>
<td>5.5 cm-long, annular; AdCa</td>
<td>A</td>
<td>1.85</td>
<td>7975</td>
</tr>
<tr>
<td>C18</td>
<td>Sigm. colon</td>
<td>5 × 4 × 4 cm; AdCa</td>
<td>B</td>
<td>1.80</td>
<td>1353</td>
</tr>
<tr>
<td>C19</td>
<td>Desc. colon</td>
<td>2.5 cm-long, annular; AdCa</td>
<td>B</td>
<td>1.86</td>
<td>1138</td>
</tr>
<tr>
<td>C20</td>
<td>Sigm. colon</td>
<td>4 × 4 × 2.5 cm; AdCa</td>
<td>C</td>
<td></td>
<td>NA⁴</td>
</tr>
<tr>
<td>C31</td>
<td>Rectum</td>
<td>3.5 × 3.5 × 3 cm; AdCa</td>
<td>B-C</td>
<td>1.79</td>
<td>1998</td>
</tr>
</tbody>
</table>

* Desc., descending; AdCa, adenocarcinoma; Trans., transverse; Sigm., sigmoid; Asc., ascending.

² Stool sample was unavailable before Picolax clearance of intestinal tract. NA, not applicable.

³ Adenomatous polyps were present as well.

⁴ Samples were excluded due to loss of material during cell isolation. excl, excluded; NA, not applicable.

⁵ The tumor was initially believed to be a polyp; however, a biopsy taken during colonoscopy revealed cancer.

seems to be obvious, the mechanisms of the phenomenon are not. It is reasonable to conclude that elevated SDNAI values in cancer patients result from increased numbers of exfoliated epithelial cells, which are recovered from the stool surface. However it is unknown whether the cells are predominantly exfoliated from the tumor surface or overall cell production and exfoliation in the large bowel are increased. Excessive exfoliation from the tumor surface and adjacent areas that are often subject to perifocal inflammation is very likely. Even simple mechanical contact between tumor and feces may enhance cell sloughing because tumors normally protrude into the gut lumen and have much thinner protective mucous gel layer (22). Moreover tumor cells, especially those that have already lost their wild-type p53 tumor suppressor gene, may be more resistant to hypoxia (23). Furthermore, polyploidy, often observed in neoplasia, may contribute to SDNAI elevation. On the other hand, in most cases the tumor surface seems to be much too small to provide massive exfoliation needed to dramatically raise SDNAI, and increased proliferation of nontumor colonic epithelium in colorectal cancer patients has been reported repeatedly (24–26), as well as decreased apoptosis (27). The existence of such a “field effect,” which may be mediated through complex endocrine and immune mechanisms acting in the gut (28, 29), is supported by the absence of any clear difference between SDNAI values in patients with cancers of proximal and distal colon. At the molecular level, stimulation of exfoliation may involve altered interactions in the β-catenin-E cadherin-APC protein system controlling epithelial cell adhesion and migration (30) or shifts in the extracellular matrix regulatory role (31). Moreover, changes in exfoliation, especially those occurring during transition from adenoma to carcinoma, may be related to the phenomenon of “field cancerization” first described by Slaughter et al. (32) in oral squamous epithelium and recently supported by genetic evidence in head and neck tumors (33) and colorectal cancers (34). All of the discussed mechanisms may be involved together in the SDNAI increase. The field effect hypothesis better explains the lack of correlation between SDNAI values and tumor size or localization.

The preliminary results of the present study suggest that SDNAI values over 700 indicate the need for further investiga-
tion, whereas indexes below 500 may be considered normal. The cutoff value of 700 is 3-fold the mean for the younger age group, and a question may be raised on the significance of high-ranking SDNAI values in young persons. Additional work should be done to identify a precise “threshold” SDNAI level, which could be used as a diagnostic criterion. The use of exfoliated colonocytes isolated from feces should not be limited by the SDNAI determination. Various analytical approaches can be applied to the cells and DNA, RNA, and proteins extracted from them to assess the presence of molecular markers of malignancy. In addition to gene mutation determination (K-ras, APC, and p53), such methods as detection of telomerase activity, which is known to be associated with cancer and is detectable in exfoliated malignant epithelial cells (35–37), should be considered.

Other conditions, especially inflammatory changes in the bowel, in particular ulcerative colitis, may result in enhanced colonocyte exfoliation, leading to increased SDNAI values. Many of these conditions are associated with typical clinical manifestations; nevertheless, their effect on SDNAI values requires elucidation to provide guidelines for the use of a SDNAI-based test for colorectal cancer screening.

ACKNOWLEDGMENTS

We thank Dr. T. Bandaletova for histopathology consultation and R. Hughes for assistance in some exfoliated cell isolations. The help of patients and volunteers is gratefully acknowledged.

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