Molecular Screening Testing for Colorectal Cancer

Bernard Levin

Worldwide, colorectal cancer is diagnosed in ~1 million individuals each year, accounting for >9% of all new cancer cases (1). Approximately 529,000 people worldwide die of this disease annually. In 2006, it is estimated that 146,000 cases of colorectal cancer will occur in the United States with 55,000 deaths (2). Colorectal cancer is an excellent candidate for population-based screening because it is relatively common (5% lifetime risk in the United States). Most nonfamilial cases develop slowly over the years, and there are acceptable screening tests. Screening for colorectal cancer decreases both the mortality and the incidence and is recommended for those who are ages ≥50 years. Although interest in screening has increased, only 53% of the U.S. population who are ages ≥50 years have undergone screening (3). Currently available screening test options include fecal occult blood tests (guaiac or immunochemical methods), flexible sigmoidoscopy, colonoscopy, and double-contrast barium enema. Virtual colonoscopy (computerized tomographic and magnetic resonance colonoscopy) and molecular testing of stool and blood have recently been introduced and are still undergoing improvements and validation.

Non-DNA-Based Stool Tests

The value of fecal occult blood test screening is supported by the highest level of evidence, namely four randomized, controlled trials of screening (4). On an intention to screen analysis, annual screening with a guaiac fecal occult blood test reduces mortality by 35% and biennial screening by 20%. Nevertheless, the specificity of guaiac fecal occult blood testing is limited by false-positive reactions to dietary compounds, medications, and gastrointestinal bleeding from sites above the colon. Attempts to enhance the effectiveness of screening have resulted in the development of immunochemical tests for fecal occult blood. Fecal immunochemical tests seem to offer more advantages than do guaiac fecal occult blood tests, including greater sensitivity for cancers and adenomas and ease of use (4).

A variety of stool proteins have been studied as potential screening tools for colorectal neoplasia. These include transferrin, albumin, and α-1 antitrypsin (5, 6). None of these tests offer any specific advantages over tests for fecal occult blood.

Detection of Colonocytes

It has been shown that colonocytes can be recovered from the surface of stool from healthy volunteers (7) and exfoliated cells from colorectal cancers (8). However, the number of malignant colonocytes obtained is usually low; thus, it would be advantageous to be able to differentiate between normal and neoplastic colonocytes. One example of such an approach has been the quantitation of minichromosome maintenance proteins that are indicators of the abnormal cell cycle entry that characterize neoplastic cells. Although initial results are promising, much additional work is required (9).

Molecular Testing

Advances in our understanding of the molecular genetic changes involved in neoplastic transformation have led to new noninvasive methods to detect tumors (10, 11). These approaches detect mutant forms of the oncogenes and tumor-suppressor genes that have been shed by the tumors and are associated with the initiation and progression of these neoplasms. Early examples involved the examination of urine and stool to detect those acquired gene abnormalities (12, 13). More recently, mutant DNA molecules have been identified in plasma (14).

Fecal DNA Testing

Mutations in oncogenes and tumor-suppressor genes have been identified that are associated with the progression from normal mucosa to adenomatous polyps and to colorectal carcinomas. These acquired genetic abnormalities include inactivation of the adenomatous polyposis coli (APC) gene, activation of the K-ras gene, detection of the deleted in colon cancer gene, and inactivation of p53.

These molecular changes commonly involve chromosomal instability with chromosomal deletions, duplications, and rearrangements that result in aneuploidy (15). Other mechanisms of colorectal carcinogenesis involve increased rates of mutation, often in tandemly repeated DNA sequences (microsatellite instability) or epigenetic instability (CpG island methylator phenotype) in which genes are inappropriately silenced by promoter hypermethylation (16). Epithelial cells containing these genetic abnormalities are constantly shed into the lumen. PCR technology enables the separation of human DNA from bacterial DNA in the stool.

There are technical obstacles to the recovery and analysis of human DNA from stool. Human DNA is present in concentrations accounting for ~0.01% to 0.0% of total stool DNA (17) recoverable, and tumor DNA is highly heterogeneous. To maximize sensitivity of detecting mutant DNA as a screening test for colorectal neoplasia, it is important to maximize the recovery of DNA from stool. Insufficient recovery diminishes the effectiveness of PCR, leading to false-negative results. The most recent improvement for enrichment of DNA recovery is based on sequence-sequence capture with acrylamide gel–immobilized capture probes. This, in turn, has led to enhanced assay sensitivity (18).
Long DNA

Long lengths of DNA occur more abundantly in stool samples from patients with colorectal cancers owing to the presence of neoplastic cells being shed into the colonic lumen without undergoing apoptosis (19). Normally, DNA is broken down during apoptosis by endonucleases into 180 to 210 bp fragments; however, in colon cancer, because of deficient apoptosis, long DNA can be identified and has been used for cancer detection either alone (19) or in combination with other markers (20).

Long DNA is susceptible to degradation if incubated at room temperature, which can diminish its value as a screening assay under "field" conditions of variable storage and handling. The addition of stabilization sensitivity buffer can prevent such loss of assay sensitivity (21).

DNA Markers

Tests for specific markers have been developed based on the profile of mutations that occur commonly at different stages of the adenoma-carcinoma sequence (Fig. 1). To assess the sensitivity and specificity for detection of mutant DNA in stool, studies have first focused on the frequency of mutations in surgically resected colorectal adenomatous polyps or cancer and then assessed their presence in the stool (Table 1).

K-ras mutations were the first DNA markers tested by Sidransky et al. (13) in 1992. Mutations were detected in stool in eight of nine neoplasms (seven potentially curable cancers and two adenomas). Overall, K-ras mutations (usually on codons 12 and 13) are variably present in 40% to 80% of colorectal cancers and advanced adenomas (Table 2) and are not observed in smaller adenomas (22). Furthermore, mutant K-ras can arise from nonneoplastic sources, such as hyperplastic aberrant crypt foci (23) and pancreatic hyperplasia (24).

APC mutations occur early in the adenoma-carcinoma sequence and are detectable in ~60% of both adenomas and colorectal cancers (25). Traverso et al. (26, 27) used a digital protein truncation assay based on the amplification of a small number of APC templates and the detection of truncated poly-peptides generated by the in vitro transcription and translation of PCR products. Using this assay, 61% of cancers and 50% of large adenomas were detected at a specificity of 100%.

Tp53 mutations occur late in colorectal carcinogenesis, being found in 50% to 70% of colorectal cancers but not in adenomas. Eguchi et al. (28) tested for mutations of exons 5 to 8 of Tp53 in stool and reported a sensitivity of 28% in a small number of patients.

Microsatellite instability can be detected in over 90% of carcinomas and 80% of adenomas in hereditary nonpolyposis colorectal cancer, but in only 15% of sporadic carcinomas and 5% of adenomas. In potentially premalignant lesions such as serrated adenoma, abnormalities of the microsatellite instability pathway have been described (29). Sporadic carcinomas exhibiting microsatellite instability are generally right sided. BAT26, mononucleotide tract microsatellite region, is the most commonly used marker. Traverso et al. (26) identified BAT26 alterations in 28 of 46 proximal colorectal cancers, and identical abnormalities were in the stool DNA of 17 of these patients (sensitivity 40%). This test may be especially useful in individuals with hereditary nonpolyposis colorectal cancer as an adjunct to colonoscopic screening.

Hypermethylated DNA

Hypermethylation of CpG islands and genome hypermethylation may occur early in neoplastic transformation in the colon

Table 1. Presence of common gene mutations in sporadic colorectal neoplasia

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer (%)</th>
<th>Adenoma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras*</td>
<td>20-38</td>
<td></td>
</tr>
<tr>
<td>APC*</td>
<td>50-83</td>
<td>20-80</td>
</tr>
<tr>
<td>Tp53*</td>
<td>41-69</td>
<td>4-26</td>
</tr>
<tr>
<td>MSI</td>
<td>15-17</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MSI, microsatellite instability.

*Ref. 25.
Ref. 37.
Ref. 38.

Table 2. Single DNA-based stool markers

<table>
<thead>
<tr>
<th>Gene mutations</th>
<th>Sensitivity (CRC (%))</th>
<th>Specificity (Adenomas (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sidransky et al., 1992 (13)</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Ratto et al., 1996 (39)</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>Villa et al., 1996 (40)</td>
<td>80</td>
<td>96</td>
</tr>
<tr>
<td>Puig et al., 2000 (41)</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>Wan et al., 2004 (42)</td>
<td>56</td>
<td>95</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eguchi et al., 1996 (28)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traverso et al., 2002 (26)</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>MSI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traverso et al., 2002 (27)</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>Epigenetic mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Müller et al., 2004 (23)</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Chen et al., 2005 (32)</td>
<td>46</td>
<td>90</td>
</tr>
</tbody>
</table>

Abbreviation: CRC, colorectal cancer.
Recent studies have identified potential targets that could be incorporated into multitarget assays especially as an advantage in detecting proximal lesions (31). Chen et al. (32) have recently presented data on a normally unexpressed and unmethylated gene, vimentin, that becomes methylated in 53% to 83% of colon cancers. The biomarker is equivalently sensitive for early- and late-stage cancers with a sensitivity of 46% and specificity of 90%. Other studies have shown that methylated SFRP2 DNA can be detected in the fecal DNA of colon cancer patients with a sensitivity of 77% and a specificity of 77% (23).

**Multitarget Assays**

Because the molecular changes in colorectal neoplasia are heterogeneous, multiple DNA alterations must be targeted to enhance sensitivity. Furthermore, it is important for each assay within a marker panel to have high specificity to avoid false-positive results. Early studies using three markers (TP53, BAT26, and K-ras) were used to detect tumor-associated mutations in the stool before a molecular analyses of the paired tumors. The three genetic markers together detected 71% of the 51 patients (confidence interval, 56-83%) with colorectal cancers and 36 (92%) of 39 individuals whose tumor had a DNA abnormality (33).

Later, small trials using multiple markers (Pre-Gen-Plus) reported test sensitivity from 62% to 91% for adenocarcinoma and 27% to 82% for adenoma detection with specificities ranging from 93% to 98% (Table 3). A recent study, with colonoscopy as a reference standard, assessed in a prospective screening trial the performance of a panel of 21 DNA targets in 4,404 average risk asymptomatic individuals. Sensitivity for colorectal cancer (n = 31) was 52% and sensitivity for colorectal cancer or advanced adenoma (n = 418) was 18%. Specificity was 95% (34).

In a recent multicenter trial done on a single stool specimen and with colonoscopy as a reference standard, the molecular assays detected 35% of colorectal cancers or high-grade dysplasia (n = 23) with a specificity of 98%. In this assay, it is likely that poor detectability of long DNA was related to degradation in storage, an obstacle overcome by subsequent improvements in technique (18). In a recent study, Itzkowitz et al. (35) presented results of a novel fecal DNA test incorporating two markers, viz. a combination of a marker of DNA integrity (DIA) and a marker of vimentin methylation (V29). In a series of 122 normal individuals and 40 with known colorectal cancer, sensitivity for colorectal cancer was 87.5% with 82.0% specificity. Further studies will be needed to determine how these promising results can be translated into clinical use.

**Plasma Markers**

It is clear that the convenience and simplicity of a plasma assay would be considerable. Recently, Diehl et al. (14) described the results of a novel assay for circulating APC. Mutant APC molecules were detectable in over 60% of patients with early, potentially curable colorectal cancers but not in individuals with adenomas. This approach may be a useful adjunct if further validated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Marker panel</th>
<th>Test sensitivity, % (n)</th>
<th>Specificity, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahlquist et al., 2000 (43)</td>
<td>APC, K-ras, p53; MSI; long DNA</td>
<td>91</td>
<td>82</td>
</tr>
<tr>
<td>Tagore et al., 2000 (44)</td>
<td>APC, K-ras, p53, MSI; long DNA</td>
<td>63</td>
<td>57</td>
</tr>
<tr>
<td>Syngal et al., 2002 (45)</td>
<td>APC, K-ras, p53; MSI; long DNA</td>
<td>62</td>
<td>27</td>
</tr>
<tr>
<td>Syngal et al., 2003 (46)</td>
<td>APC, K-ras, p53; MSI; long DNA</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Brand et al., 2002 (47)</td>
<td>APC, K-ras, p53; MSI; long DNA</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Calistri et al., 2003 (48)</td>
<td>APC, K-ras, p53; MSI; long DNA</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Imperiale et al., 2004 (34)</td>
<td></td>
<td>71</td>
<td>97</td>
</tr>
<tr>
<td>Ahlquist et al., 2005 (49)</td>
<td></td>
<td>67</td>
<td>97</td>
</tr>
</tbody>
</table>

Abbreviation: LOH, loss of heterozygosity.
References

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