

Noninvasive molecular biomarkers for the detection of colorectal cancer

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Colorectal cancer (CRC) is the third most common malignancy in the world. Because CRC develops slowly from removable precancerous lesions, detection of the disease at an early stage during regular health examinations can reduce both the incidence and mortality of the disease. Although sigmoidoscopy offers significant improvements in the detection rate of CRC, its diagnostic value is limited by its high costs and inconvenience. Therefore, there is a compelling need for the identification of noninvasive biomarkers that can enable earlier detection of CRC. Accordingly, many validation studies have been conducted to evaluate genetic, epigenetic or protein markers that can be detected in the stool or in serum. Currently, the fecal-occult blood test is the most widely used method of screening for CRC. However, advances in genomics and proteomics combined with developments in other relevant fields will lead to the discovery of novel non invasive biomarkers whose usefulness will be tested in larger validation studies. Here, non-invasive molecular biomarkers that are currently used in clinical settings and have the potential for use as CRC biomarkers are discussed. [BMB reports 2008; 41(10): 685-692]

Introduction

Colorectal cancer (CRC) is the third most common malignancy in the world. In addition, there are approximately 1,000,000 new cases of CRC and 500,000 deaths associated with CRC each year. Indeed, CRC represents one of the primary causes of cancer deaths in Europe and the USA (1). In Korea, CRC is the fourth leading cause of mortality by cancer, and its incidence is increasing (2). CRC is believed to develop slowly via a progressive accumulation of genetic mutations; therefore, the risk of recurrence and subsequent death due to CRC is closely related to the stage of the disease at the time of primary

diagnosis. Recent studies have shown that shifting the detection of the disease to an earlier stage via mass screening and intervening at this stage can reduce the risk of death from CRC (3, 4). These findings strongly demonstrate the clinical need for biomarkers for early detection of CRC.

Biomarkers are substances that are used as indicators of a biological state. Accordingly, biomarkers have characteristics that enable them to be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (5). One of the key requirements of a test for CRC is that it must allow detection of the disease at earlier stages so that the disease can be cured effectively. Accordingly, such tests should have high sensitivity and specificity while producing a low number of false-negative and false-positive results to prevent subjecting healthy individuals to unnecessary colonoscopies. Sigmoidoscopy offers significant improvements in detection rates for CRC. However, the diagnostic value of sigmoidoscopy is limited with regards to costs, risks, and inconvenience (6, 7). Noninvasive biomarkers can be analyzed relatively easily and economically; therefore, they have the potential to greatly enhance screening acceptance. Several non-invasive tests for CRC detection are available, of which the fecal-occult bleeding test (FOBT) is the most commonly used (8-10). However, this method lacks sensitivity as well as specificity for screening an average risk population. As a result, new cancer biomarkers that will further enhance the detection of the disease and trigger follow-up colonoscopies when necessary must be developed. In addition to such screening biomarkers, prognostic markers that predict the likely course of the cancer, stratification markers that predict the likely response to a drug prior to beginning treatment, and efficacy markers that monitor the efficacy of drug treatment may eventually reduce the mortality rate of CRC.

Recent advances in genomics and proteomics have contributed to our understanding of pathways that control the growth, differentiation, and death of cells. Genomic techniques such as DNA microarray analysis and proteomic methods such as 2-dimensional electrophoresis and mass spectrometry are now commonly used to evaluate the expression profiles of genes and proteins in cells, tissues, and bodily fluids (11, 12).

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Received 22 September 2008

Keywords: Biomarker, Colorectal cancer, Early detection, Fecal-occult blood test, Noninvasive marker, Proteomics

Identification of genes or proteins that are characteristic of the development of cancer can potentially uncover biomarkers that will aid in the diagnosis of CRCs. This review will focus on potential biomarkers that have recently been discovered and may be used in the future to detect CRC using non-invasively-collected samples as well as biomarkers that are currently being used in clinical settings (Table 1).

Fecal markers

Fecal hemoglobin

Stool-based screening for CRC is simple, inexpensive and the least invasive method of screening available (13). FOBT, which is the most widely used screening modality for CRC, detects hemoglobin enzymatically or immunologically (14). Enzymatic FOBT measures the peroxidase-like activity of hemoglobin that originates from any source; therefore, it is susceptible to both colorectal and upper gastrointestinal bleeding. In addition, the ingestion of certain foods (red meats, fruits and vegetables) and medicines (non-steroidal anti-inflammatory drugs) can also yield false-positive results. Immunological FOBT uses antibodies that specifically detect human hemoglobin; therefore, it is not impacted by plant peroxidase in the diet. An important limitation of the FOBT is its relatively poor sensitivity at detecting early-stage lesions. In addition, FOBT shows low sensitivity for the detection of both adenomas (~10%) and CRCs (40-85%). Finally, large randomized clinical trials have shown that FOBT is not very reliable and that

they reduce cancer mortality by only 30% (3, 15).

Genes and epigenetic markers

Colonocytes, which are shed into the fecal stream, provide informative material that can be used to detect genes and epigenetic markers in feces (16). Unlike fecal blood, which is shed only intermittently, it is believed that colonocytes are shed continuously. Furthermore, the shedding of colonocytes from CRC occurs more frequently than from normal colonic epithelium. Fecal colonocytes are assessed by analyzing DNA mutations for targets such as *K-ras*, *p53*, and adenomatous polyposis coli (*APC*), by analyzing epigenetic markers such as microsatellite instability (MSI), or by measuring unfragmented long-form DNA (L-DNA).

K-ras, which encodes a Ras family protein, functions as a guanine nucleotide binding protein that is involved in a signal transduction pathway that includes the phosphatidylinositol-3-kinase and serine/threonine protein kinase B pathways (17). *K-ras* mutations have been found in 40-50% of sporadic colon cancers and adenomas (18). In addition, studies have demonstrated that *K-ras* mutations are present in aberrant crypt foci (a suspected pre-cancerous lesion) in 13-95% of all CRC cases (19-21), which suggests that *K-ras* mutations may be an important early event in tumorigenesis.

p53 encodes a tumor suppressor protein that regulates the expression of genes involved in apoptosis, angiogenesis, the cell cycle and maintenance of the genome (22). Approximately 50% of all human cancers contain mutated *p53* genes, and 30-60% of

Table 1. Noninvasive molecular biomarkers for the detection of CRC

Analyte	Subject	Type	Status
Fecal hemoglobin	Stool	Protein	IU
<i>K-ras</i>	Stool	DNA	CV
<i>APC</i>	Stool	DNA	CV
L-DNA	Stool	DNA	CV
<i>p53</i>	Stool	DNA	CV
CEA	Serum	Protein	IU
CA19.9	Serum	Carbohydrate	IU
TIMP-1	Serum	Protein	CV
Spondin-2, DcR3, Trail-R2, Reg IV, MIC 1	Serum	Protein	PD
PSME3	Serum	Protein	PD
NNMT	Serum	Protein	PD
CRMP-2	Serum	Protein	PD
SELDI (apolipoprotein C1, C3a-desArg, alpha1-antitrypsin, transferrin)	Serum	Protein	PD
HNP 1-3	Serum	Protein	PD
MIF	Serum	Protein	PD
M-CSF	Serum	Protein	PD
M2-PK	Serum	Protein	PD
Prolactin	Serum	Protein	PD
Septin 9	Plasma	DNA	PD
Five-gene panel (<i>CDA</i> , <i>BANK1</i> , <i>BCNP1</i> , <i>MS4A1</i> , <i>MGC20553</i>)	WBC	DNA	PD
CCSA-2, -3 -4	Serum	Protein	PD
MMP-9, -7	Serum	Protein	PD
Laminin	Serum	Protein	PD

IU, in use; CV, clinical validation; PD, preclinical development

CRCs have demonstrated mutations in the gene that vary based on the stage, grade and location of the cancer (23). These mutations appear to be formed relatively late in the genesis of CRC, and altered *p53* has only a modest impact on outcome. Therefore, the relatively low mutation rate of *p53* in early-stage tumors limits its sole use in DNA-based detection of CRC.

APC protein is another tumor suppressor that assembles on a scaffold protein, axin, with β -catenin and glycogen synthase kinase 3 β to coordinate the regulation of β -catenin signaling (24). Inactivation of the APC protein is responsible for both inherited and sporadic forms of CRC. Like *K-ras*, mutation of *APC* appears to be an early-genetic event in the progression from adenoma to carcinoma, which indicates its potential for use as a screening marker. However, unlike *K-ras*, the mutations are distributed throughout the coding region, thereby making it technically difficult and time consuming to detect all of the potential mutations during screening programs for CRC (21, 25, 26).

Microsatellites are stretches of short DNA sequences that contain a motif of 1-5 nucleotides that are tandemly repeated (27). The most common microsatellite in human DNA is a dinucleotide repeat of cytosine and adenine. These tandem repeats occur throughout the human genome. MSI occurs when microsatellites undergo a change in length that covers approximately 15% of the CRC. MSI tumors show a better prognosis than stage-matched microsatellite stable tumors (28). In sporadic CRCs, the development of MSI most commonly occurs due to epigenetic silencing of the DNA mismatch repair gene, *MLH1* (29). There are several MSI markers, among which *BAT26* is probably the most widely used.

Shedding of colonocytes is a normal consequence of exfoliation (30). Untransformed colonocytes are shed continuously from the colonic mucosa. These cells usually undergo apoptosis, which is characterized by DNA fragmentation and laddering. Conversely, malignant colonocytes shed from CRC tumors have a decreased rate of apoptosis relative to normal colonocytes, which facilitates detection of intact genomic DNA (L-DNA) as a potential stool-based marker. Boynton *et al.* (31) amplified six genomic fragments of different length from each of four different genetic loci (*APC*, *p53*, *BRCA1*, and *BRCA2*) using fecal specimens collected from 25 CRC patients and 77 controls. In their study, when a positive L-DNA was defined as >18 bands detected from a possible 24 bands (4 loci \times 6 fragments), the specificity for CRC detection was 97% and the sensitivity was 57%.

A large population-based study conducted by Imperiale *et al.* revealed that a fecal DNA panel consisting of 21 mutations (3 in the *K-ras* gene, 10 in the *APC* gene, and 8 in the *p53* gene; the MSI marker *BAT-26*; and L-DNA) detects a greater proportion of important colorectal neoplasia than FOBT without compromising specificity (32). In addition, the sensitivity of the fecal DNA panel was 52% for invasive cancers and 41% for invasive cancers plus adenomas with high-grade dysplasia, whereas that of the FOBT was 13% for the former and 14% for

the latter. In subjects with negative findings on colonoscopy, the DNA panel had a specificity of 94%, whereas FOBT had a specificity of 95%. Taken together, the results of this study clearly demonstrate that the DNA panel has a higher sensitivity than FOBT without reduced specificity.

Serum or blood markers

CEA

Carcinoembryonic antigen (CEA) is a high molecular weight glycoprotein that belongs to the immunoglobulin superfamily. The carboxy-terminal of CEA contains a hydrophobic region that is modified to provide a glycosyl phosphatidylinositol link to the cell membrane. Although its presence can be determined in biopsy samples, it is usually identified in serum. This protein has been used for many years as a biomarker of CRCs as well as for other cancers (33). Specifically, high CEA levels are associated with cancer progression, and levels of the marker are expected to fall following cancer surgery (34). However, in the absence of cancer, high CEA levels may also occur in response to other conditions such as hepatitis, inflammatory bowel disease, pancreatitis, and obstructive pulmonary disease. Furthermore, CEA may not be detected when cancer is in advanced stages. As a result, CEA does not provide sufficient sensitivity and reliability for the early detection of cancer. Indeed, using a cut-off point of 2.5 ng/ml results in the sensitivity of CEA for early CRC (i.e., Dukes' A and B disease) being only 30-40% with a specificity of 87% (9). Therefore, the NIH does not recommend that CEA be used to screen for early CRC (33). Instead, the potential value of the CEA test lies in its use to measure the course of the progression of cancer as a prognostic marker once it has been diagnosed, with higher CEA levels being indicative of greater disease severity and a poorer prognosis.

CA 19-9

Carbohydrate antigen 19-9 (CA 19-9), which is the second most investigated gastrointestinal tumor marker, is known to be a sialylated Lewis-a antigen (35). CA 19-9 was originally defined by a monoclonal antibody produced by a hybridoma prepared from the spleen cells of a mouse that had been immunized with the human colorectal carcinoma cell line, SW 1116. Although CA 19-9 is the best marker available for pancreatic adenocarcinoma, it is less sensitive than CEA for CRC and provides less information than CEA when used for monitoring patients that have already been diagnosed with CRC (36). Other carbohydrate antigens such as CA 50, CA 195, CA 242, CA M26, CA M25, CA M43 and CA 72-4 have also been evaluated extensively (37); however, due to their observed sensitivity, stage dependency and specificity, these antigens are not useful markers for the detection of CRC.

Tissue inhibitor of metalloproteinase type 1

Tissue inhibitor of metalloproteinase type 1 (TIMP-1) is a mul-

tifunctional glycoprotein that inhibits most matrix metalloproteinases (MMPs). The total levels of TIMP-1 have been shown to be significantly higher in patients with both colonic and rectal cancers, while healthy blood donors have a low and very narrow range of plasma TIMP-1 levels (38, 39). More importantly, TIMP-1 is capable of detecting early stage colon cancer with a sensitivity and specificity comparable to those obtained when detecting late stage cancers. Conversely, patients with colon adenomas, inflammatory bowel diseases or primary breast cancer do not show increased plasma levels of total TIMP-1 (40). Preoperative TIMP-1 levels have been proposed as a stage-independent prognostic marker for CRC in multivariate statistical analyses in two separate high-powered studies (41, 42). However, the results of these studies revealed that elevated levels of TIMP-1 were not restricted to advanced stages of CRC alone; therefore, larger studies are required to validate the use of TIMP-1 for early diagnosis and evaluation of the prognosis of CRC.

Five-serum-marker panel (spondin-2, DcR3, Trail-R2, Reg IV, MIC 1)

diaDexus Inc. recently evaluated four serum biomarkers, spondin-2, tumor necrosis factor receptor superfamily member 6B (DcR3), TRAIL receptor 2 (TRAIL-R2) and Reg IV in 600 serum samples in collaboration with the Mayo Clinic (43). All four markers, as well as a fifth marker, macrophage inhibitory cytokine 1 (MIC1), were found to be elevated in patients with CRC when compared to normal controls and patients with benign disease. In addition, this five-serum marker panel offers better sensitivity and specificity than CEA. Accordingly, the company is currently developing biomarker panels that include the 5 markers as diagnostic modalities to improve the detection rate of early stage CRC.

Nicotinamide N-methyltransferase and proteasome activator complex subunit 3

Roche Diagnostics GmbH utilized two-dimensional gel electrophoresis and mass spectrometry to analyze 16 matched CRC and adjacent normal tissue samples. Proteins found to be elevated in cancer tissue were then further validated with serum samples. Elevated levels of nicotinamide N-methyltransferase (NNMT) and proteasome activator complex subunit 3 (PSME3), which are not predicted to be secreted, were found in serum from patients with CRC (44, 45). Validation studies using 109 CRC samples, 317 healthy control samples, and 87 samples from patients with benign bowel diseases revealed that the diagnostic accuracy of PSME3 was similar to that of CEA, and that NNMT was better than CEA at detecting CRC. However, the abundance of PSME3 is less stage-dependent than CEA.

Collapsin response mediator protein-2

Although direct analysis of human samples may sometimes be very challenging, discovering candidate biomarkers from can-

cer cell lines and subsequent validation in human samples is plausible (46, 47). By analyzing the secretomes of 21 cancer cell lines derived from 12 cancer types, Wu *et al.* identified collapsin response mediator protein-2 (CRMP-2) and evaluated it as a potential CRC biomarker in the serums of 201 CRC patients and 210 healthy controls (48). The use of CRMP-2 alone showed better sensitivity but poorer specificity than CEA. However, combined detection using CEA and CRMP-2 produced better sensitivity (77%) and specificity (95%) than detection using either of these markers alone (43 and 61% sensitivity, respectively; 87 and 65% specificity, respectively). Therefore, CRMP-2 may be a valuable serum marker when used in combination with CEA.

Six SELDI peaks corresponding to apolipoprotein C1, complement C3a-desArg, α 1-antitrypsin and transferrin

Surface-enhanced laser desorption/ionization (SELDI) mass spectrometry is a technology that can produce proteomic fingerprints from biological samples using a relatively high-throughput platform (49). In this method, samples are applied to proteinchip arrays coated with chromatographic surfaces and then analyzed by mass spectrometry. Martin *et al.* used SELDI to investigate the serum proteome of 62 CRC patients and 31 noncancer subjects (50). They identified four proteins with diagnostic potential: apolipoprotein C1, complement C3a-desArg (the stable form of C3a anaphylatoxin), α 1-antitrypsin and transferrin. Artificial neural networks trained using only the intensities of six SELDI peaks corresponding to the identified proteins were able to classify the patients enrolled in the study with 95% sensitivity and 91% specificity, while the use of CEA to evaluate the same sample set showed 54% sensitivity and 93% specificity.

Habermann *et al.* demonstrated that C3a-desArg is present at significantly higher levels in serum from patients with colorectal adenomas and carcinomas than in serum from healthy individuals (51). Specifically, in a blinded validation study ($n = 59$), the use of C3a-desArg alone predicted the presence of colorectal malignancy with a sensitivity of 97% and a specificity of 96%. C3a-desArg and other proteins corresponding to SELDI peaks are common serum proteins, and changes in their concentrations most likely reflect epiphenomena rather than secretion by cancer cells.

α -Defensins

Two independent approaches using SELDI-TOF to analyze the protein profiles of colon cancer serum and protein profiles of CRC tumors demonstrated that human neutrophil peptides (HNP)-1, HNP-2 and HNP-3, also known as α -defensin-1, α -defensin-2, and α -defensin-3, are up-regulated in patients with CRC (52, 53). Indeed, the HNP1-3 level in the serum of 48 CRC patients and 42 normal controls was capable of identifying CRC with a sensitivity of 69% and a specificity of 100%. However, a larger study is required to refine and validate the diagnostic accuracy of these findings.

Macrophage migration inhibitory factor

Based on an observation that the gene expression level of macrophage migration inhibitory factor (MIF) is elevated in CRC tissues, our group has evaluated the use of the protein as a potential biomarker for CRC. In an analysis of serum samples of 129 patients with colon cancer and 53 healthy control subjects, the serum MIF level was found to be significantly increased in patients with CRC (54). Although the specificity of MIF is not as high as that of CEA (90.6% vs. 100.0%), MIF is more sensitive during early cancer detection (47.3% vs. 29.5%), which suggests that MIF may be used as a diagnostic marker in CRC.

Macrophage-colony stimulating factor

The serum levels of both macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor are significantly higher in CRC patients than in healthy subjects (55, 56). In addition, serum levels of M-CSF are more associated with lymph node metastasis than CEA and CA 19-9, which suggests that serum M-CSF elevation in CRC patients might help predict the risk of lymph node metastasis of this tumor. Finally, M-CSF appears to offer additional information to that presented by classic prognostic factors.

Prolactin

Prolactin, which is a hormone with multiple biological actions that is synthesized by the anterior pituitary gland, is elevated in patients with CRC. A study that evaluated 47 CRC patients and 51 healthy controls revealed that prolactin can predict CRC with a sensitivity and specificity of 77% and 98%, respectively (57).

M2-pyruvate kinase

M2-pyruvate kinase is an isoform of glycolytic enzyme pyruvate kinase. Although the protein is a cytosolic enzyme, it is liberated into circulation via a mechanism that is not yet known. However, it has been suggested that M2-pyruvate kinase is released into circulation from dying cancer cells. Therefore, M2-pyruvate may be a useful marker for the detection of CRC. In addition, two independent studies revealed that the use of M2-pyruvate kinase for the detection of CRC has a sensitivity of 48-58% and a specificity of 90-95%. Furthermore, when combined with CEA, the sensitivity of M2-pyruvate increases without decreasing the specificity (58, 59).

Methylated septin-9 DNA

The assessment of epigenetic events is one of the most promising means of identifying biomarker candidates for the early detection of cancer. DNA methylation in which cytosines within the palindromic dinucleotide 5'-CpG-3' sequence are methylated shapes the chromatin structure of DNA according to its functional state (60). The cancer genome is frequently characterized by hypermethylation of specific genes. Therefore,

Epigenomics AG has developed a blood-test for CRC that is based on methylation of *SEPT9*, *NGFR* and *TMEEF2* (61). To evaluate this test, free-floating DNA was extracted from plasma samples of 133 CRC patients and 179 healthy controls in the same age range, and the methylation levels were then measured using restriction enzyme-based qPCR. The biomarker with the highest performance was found to be *SEPT9*, which was capable of detecting CRC with a specificity and sensitivity of 95% and 52%, respectively, when a cutoff of 0.011 µg/L of methylated *SEPT9* DNA was used.

Five gene markers in whole blood

Gene expression patterns in the peripheral blood reflect changes that occur within the cells and tissues of the body (62). Han *et al.* extracted total RNA from the white blood cells of peripheral blood and identified differentially regulated genes using a microarray (63). Specifically, they used a panel comprised of five genes including B-cell scaffold protein with ankyrin repeats 1 (*BANK1*), B-cell novel protein 1 (*BCNP1*), cytidine deaminase (*CDS*), FERM domain containing 3 (*MGC20553*), and membrane-spanning 4-domains, subfamily A, member 1 (*MS4A1*) to detect CRC and found that this test had a sensitivity of 88-94% and a specificity of 64-77%.

Other markers

Three proteins, colon cancer-specific antigen (CCSA)-2, CCSA-3 and CCSA-4, have shown promise as markers for the detection of CRC. Getzenberg *et al.* identified several nuclear matrix proteins from CRC cell lines and tested some of them to determine if they would be useful as cancer biomarkers (64). Using a cutoff value of 2 µg/ml for CCSA-3, both CRC and advanced adenoma were detected with 89% sensitivity and 82% specificity. In addition, the sensitivity and specificity was 85% and 91%, respectively, when CCSA-4 was used with a cutoff value of 0.3 µg/ml. Finally, the use of CCSA-2 at a cutoff of 10.8 µg/ml had an overall specificity of 78% and sensitivity of 97% when used to separate individuals with advanced adenomas and CRC from normal, hyperplastic, and nonadvanced adenoma populations (65). Although the initial studies have had promising outcomes, the molecular identities of the three proteins have not yet been publically disclosed.

Remodeling of the extracellular matrix is important in the development of cancers, and several extracellular matrix proteins that can be liberated into circulation have been evaluated as potential biomarkers. The results of these evaluations have revealed that the serum levels of MMP9 and MMP7 depend on the presence of colorectal malignancy (66, 67). In addition, it has been suggested that serum laminin and MMP7 can be used as independent prognostic markers of CRC (67, 68).

Conclusion and perspectives

FOBT is currently the only screening modality for CRC. DNA-based fecal markers are promising but are not widely

used in clinical settings. In addition, a lack of sensitivity and specificity preclude the use of all existing serum markers for the early detection of CRC. CEA is used to monitor therapy in advanced CRC, and the preoperative level of CEA is used to provide prognostic information. However, there is insufficient evidence for routine use of other classic serum markers such as carbohydrate antigens and TIMP-1. Therefore, large-scale validation studies are required to evaluate the potential for the use of biomarkers that have recently been discovered through omics technology.

Genomics, proteomics, and a combination of both of these methods play major roles in the discovery of biomarkers. As with many other methods, each approach has its advantages and disadvantages. However, in light of the fact that many important functions of proteins require post-translational modification or their interaction with other protein(s), proteomics will continue to play a dominant role in the biomarker field. Clinical proteomics as a principal tool for the detection of differentially expressed proteins in samples from CRC patients versus healthy donors is required to significantly increase the repertoire of candidate biomarkers. In addition, steps should be taken to design adequate clinical trials and build a business model that can be used for the commercialization of newly discovered biomarkers. These efforts will hasten the translation of proteomic discoveries into clinical practice in the not-so-distant future.

Acknowledgements

This study was supported by grants from the Functional Proteomics Center of the 21st Century Frontier R&D Program funded by the Korean Ministry of Education, Science and Technology, Republic of Korea.

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