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REVIEW

# Use of blood-based biomarkers for early diagnosis and surveillance of colorectal cancer

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## Abstract

Early screening for colorectal cancer (CRC) holds the key to combat and control the increasing global burden of CRC morbidity and mortality. However, the current available screening modalities are severely inadequate because of their high cost and cumbersome preparatory procedures that ultimately lead to a low participation rate. People simply do not like to have colonoscopies. It would be ideal, therefore, to develop an alternative modality based on blood biomarkers as the first line screening test. This will allow for the differentiation of the general population from high risk individuals. Colonoscopy would then become the secondary test, to further screen the high risk segment of the population. This will encourage participation and therefore help to reach the goal of early detection and thereby reduce the anticipated increasing global CRC incidence rate. A blood-based screening test is an appealing alternative as it is non-invasive and poses minimal risk to patients. It is easy to perform, can be repeated at shorter intervals, and therefore would likely lead to a much higher participation rate. This review surveys various blood-based test strategies currently under investigation, discusses the potency of what is available, and assesses how new technology may contribute to future test design.



**Key words:** Colorectal neoplasms; Early detection of cancer; Colonoscopy; Biological markers; Blood; Messenger RNA; MicroRNA; Long non-coding RNA; DNA methylation; Microsatellite instability; Loss of heterozygosity; High-throughput nucleotide sequencing; Mass spectrometry; Real-time polymerase chain reaction; Microarray analysis

**Core tip:** Current colorectal cancer screening modalities are severely inadequate for global application because of high costs and a low participation rate. The alternative is to develop a blood-based screening test based on biomarkers which can replace colonoscopy as a first-line screening tool. The blood-based test should identify the high risk population, which will then be followed by colonoscopy as a secondary test. This review surveys the various experimental approaches and latest research into ideal biomarkers for the initial screening test, the pros and cons of each method and their potential to lead to a future screening test.

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## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and fourth most common cause of cancer death in the world[1]. It is anticipated that as global communities become more developed and the world population ages, the morbidity and mortality rates due to CRC will increase substantially[2]. Although a number of early detection screening modalities have been used extensively in developed nations to lower the incidence and mortality rate, their overall high cost and low participation rate render them to be ineffective in controlling CRC on the global scale. Therefore, an alternative first line screening modality that has high sensitivity, high specificity, is relatively inexpensive and easily implemented, is urgently needed to help reduce the expected increase in global CRC burden. The main purpose of this review is to investigate the potential application of blood-based biomarkers in early diagnosis and surveillance of CRC cases.

## URGENT NEED FOR A NEW CRC SCREENING MODALITY

Cancer is the leading cause of death in countries with a very high human development index and is poised to become a major cause of morbidity and mortality in every region of the world in the next few decades[3]. The United Nations has forecasted that the global population will reach 7.2 billion by July 2013, but population growth will slow in the next few decades, reaching 9.6 billion in 2050 and 10.9 billion in 2100 according to the medium-variant projection[4]. The United Nations report further delineated that the population growth will trend toward a balance between declining fertility rate and increasing population longevity. The increase in the aged population is expected to translate into an increasing global burden of cancer incidence[3,5]. In particular, it is anticipated that when the global population as a whole becomes more developed through rapid societal and economic changes, infection-related cancers (*i.e.,* cervical, stomach and liver cancers) will continue to decline but will be replaced with an increasing number of cancers associated with reproductive, dietary, and hormonal factors (*i.e.,* breast, colorectal, lung, and prostate cancers) as is typically found in high human development index regions.

Therefore, it is crucial to develop an early diagnostic modality for CRC that can be adaptable, economical, and implemented en masse by the global community.

### *Current screening options and their pros and cons*

In the United States, CRC is the third most common cancer diagnosed among men and women and the second leading cause of cancer death with the estimation of 142280 new cases and 50830 deaths in 2013[6]. The five-year survival rate is 90% for cancer found localized or confined to the bowel wall, 70% for cancer with lymph node involvement, and 10% for cancer that has metastasized. Clearly, these numbers demonstrate that screening and early detection would lead to better survival, prognosis, treatment options, and hence quality of life. In 1980, the American Cancer Society (ACS) issued a formal guideline for CRC screening in average-risk adults, including an annual digital rectal exam and stool guaiac slide test in addition to the performance of a sigmoidoscopy every three to five years[7]. Since the guideline was issued, the cancer morbidity and mortality rates, which peaked around 1985 in the United States, have been in steady decline[6]. It is conceivable that the decline of CRC rates is at least partially attributable to the implementation of early screening and surveillance programs[8].

As of 2008, the basic screening modalities remain remarkably similar to those used in 1980 when the original guideline was issued, even when taking into account the development of newer technology in subsequent years[8]. In general, ACS, American College of Radiology (ACR), and the United States Preventive Services Task Force (USPSTF)[9] all agree on and emphasize the importance of CRC screening[8,10-12]. The recommended CRC screening modalities can be roughly divided into two different categories: fecal tests and direct structural exams.

The fecal tests are essentially "blood in the stool" tests. They can be performed using either a hemoglobin test [the guaiac-based Fecal Occult Blood Test (gFOBT)] or a newer and more sensitive version of an antibody-based globin test, known as the immunochemical FOBT or Fecal Immunochemical Test (FIT)[13]. In general, the gFOBT test is a non-invasive, inexpensive and easily applicable screening test which patients can readily perform in the comfort of their own home. Specimens from a FIT must be submitted to a laboratory for testing. The fecal tests help to reduce the risk of CRC death but has no effect on all-cause mortality[14]. They are not specific tests for CRC markers, and if found positive, the presence of CRC must still be confirmed by a direct structural exam such as colonoscopy or imaging procedures[15]. The fecal tests have high false positive rate for detecting CRC as gastrointestinal bleeding may occur in other conditions like colitis and hemorrhoids[16-18]. This, therefore, increases the burden of unnecessary colonoscopies and anxiety among patients[19]. It also may not detect precancerous lesions or early stage adenomas as bleeding may not be readily detectable in the presence of these conditions[20,21]. Regarding the fecal tests in general, the opportunity for CRC prevention is both limited and incidental and they are therefore not recommended as the solo screening test for CRC[8].

Direct structural exams include endoscopic procedures, such as flexible sigmoidoscopy and colonoscopy, and imaging procedures, such as double-contrast barium enema and computed tomographic colonography. In general, both flexible sigmoidoscopy and colonoscopy are invasive procedures using a colonoscope. Sigmoidoscopy is a small-scale colonoscopy which can be performed with a simple preparation without sedation, and

is used to examine the lower half of the colon lumen as opposed to the entire colon. The complete colonoscopy allows direct mucosal inspection of the entire colon from the appendiceal orifice to the dentate line. Same-session biopsy sampling or definitive treatment by polypectomy in the case of precancerous polyps and some early-stage cancers can also be performed. The double-contrast barium enema and computed tomographic colonography are both imaging examinations of the colon in its entirety and are either noninvasive or minimally invasive. However, although they allow for complete examination of the colon, there is no opportunity for biopsy or polypectomy and must therefore be followed up by therapeutic colonoscopy when polyps are found.

### *Inadequacy of colonoscopy*

In the United States, colonoscopy has become the gold standard of CRC screening. It is one of the critical screening procedures recommended by ACS, ACR, and USPSTF, and it is also recommended by the American College of Gastroenterology as the preferred screening test[22]. The principal benefit of colonoscopy is that it allows for a full structural examination of the colon and rectum in a single session and for the detection of colorectal polyps and cancers accompanied by biopsy or polypectomy. Therefore, it has been performed with much higher frequency than all other procedures[23].

However, even in the United States where the technology and procedure are widely available, the colorectal screening participation is still low among average-risk adults in the range of 29.8% to 55.2%[24]. The participation rate is also surprisingly low at 40% for people at increased risk of CRC[25,26]. The majority of United States adults are not receiving regular age- and risk-appropriate screening due to concerns of cost, risk, and the discomfort and cumbersome preparation associated with the procedure[27-29]. The same is true in other European and Asian nations[2,30-32].

Although colonoscopy is the most effective screening method for CRC, there are various reported risks associated with the procedure, including bleeding (1.64 per 1000 patients), perforation (0.85 per 1000), death (0.074 per 1000), missed adenoma (6%-12%), and missed cancer (5%)[33]. The observed rate of missed polyps and/or cancer are largely due to variations in polyp size and other factors such as sub-optimal bowel preparation, experience of the endoscopists, and patient anatomical variations[34]. When it is taken into consideration that the guideline for the average-risk adult is to undergo colonoscopy every 10 years beginning at age 50[8,22] coupled with the rate of missed polyps being between 6% and 12%, there is still risk of developing CRC even when regular colonoscopy screening guidelines are followed.

### *Importance of an alternative screening method for CRC*

The goals of any test are to detect disease early, improve duration and quality of life, reduce mortality and/or morbidity, and augment patient participation for that disease process-all at a very low risk and cost. To this end, the current CRC screening modality based on colonoscopy is severely inadequate. Despite all of the benefits that colonoscopy can offer as a screening procedure for CRC, concerns about its cost, risks, cumbersome preparatory procedure, and willingness of the general public to participate seriously compromise its effect in undermining the global CRC burden[35-37].

In an ideal world, the first line screening should be performed to identify a high risk segment of the population and then use a more extensive test (colonoscopy) on this sub group to reduce incidence of advanced diseases. In other words, it is crucial for the first line screening program to separate the following three entities: the general population (average risk), high risk group, and cancer group. Despite its non-specific nature, the simple FIT, when coupled with colonoscopy, has helped to dramatically reduce cancer incidence and number of deaths - In 100000 average risk patients, this screening has helped to reduce the number of cancer cases from 4875 to 1393, and number of cancer deaths from 1782 to 457[38]. Therefore, a more effective and sensitive blood-based biomarker test, supported by evidence from larger studies with solid results, can readily replace the stool-based test.

In order to establish a screening test, it must be evaluated for the following elements: frequency of performance, risk of complications, limitations, and false positive and negative rates. A blood-based test could be ideally used as a first line screening if all these elements were reliably determined and optimized. Colonoscopy would then become the secondary test, not the primary one. There will be greater willingness, by physicians and patients alike, to perform a blood test every several years than to justify the bowel preparation and complications of colonoscopy every 5-10 years.

## BLOOD-BASED BIOMARKER FOR SCREENING CRC

Blood vessels are the human body's internal superhighways, for transporting nutrients to all cells in the body and carrying away waste products to avoid toxin buildup. Furthermore, they are also the body's chief communication channel into which signaling molecules such as hormones and cytokines are secreted and released in order to regulate a cascade of effector cell functions on distant sites. It would be ideal, therefore, to take advantage of this superhighway, with all of its abundant signaling molecules, to gauge a patient's health status.

The idea of a blood-based molecular test is appealing because the specimens can be obtained readily in a non-invasive manner, and it can be easily performed for any patient with minimal risk. If it were available, a blood-based test for CRC would reduce the overall cost, risk, and low patient participation issues that are typically associated with colonoscopy[39]. The key to developing a

useful blood-based molecular test is to find specific molecular indicators in the blood that are sensitive and specific for the detection of CRC. These indicators can be present in plasma or serum, and any form of molecules, including RNA, DNA, and protein[40-44].

Recent advances in the development of molecular diagnostic technology have allowed an expanding list of potential new screening modalities based on blood specimens to emerge. The available technologies, their current status, and their potential application will be discussed in further detail below.

### Circulating RNA markers

RNA was originally thought to be highly labile, easily degradable, and therefore not likely to be stable or detectable outside of the protective cellular environment. However, numerous recent studies have shown that RNA are actually stable outside of cells[45,46], and all species of RNA, including both coding messenger RNA (mRNA)[47] and non-coding RNA, which includes microRNA (miRNA) and long non-coding RNA (lncRNA)[48,49], can be extracted and detected in the circulating blood plasma, serum, and other bodily fluids[50-52]. Furthermore, RNA expression is highly regulated in normal state but becomes increasingly dysregulated in a pathological state such as cancer[48,53]. Therefore, numerous studies have focused on profiling RNA expression, which may correspond to cancer state, and finding the indicator biomarkers for cancers[54-57].

### mRNA markers

Various research groups have investigated the potential use of circulating mRNA as markers for cancer. The general experimental strategy is to employ microarray technology for mRNA expression profiling, which is then followed by validation using real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The specimens used are either mRNA extracted directly from blood serum/plasma or from peripheral blood cells[58]. Kopreski *et al*[47] demonstrated the possibility of detecting tumor mRNA, tyrosinase, in the serum of malignant melanoma patients although the result remains controversial[59]. Tsouma *et al*[60] extracted RNA from peripheral blood cells and used the multiplex RT-qPCR technology to determine the expression of three transcripts (carcinoembryonic antigen, cytokeratin 20 and epidermal growth factor receptor) to determine the disease stage and overall survival of CRC patients. DePrimo *et al*[61] and Twine *et al*[62] performed microarray-based mRNA expression profiling in peripheral blood mononuclear cells in 2003 and proposed some potential markers. However, this research generally remained at a proof-of-concept or pilot study stage, and further follow-up study has been sparse as the strategy they originally employed is now gradually being replaced by the new technology of Next Generation Sequencing (NGS), which will be discussed in more detail later.

### ColonSentry as CRC screening or risk-assessment test?

Marshall *et al*[63] from GeneNews Ltd. developed a blood-based test using a seven-gene biomarker panel (*ANXA3, CLEC4D, LMNB1, PRRG4, TNFAIP6, VNN1* and *IL2RB*) testing RNA extracted from peripheral blood cells. This seven-gene panel was derived from a 196-gene expression profile using 112 CRC patients (including those with stage Ⅰ, Ⅱ, Ⅲ, and Ⅳ disease) and 120 controls. The panel was confirmed using 202 CRC patients (from all stages) and 208 controls, all from the Canadian population. They reported a sensitivity of 72% and specificity of 70% for this initial study. Then, they validated the seven-gene profile using 99 CRC patients (presumably from all stages) and 111 controls from the Malaysian population and reported 61% sensitivity and 77% specificity[64]. The researchers further validated their panel with an even larger population of 314 CRC patients (from all stages) and 328 controls from Canada and the United States, and they reported a sensitivity of 78% and specificity of 66%[65]. GeneNews now offers the ColonSentry test, presumably based on this seven-gene profile, as the world's first commercially available blood test for colon cancer screening, which is licensed to Enzo Clinical Labs of Enzo Biochem for the United States market. The test has recently been approved by the New York State Department of Health as a test to determine a person's risk of having CRC[66].

The ColonSentry molecular diagnostic test is marketed as a risk assessment tool rather than a cancer detection test. Although the experimental design for this seven-gene profile appeared to focus on identifying a pan-CRC marker panel when it profiled and validated a total of 727 CRC patients from all stages (estimated to be 30% stage Ⅰ, 30% stage Ⅱ, 30% stage Ⅲ, and 10% stage Ⅳ), there is no mention of any study on high risk individuals, advanced adenomas (AA), or patients with colon polyps that ultimately turned cancerous. It is therefore unclear how a set of pan-CRC markers for all CRC stages can be marketed as a risk assessment test. In any case, the test is considered experimental and investigational with many independent experts still questioning its effectiveness.

### MiRNA as blood-based cancer markers

MiRNA are small non-coding RNA about 18-25 nucleotides in size[67]. A large body of publications indicates that miRNA regulate gene expression at the post-translational level in almost every biological event and play important roles in tumorigenesis, cancer development, migration and metastasis[68]. The differential expression of miRNA has been related to various cancers[69], and efforts have been made to profile the global and circulating miRNA expression patterns associated with various cancers, including breast cancer[70], lung cancer[71], lymphoma[72], ovarian cancer[73], and pancreatic cancer[74,75].

For CRC, studies have accumulated over the past five years that focus on profiling circulating blood plasma or serum miRNA and validating the findings with RT-qP-

CR. Ng *et al*[76] was the first group to profile 95 miRNA using a real-time PCR-based array on 5 CRC patients and 5 controls (presumably from the Chinese population in Hong Kong) and to validate the results with 90 CRC patients and 50 healthy controls. They identified miR-17-3p and miR-92 to be elevated significantly in CRC patients with 89% sensitivity and 70% specificity. Wang *et al*[77] profiled 742 miRNA using a miRNA microarray on 10 CRC patients and 10 normal controls from the Chinese population and validated the results with 90 CRC patients, 43 AA patients, and 58 healthy donors. They found miR-601 and miR-760 to be decreased in both CRC and AA patients when compared to healthy controls with 83.3% sensitivity and 69.1% specificity. Giráldez *et al*[78] performed a genome-wide profiling of 743 miRNA using a miRNA microarray on 21 CRC patients, 20 AA patients, and 20 healthy controls from the Spanish population, and they validated the findings using RT-qPCR with 42 CRC patients, 40 AA patients, and 53 controls. They identified a six-miRNA panel (miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335) as being able to differentiate CRC patients from healthy individuals with 78.57% sensitivity and 79.25% specificity, and miR-18a could also differentiate AA patients from healthy individuals with both 80% sensitivity and specificity. Luo *et al*[79] used a TaqMan MiRNA array to profile 667 miRNAs on 50 CRC patients and 50 controls from the German population and validated the results with new cohorts of 80 CRC patients compared to 144 controls and 50 AA patients compared to 50 controls. They identified nine miRNA (miR-18a, miR-20a, miR-21, miR-29a, miR-92a, miR-106b, miR-133a, miR-143, and miR145) to be differentially expressed in CRC patients and controls with the area under the accompanying receiver operating characteristic curve reported to be 0.745. The panel of miRNA did not, however, differentiate AA patients from the controls. Kanaan *et al*[80] screened for 380 miRNA using microfluidic TaqMan array technology on 20 CRC patients, 9 AA patients (referred to as colorectal adenomas), and 12 healthy donors of mixed racial background in the United States. They then validated the findings with a new cohort of 45 CRC patients, 16 AA patients, and 26 healthy controls; they derived an eight-miRNA panel (miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532-5p and 532-3p, and miR-652) that can distinguish AA patients from controls with 88% sensitivity and 64% specificity, and a three-miRNA panel (miR-431, miR-15b, and miR-139-3p) to differentiate stage Ⅳ CRC patients from controls with 93% sensitivity and 74% specificity. Ahmed *et al*[81] performed a profiling using miRNA microarray chips covering miRNA based on the published miRBase v17 list (presumed to be 1733 human miRNA) and validated their results using TaqMan RT-qPCR to analyze a panel of miRNA expression both in CRC patient plasma and tissues. They found nine miRNA (miR-7, miR-17-3p, miR-20a, miR-21, miR-92a, miR-96, miR-183, miR-196a and miR-214) to have increased expression and six miRNA (miR-124, miR-127-3p, miR-138, miR-143, miR-146a, and miR-222) to have reduced expression in both CRC patient plasma and tissues with 90% sensitivity and 95% specificity.

A few studies selected their miRNA markers based on published literature and re-confirmed the results with RT-qPCR assays. Huang *et al*[82] measured the levels of twelve miRNAs (miR-17-3p, -25, -29a, -92a, -134, -146a, -181d, -191, -221, -222, -223, and -320a) studied in the literature in 120 CRC patients, 37 AA patients, and 59 healthy controls from the Chinese population, and they confirmed miR-29a and miR-92a as potential indicators for CRC with 83% sensitivity and 84.7% specificity. Similarly, Liu *et al*[83] measured the levels of five miRNAs (miR-18a, -21, -31, -92a, and -106a) in serum samples from 200 CRC patients, 50 AA patients, and 80 healthy controls from the Chinese population and identified miR-92a along with miR-21 to be both significantly higher in CRC patients with 68% sensitivity and 91.2% specificity. Pu *et al*[84] measured miRNA expression levels of three target miRNAs (miR-21, -221, and -222) in 103 CRC patients and 37 controls from the Chinese population and found elevated expression of miR-221 in CRC patients with 86% sensitivity and 41% specificity. Wang *et al*[85] screened three miRNAs (miR-29a, -92a, and -17-3p) in 38 metastatic CRC and 36 primary CRC patients, assumed to be from the Chinese population, but did not utilize healthy controls. They found miR-29a to be higher in CRC patients with liver metastases than in primary CRC patients with sensitivity and specificity of 75%, and hence miR-29a may be useful in discriminating metastatic from non-metastatic CRC patients. Cheng *et al*[86] screened three miRNAs (miR-21, -92, and -141) using a cohort of 102 CRC patients and an age-matched cohort of healthy donors of mixed racial background from the United States population, validated their findings using 156 CRC patients and matched controls from the Chinese population, and found miR-141 to be higher in cases of advanced CRC (stage Ⅳ) with 90.9% sensitivity and 77.1% specificity.

As summarized in Table 1, there are a total of 38 miRNA that have been studied and proposed as potential biomarkers for CRC in the publications discussed above. In general, most of these studies focused on early stage CRC patients while some also included borderline AA patients. When pooling from all the studies mentioned here, sensitivities in the range of 68%-91% were reported, but the majority (in 9 out of 12 cases) observed sensitivities in the 83%-91% range. Reported specificities were in the range of 41%-95%, but the majority (also in 9 out of 12 cases) were in the 70%-95% range. Some miRNA, including miR-15b, miR-17-3p, miR-18a, miR-20a, miR-21, miR-29a, and miR-92a, have been proposed by more than one group of investigators. One unique miRNA, miR-21, might actually be a useful pan-cancer marker as it is similarly up regulated in other cancers[87]. However, most of these studies have not yet been evalu-

**Table 1 Potential blood microRNA markers**

| MiRNA | AA? | Ref. |
|---|---|---|
| Upregulated in primary CRC | | |
| miR-7 | | [81] |
| miR-15b | √ | [78,80] |
| miR-17-5p | √ | [80] |
| miR-17-3p | | [76,81] |
| miR-18a | √ | [78,79] |
| miR-19a | | [78] |
| miR-19b | | [78] |
| miR-20a | | [79,81] |
| miR-21 | | [79,81,83,87,160-163] |
| miR-29a | | [78,79,82] |
| miR-92a | | [76,79,81-83] |
| miR-96 | | [81] |
| miR-106b | | [79] |
| miR-133a | | [79] |
| miR-142-3p | √ | [80] |
| miR-143 | | [79] |
| miR-145 | | [79] |
| miR-183 | | [81] |
| miR-195 | √ | [80] |
| miR-196a | | [81] |
| miR-214 | | [81] |
| miR-221 | | [84] |
| miR-331 | √ | [80] |
| miR-335 | | [78] |
| miR-532-5p | √ | [80] |
| miR-532-3p | √ | [80] |
| miR-652 | √ | [80] |
| miR-1246 | | [164] |
| Upregulated in metastatic CRC | | |
| miR-15b | √ | [80] |
| miR-29a | | [85] |
| miR-139-3p | | [80] |
| miR-141 | | [86] |
| miR-431 | | [80] |
| Downregulated in primary CRC | | |
| miR-124 | | [81] |
| miR-127-3p | | [81] |
| miR-138 | | [81] |
| miR-143 | | [81] |
| miR-146a | | [81] |
| miR-222 | | [81] |
| miR-601 | √ | [77] |
| miR-760 | √ | [77] |

AA: Able to differentiate advanced adenoma; CRC: Colorectal cancer; MiRNA: MicroRNA.

ated beyond the proof-of-principle and pilot stage, and not all miRNA markers were subsequently studied and confirmed by other groups. For example, Faltejskova *et al*[88] was not able to confirm the potency of miR-17-3p, miR-29a, miR-92a, and miR-135b as biomarkers for CRC. Luo *et al*[79] and Ahmed *et al*[81] found differential miR-143 expression in their respective studies. Other potential markers such as miR-17-3p, miR-18a, miR-21, miR-92, and miR-221 were not confirmed in follow-up studies by other groups[82-84,86].

Clearly, it is comprehensible that different experimental designs, procedures and methods, endogenous controls, patient populations, instrumentation and lab personnel could contribute to the seemingly contradicting results that have been published thus far. Nevertheless, the 38 candidate miRNA markers together can be further investigated using currently available technology, such as the TaqMan RT-qPCR profile platform already utilized by some of the research groups. It is possible, therefore, to coordinate a multicenter clinical trial involving different research groups and incorporating patient populations from a wide variety of backgrounds. It would be critical to synchronize specimen collection, processing procedures, and storage conditions for the collected specimens. The experimental design should also be based on a coordinated and synchronized set of experimental procedures and instrumentation that utilize the same endogenous control(s). The validity of each of the 38 miRNA markers as a tool for diagnosing CRC can then be evaluated for their potential future application.

## NEXT GENERATION SEQUENCING

Since the first drafts of the human genome were published in 2001, sequencing technology has advanced at an ever rapid pace[89]. The cost of sequencing has decreased from about $1000 per megabase of DNA sequence when the first generation Sanger-based sequencing machine was used in 2001, down to $0.1 per megabase of DNA sequence using the next generation sequencing machine in 2013[90,91]. The cost for personal whole-genome sequencing has dropped from $100000000 in 2001 to $4000 (sequencing offered by Illumina, Inc.) in 2013, and it could possibly be driven further down to $1000 in the imminent future[92]. The availability of the NGS has revolutionized biomarker studies[93]. It is now possible to perform direct RNA sequencing (RNA-seq)[94] to sequence the whole transcriptome, which includes the entire set of all RNA molecules-coding RNA (mRNA, rRNA, tRNA) and non-coding RNA (miRNA, lncRNA, and other small RNA species)[94,95].

RNA-seq is very versatile and has been used to analyze tissue RNA biomarkers in breast cancer[96], hepatocellular carcinoma[97], lymphoma[98,99], melanoma[100,101], and prostate cancer[100]. RNA-seq has also been used to analyze gene expression signatures associated with survival[100], smoking status[102], and altered expression associated with *KRAS* mutation[103] in lung cancer. In terms of CRC, Wu *et al*[104] have performed transcriptome profiling comparing CRC, adjacent normal, and distant normal tissues and have identified 5 differentially expressed genes, including *ITGB5, COL1A1, FN1, SPP1,* and *COL3A1*, as well as alternative splicing, isoforms, and gene fusion events. It is anticipated that with the ability to extract and sequence RNA from blood plasma, more studies on blood-based RNA markers, based on RNA-seq technology, will soon emerge.

### *lncRNA markers*

Given the increased availability of RNA-seq technology, it is now possible to study the lncRNA, which was dismissed as "junk" in the past but has now been found to regulate gene expression and cellular functions[105].

LncRNA, like its miRNA counterpart, plays major roles in tumor suppression and oncogenic functions and has been found to be dysregulated in human cancers[106]. Therefore, its potential role as biomarkers for cancer and other diseases has been investigated extensively[107,108]. As an example, Prostate cancer antigen (PCA3, also known as DD3) is a non-coding RNA that is highly sensitive and now used as a biomarker for the urine diagnostic test of prostate cancer[109-111].

In terms of CRC, research is currently focused on the role of lncRNA as tissue biomarkers. Ge *et al*[112] found that Prostate cancer-associated ncRNA transcripts 1 was upregulated in CRC tissue but not in adjacent normal tissue. Zhai *et al*[113] found that long intergenic noncoding RNA-p21 was upregulated in CRC tissue, and the expression level seemingly correlated with tumor progression (higher expression in later stages). Ling *et al*[114] showed a novel lncRNA-CCAT2 was highly overexpressed in CRC, and it was shown to be promoting tumor growth, metastasis and chromosomal instability. Kogo *et al*[115] demonstrated that expression of lncRNA-HOTAIR, which is known to reprogram chromatin organization and promote breast cancer metastasis[116], is also higher in stage Ⅳ CRC patients with liver metastases. Xu *et al*[117] found the lncRNA-human metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) to be dysregulated in cancer, and the mutation on the 3' end of MALAT-1 is apparently tumorigenic. It is conceivable that RNA-seq technology can help facilitate further investigation into lncRNA functions and exploration of blood-circulating lncRNA as potential biomarkers for CRC and other cancers in the future.

## BLOOD-BASED CIRCULATING DNA MARKERS

The presence of tumor DNA in circulating blood (plasma or serum) has been documented dating back to 1977[118]. Cell-free DNA (cfDNA) was thought to be released from either apoptotic or necrotic cancer cells, from direct secretion or as a byproduct of phagocytosis from macrophages or other scavenger cells[119,120]. Originally, it received little attention, but with recent advances in next generation sequencing (NGS) technology, it has been explored extensively for the potential application to cancer detection[121]. In general, the studies of cfDNA as cancer biomarkers focus on monitoring the presence of promoter hypermethylation, aberrant tumor DNA mutation, microsatellite alternations, and mitochondria DNA in blood circulation. The validity of each approach will be discussed below.

### *Aberrant DNA methylation as markers*

Aberrant DNA methylation has been associated with tumorigenesis as a consequence of the alteration it causes in gene expression[122,123]. For example, hypermethylation of tumor suppressor promoter genes would cause inappropriate gene silencing and therefore lead to cancer[124]. In general, DNA methylation is thought to be associated with an early event in tumorigenesis and has therefore been proposed as a potential early cancer detection marker[123,125]. The research strategy typically focuses on using methylation specific PCR (MSP) to study hypermethylation of methylation sites, in CpG dinucleotides or in CpG islands, in the promoters of tumor suppressor genes[124,126]. In the context of CRC, Nakayama *et al*[127] and Lecomte *et al*[128] both monitored the hypermethylation of the promoter of tumor suppressor gene *p16* and found the plasma in 21 of 31 (68%) patients and 31 of 45 (69%) patients, respectively, to be positive. Grady *et al*[129] found aberrant hypermethylation of the human MutL homolog 1 (*hMLH1*) promoter in the sera of 9 out of 19 (47%) cases of CRC. Leung *et al*[130] monitored promoter hypermethylation in three genes, adenomatous polyposis coli (*APC*), *hMLH1,* and *helicase-like transcription factor,* and found at least one of the three genes with methylated promoter DNA in the sera of 28 out of 49 (57%) CRC patients. Additional genes monitored for tumor-related promoter hypermethylation, including the putative metastasis suppressor gene *death-associated protein kinase*, the detoxification gene *glutathione S-transferase P1*, the DNA repair gene *O*$^6$*-methylguanine-DNA-methyltransferase,* and *p14-ARF* in other cancers exhibit a detection rate that is generally in the range of 42% to 73%[131-133]. It is conceivable that NGS technology can be coupled with MSP to identify a pool of tumor suppressing genes silenced in association with early stage CRC and AA, test their corresponding promoter methylation, and generate a set of candidate markers based on epigenetic changes as a screening panel for CRC in the future.

### *Aberrant tumor DNA mutation markers*

The NGS technology has been employed for somatic mutation analysis in CRC[134], particularly on several high mutation frequency genes, such as *K-RAS*[128,135,136], *TP53*[137], and *APC*[138]. However, the percentage of circulating tumor DNA is relatively low when compared to wild-type DNA[139]. For example, Diehl *et al*[138] has shown that in advanced CRC, the mutated *APC* DNA fragment is found to be in the range of 1.9% to 27% of cfDNA but only 0.01% to 0.12% in early stage CRC. Even with direct sequencing technology, it does not allow reliable detection of less than 25% mutant signal in a background of wild-type DNA[140]. Furthermore, the tumor-associated mutations are often unique with each patient[141,142], and therefore, based on the current available technology, it is less likely to develop a low cost and highly sensitive comprehensive test to cover all somatic mutations for early cancer detection.

### *Microsatellite alterations as markers*

Microsatellite alterations, which include microsatellite instability (MSI) and loss of heterozygosity (LOH), are known to be associated with tumorigenesis and cancer

progression and therefore were proposed as potential tumor markers detectable in cfDNA[143]. MSI analysis focuses on measuring the specific polymorphic tetranucleotide repeat and/or dinucleotide markers that are located in regions frequently shifted or altered in cancer, and LOH analysis focuses on the loss of specific chromosomal regions bearing tumor suppressors. Hibi *et al*[141] examined microsatellite alterations and found LOH or microsatellite shift of at least one locus (18a, 17p, and 8p) in 35 of 44 (80%) primary CRC tumors, but none of the LOH or microsatellite shifts were detected in the corresponding serum DNA. Several other groups focused on different cancers with most success in metastatic cancers[143,144]. In general, microsatellite alteration analysis exhibits relatively low sensitivity and specificity in detecting early stage cancer.

### *Circulating mitochondrial DNA as markers*

There are generally a few hundreds of copies of mitochondrial DNA in each cell[145]. Due to its multi-copy nature, mtDNA is frequently found to be heteroplasmic, with a heterogeneous mixture of polymorphic variants. In cancer cells, mtDNA harbor further heteroplasmic alterations associated specifically with cancer, especially in the highly variable D-loop (displacement loop) region. With the NGS, the approaches generally focused on either differential copy number of mtDNA versus gDNA, or mtDNA alteration and tumor-associated mtDNA mutations[146]. For CRC, Hibi *et al*[147] has studied mtDNA alternation in early CRC patients and found that 7 out of 77 (9%) CRC tissues contained true somatic mutations in the D-loop region, but only one out of these 7 positive patients (14%) were noted to have mtDNA alterations in their serum DNA. Due to of the relatively low detection rate of early stage cancer, most studies therefore focused on its potential application in metastatic cancers[148-153].

## IDENTIFICATION OF BLOOD-BASED PROTEIN MARKERS

The study of blood-based protein markers in general focuses on proteins secreted, shed or leaking from cancer cells into the blood stream. This is generally referred to as "cancer secretome"[154]. The cancer secretome can be studied comprehensively by several mass spectrometric technologies. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and HPLC-electrospray ionization mass spectrometry (ESI-MS) analyze biomolecules in biological fluids[155,156]. Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) can be used as a serum protein profiler to identify new biomarkers[157]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can fractionate and identify the specific molecules of interest[154]. There is also an Aptamer proteomic technology that can be used to identify biomarkers for cancer[158]. Many candidate protein biomarkers have been generated based on these technologies.

However, the application of these technologies remains research-oriented. The potency of their translational capability in clinical and diagnostic application requires further investigation[159].

## CONCLUSION

Early screening of CRC is clearly the most effective way to combat the anticipated increase of global CRC morbidity and mortality. Despite all recent technological advances, the currently available screening modalities remain archaically similar to 33 years ago. The most effective screening modality today is through the invasive procedure of colonoscopy. However, even in the United States, where the procedure is widely available and publicized, covered by most medical insurance plans, and recommended by medical professionals and practitioners, the participation rate is still pathetically low. It is conceivable that the participation rate would not fare better even if it were widely available on a global scale. Clearly, a new first line CRC screening procedure that is inexpensive, low risk, highly sensitive, and does not require cumbersome preparation is desirable.

A blood-based screening test for CRC would be an attractive alternative to colonoscopy if it were available because it is essentially non-invasive and relatively painless to the patient. Ideally, a blood-based test can be a useful first line screening tool for the general population at average risk, thereby separating out high risk and CRC patient groups. However, for patients with known high risk factors, including family history of CRC, familial adenomatous polyposis, hereditary nonpolyposis CRC, inflammatory bowel disease, history of polyps, or previous CRC, colonoscopy should still be the primary method of screening and follow-up starting at age 50, although a blood-based test can still be used for screening these patients earlier at age 40. In short, circumstances under which a blood-based screening test is used should be determined based on the sensitivity and specificity of the methodology developed in the future.

The key to establishing a good blood-based test is to find highly sensitive and specific biomarkers in the blood. As discussed in this review, various types of biomarkers have been proposed and explored by many research groups to varying degrees. Table 2 summarizes the sensitivity, specificity, and estimated cost for the types of stool-based tests, structural exams, and potential blood-based tests as discussed in this review. The ColonSentry® seven-gene mRNA biomarker panel is the first commercially available blood test that is supposed to determine the risk of developing CRC. The sensitivity and specificity for this "risk assessment" are 78% and 66% respectively. As shown in Table 2, among all the biomarker types, the miRNA markers demonstrated the greatest potential because most publications reported a relatively high sensitivity (83%-91%) and specificity (70%-95%) rate, utilized mostly AA and early stage CRC patient, and

**Table 2 Comparison of colorectal cancer screening tests**

| Test name | Cost | Procedure type | Prep? | Sensitivity | Specificity | Note | Ref. |
|---|---|---|---|---|---|---|---|
| gFOBT | $5[3] | Stool test | Yes[1] | 12%[2] and 40% | 98% | Hemoccult Ⅱ | [165] |
| iFOBT/FIT | $22[3] | Stool test | Yes[1] | 22%[2] and 70% | 95% | | [165] |
| Fx. Sigmoidoscopy | $500-$750[3] | Invasive | Yes | 95%[2] and 95% | 92% | | [165] |
| Colonoscopy | $800-$1600[3] | Invasive | Yes | 95%[2] and 98% | 90% | | [165,166] |
| DCBE | $250-$500[3] | X-ray | Yes | 48%[2] | 90% | Not recommended by USPSTF | [166] |
| CTC | $400-$800[3] | CT-scan | Yes | 59%[2] | 96% | Not recommended by USPSTF | [166] |
| Blood-based test | | | | | | | |
| ColonSentry® | $350 | blood-test | No | 78% | 66% | GeneNews/Enzo Biochem | [66] |
| MiRNA (5-gene) | Est. $250[4] | blood-test | No | Est. 83%-91% | Est. 70%-95% | | |
| LncRNA (1-gene) | $385.00[5] | blood-test | No | N/A | N/A | | |
| DNA methylation | Est. $250[4] | blood-test | No | Est. 42%-73% | Est. 42%-73% | | |

[1]Required to clean colon; [2]For detecting advanced adenoma at ⩾ 10 mm; [3]Cost estimated from Colon Cancer Alliance website (http://www.ccalliance.org/index.html); [4]Cost estimated based on The Valley Hospital Histology Lab charge; [5]Cost estimated based on PCA3 test offered by GD Specialized Diagnostics. Fx. Sigmoidoscopy: Flexible Sigmoidoscopy; DCBE: Double-contrast barium enema; CTC: Computed tomographic colonography; USPSTF: United States Preventive Services Task Force; gFOBT: Guaiac-based Fecal Occult Blood Test; iFOBT: Immunochemical Fecal Occult Blood Test; FIT: Fecal Immunochemical Test; MiRNA: MicroRNA; LncRNA: Long non-coding RNA; CT: Computed tomography; N/A: Data not available; PCA3: Prostate cancer antigen 3.

studied a wide variety of patient populations. Therefore, a multi-center clinical trial with synchronized experimental procedures that tested all 38 miRNA listed in Table 1 could be considered. On the other hand, the aberrant DNA methylation analyses on promoters of tumor suppressors also demonstrated a high potential to be developed into a cancer screening test. With available NGS technology and MSP showing relatively high sensitivity and specificity (42%-73%), it is now possible to explore more tumor-specific promoters, which might have higher sensitivity and specificity and eventually be developed into a screening test.

On the other hand, although research studies of lncRNA markers using NGS are still at the early stage, it has a great potential to be developed into a CRC screening test as well. It is especially encouraging to see one of the lncRNA, PCA3, is now used routinely as a prognostic marker for prostate cancer. With the wider availability of NGS, it is anticipated that more studies will be undertaken to generate new candidate genes and biomarkers, which would possibly lead to a future diagnostic test for CRC.

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