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Fecal miRNAS as Biomarkers for the Detection of Colorectal Cancer

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Abstract

As the third most common type of cancer, colorectal cancer (CRC) is a leading cause of cancer-related morbidity and mortality worldwide. A major priority in the management of CRC is screening. Therefore, the search for new noninvasive biomarkers to facilitate the early detection of CRC is particularly warranted. The detection of molecular markers from fecal samples is a potential strategy for CRC screening. MicroRNAs (miRNAs) are a group of highly conserved endogenous short non-coding RNA transcripts; they play critical roles in carcinogenesis, and aberrant expression of miRNAs has been observed in various types of cancer, including CRC. Their unique stability makes fecal miRNAs promising as biomarkers for the early diagnosis of CRC. In this review, we explored the literature and summarized the role miRNAs play in CRC, focusing specifically on the potential diagnostic utility of fecal miRNA as biomarkers.

Keywords: Colorectal cancer; Fecal; MicroRNA; Biomarker

Colorectal Cancer

CRC incidence and screening

As the third most common type of cancer, colorectal cancer (CRC) is a leading cause of cancer-related morbidity and mortality worldwide. In Europe, the incidence and mortality of CRC is second only to breast and lung cancer, respectively, and comprises nearly 13% of all cancer cases [1]. The American Cancer Society (ACS) estimates that approximately 1.4 million new cases of CRC will be diagnosed globally, and more than 0.5 million people will die from this disease in 2012 [2,3]. Fortunately, CRC is regarded as a preventable disease when the preneoplastic lesions are detected early [4].

The major priority for the management of CRC is screening. The mortality rates of CRC have declined modestly, largely due to the application of proper screening tests for early detection [5]. According to clinical statistic reports, the five-year survival rate could reach 80% if CRC patients were diagnosed during the early stages of the disease, thereby allowing curative surgery to be performed. The screening guidelines for the early detection of adenomatous polyps and CRC in average-risk populations were updated in 2008 [6]. Following these guidelines, individuals aged 50–75 years who present an average risk for CRC should be screened using one or more of the following methods: 1) annual high-sensitivity guaiac-based fecal occult blood test (gFOBT) and/or fecal immunochemical test (FIT), following the manufacturer's recommendations for fecal specimen collection; 2) flexible sigmoidoscopy (FSIG) every 5 years or combination of FSIG performed every 5 years with a highly sensitive gFOBT or FIT performed annually; 3) colonoscopy every 10 years; 4) double-contrast barium enema every 5 years; or 5) computed tomography (CT) colonography every 5 years [3,7].

Many screening assays have been implemented in the health care system for the early detection of CRC. Conventional CRC screening largely relies on the fecal occult blood test (FOBT) and sigmoidoscopy/colonoscopy [8]. Unfortunately, these methods suffer from low cost-effectiveness and limited diagnostic accuracy. Although colonoscopy is regarded as the gold standard for the diagnosis of CRC, this procedure is operator-dependent and costly and introduces the risk of major complications [9]. In contrast, FOBT is the most widely adopted noninvasive CRC screening method and is responsible for a 15-33% reduction in CRC-related mortality [10]. However, FOBT suffers from several crucial limitations, including a low diagnostic accuracy for detecting CRC (33-50% sensitivity) and adenomas (11% sensitivity) [4,11,12]. Therefore, a highly accurate, affordable, and non-invasive test for the early detection of CRC is urgently needed.

CRC Biomarkers

CRC-associated protein-based markers

Over the past few decades, a variety of molecular biomarkers have been investigated for CRC detection [13-15]. Among these biomarkers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are the two best-characterized serum protein markers. Studies have shown that high CEA levels are associated with cancer progression; however, its utility in CRC screening is limited because the serum level does not typically become elevated until the tumor has penetrated the serosal membrane [16]. CA19-9 is another well-known gastrointestinal (GI) track tumor marker, which becomes elevated in many types of GI disease and cancer. Although CA19-9 increased in 67% of a sample of stage IV CRC patients, it could neither be used to detect stage I tumors nor to distinguish CRC from other GI tumors, thus rendering it unqualified for the early detection of CRC [17].

Other potential CRC detection protein markers have also been reported. A diagnostic ELISA, for example, based on a combination of mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3) and activin receptor type-2B (ACVR2B) protein, has been shown to distinguish patients with CRC from healthy individuals with a high level of diagnostic power [18]. Furthermore, mounting evidence shows the existence of circulating tumor-associated autoantibodies that are correlated with cancer progression. Our group has exploited and reported several potential CRC-associated autoantibodies for the diagnosis of CRC [19-21]. In addition to

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protein-based markers, nucleic acid-based markers, such as somatic gene mutations and tumor-associated epigenetic methylation changes in free circulating DNA of tumor origin, have also been thoroughly investigated and shown to be highly sensitive to the presence of CRC [22-24]. However, none of these purported markers have currently passed strict validation tests to confirm their diagnostic power in the early detection of CRC.

MicroRNA

MicroRNAs (miRNAs) are a group of evolutionarily conserved endogenous short non-coding RNA molecules that are encoded in the genome [25-28]. The discovery of miRNA was made in studies of the nematode *Caenorhabditis elegans* by Victor Ambros and colleagues in 1993 [29-31]. These authors found a short RNA fragment that could bind to the 3' untranslated region (UTR) of the *lin-14* mRNA and subsequently repress its translation [31]. To date, more than 1600 mature miRNAs have been discovered in the Homo sapiens genome (http://www.mirbase.org). Bioinformatics studies have estimated that up to 30% of human protein-encoding genes may be regulated by miRNAs [32]. A single miRNA can negatively regulate the expression of hundreds of post-transcriptional targets and governs a variety of biological processes, including cellular development, differentiation, proliferation and apoptosis by either the repression of translation or the promotion of target mRNA degradation [28,33-38].

MicroRNA Biogenesis and Function

It is estimated that approximately 3% of human genes encode miRNAs. The formation of miRNAs is a complex process that involves several steps. First, the miRNA gene is transcribed in the nucleus by RNA polymerase II into a primary transcript (pri-miRNA). It is cleaved by the RNase III enzyme complex (Drosha/Pasha) into a 70-nt-long nucleotide hairpin structure that serves as a precursor miRNA (pre-miRNA). Finally, the pre-miRNA is transported to the cytoplasm by the RanGTP-dependent transporter exportin 5 and then is further processed by another endonuclease enzyme RNase III (Dicer) to become mature miRNA [28,39]. The transcripts are then incorporated into the RNA-induced silencing complex (RISC) with the central part of the complex formed by proteins of the transactivation responsive RNA-binding protein (TRBP) family and the Argonaute family [28]. The miRNA–RISC complex targets messenger RNAs for translational repression or mRNA for cleavage, depending on the level of complementarity between the miRNA and the 3-UTR of the target mRNA [37,38,40].

MicroRNA and Colorectal Cancer

Involvement of microRNA in the pathogenesis of colorectal cancer

Cumulative evidence suggests that miRNAs play a critical role in carcinogenesis, and aberrant expression of miRNA has been observed in various types of cancer [28,41-44]. Recent efforts toward biological characterization of miRNAs have demonstrated that some miRNAs are closely associated with the development of CRC. Both over- and under-expression of specific miRNAs have been identified in CRC patients [45]. For example, Michael et al. reported for the first time that the expression of miR-143 and miR-145 were reduced in CRC [46]. Dozens of miRNAs have now been identified with potential biological and clinical relevance in CRC [47].

Fearon and Vogelstein [48] proposed a genetic model to describe the possible genetic mechanisms of colorectal carcinoma development. According to this model, the tumorigenesis of CRC proceeds through a series of genetic alterations involving oncogenes and tumor suppressor genes, as well as several crucial biological events, such as WNT pathway activation, EGFR signaling activation, TGFβ inactivation, *APC* and *TP53* gene-inactivating mutations, *KRAS* and *BRAF* gene-activating mutations, have been observed during colorectal tumor development [48,49]. All of these processes seem to be affected by miRNA regulation. For example, a low expression level of miR-143 was associated with a large primary CRC tumor size, and one of the key targets of miR-143 is the *KRAS* oncogene [50,51]. Furthermore, miR-34b/c is known to be an important mediator of p53-dependent tumor suppression, and miR-34b/c levels were decreased in CRC tissue relative to the corresponding normal tissue [52].

MicroRNA as a colorectal cancer biomarker

MicroRNA offers a new opportunity for the molecular diagnosis of cancer. Aberrant miRNA expression profiles have been identified that are clinically relevant to CRC [46,53-59]; therefore, circulating miRNAs have emerged as potential screening biomarkers for CRC [60]. For example, Huang et al. [61] also reported that combining ROC analyses using two miRNA markers (miR-29a and miR-92a) revealed that an elevated AUC of 0.883 could be used to discriminate CRC cases from healthy individuals and Ng et al. [62] determined that the expression of miR-92 is significantly elevated in the plasma of CRC patients.

Stool is a Superior Specimen Source for CRC Screening

Fecal matter comes into direct contact with the luminal surface of the colon and includes both colonocytes and tumor cells if carcinoma is present. These cells theoretically carry disease information and continually shed and enter the stool. Consequently, it is reasonable to assume that testing for these shedding tumor cells in a fecal specimen offers the possibility of increased diagnostic efficiency compared to FOBT. In addition, it has been postulated that the earliest detectable tumor cells and/or neoplastic molecule changes of CRC may be present in fecal specimens rather than in blood, indicating that fecal material makes an ideal specimen source for bowel disease screening [63-65].

MicroRNA Stability in Clinical Specimens

Fecal miRNA testing offers advantages for pre-cancerous lesion screening; however, the GI tract environment is much more complex than that of blood; thus, the stability of markers in fecal specimens is a major concern when evaluating the usefulness of these markers for clinical practice. The feasibility of using fecal miRNAs as CRC screening markers has recently been evaluated [66]. The evidence showed that in contrast with the fast degradation of mRNA and protein, endogenous miRNA transcripts were found to be more stable in a broad range of clinical specimens [61,67-69]. Chen et al. [51] demonstrated that circulating miRNAs are well protected from RNase and remain stable after being subjected to harsh conditions, and Ahmed et al. reported that miRNAs possess good stability in fecal specimens [66,70]. Furthermore, detection protocols, including stool preparation, stool miRNA extraction and quantitative analysis, have also been developed [71].

Fecal miRNAs as Diagnostic Markers

Although only limited data have focused on the usefulness of fecal miRNAs, several changes in miRNA expression profiles have been identified as having clinical relevance and have been successively detected in the stool specimens of CRC patients (Table 1). Wu et al. reported that the stool levels of miR-92a and miR-21 were significantly

Study	Target miRNA	Expression Level CRC *VS.* HC	Sample Size (n)			For CRC Detection	Internal Control	Reference
			CRC	Polyps/ Adenoma	HC	Sensitivity /Specificity		
Wu et al. [72]	miR-92a miR-21	Up-regulated	88	57 (polyps)	101	71.6/73.3 56.1/73.3 (For polyps)	U6 snRNA	[72]
Li et al. [74]	miR-143 miR-145	Down-regulated	38		13		miR-16	[74]
Kalimutho et al. [69]	miR144	Up-regulated	35		40		miR-378	[69]
Link et al. [71]	miR-21 miR-106a	Up-regulated	10	9 (adenoma)	8		miR-16/ miR-26b	[71]
Koga et al. [73]	miR-17-92 cluster miR-135	Up-regulated	197		119	69.5/81.5 46.2/95	U6 snRNA	[73]
Ahmed et al. [66]	14 types miRNAs	Up-regulated miR-21,-106a, -96, -203, -20a, -326 -92 Down-regulated miR-320, -126, -143, -484-5p, -16, -145 -125b	15		5		Total RNA/ 18S rRNA	[66]

Abbreviations: CRC: Colorectal Cancer; miRNA: microRNA; snRNA: small nuclear RNA; HC: Healthy Control.

Table 1: Fecal-based miRNAs as potential diagnostic markers for CRC.

higher in CRC patients compared to normal controls. Based on different cut-off values, each of the miRNAs had a sensitivity and specificity of nearly 50% and 80% for CRC diagnosis, respectively [72]. Koga et al. analyzed miRNA expression from exfoliated colonocytes isolated from CRC patient feces and reported that the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92a) and miR-135 were significantly increased in CRC patients, with sensitivities of 69.5% and 46.2% and specificities of 81.5 and 95.0%, respectively [73]. Link et al. found that fecal miR-21 and miR-106a were significantly increased in adenoma and CRC patients compared to healthy controls [71]. Kalimutho et al. reported that an increased miR-144 level in fecal specimens can serve as a novel diagnostic marker for CRC [73]. One latest study demonstrated that the concentrations of miR-143 and miR-145 in the feces of CRC patients were lower than in those of the healthy persons ($p<0.005$) [74].

Conclusions

MicroRNA research has grown tremendously, and studies have indicated that several miRNAs are dysregulated in CRC and have been linked to the pathological pathways of tumor development. The unique stability of these transcripts combined with their disease-specific profile suggests that fecal miRNAs show promise as biomarkers for GI tract disease screening. Although fecal miRNA screening provides new hope for the early detection of CRC, certain issues must be addressed before these markers can be applied to clinical practice. One such issue involves the internal control. The variability of miRNA quantification was more significant among the fecal samples than for other types of clinical samples [66]. To address this problem, a suitable internal control must be selected for miRNA concentration normalization. Although several small RNA species are recommended for normalizing the miRNA expression in tissues and cell lines, no suitable internal control is currently available for stool miRNA testing. Considering the example of the miRNA RNU6B, the concentration of the transcript is not correlated with the total RNA concentration in the stool, indicating that it is not qualified to serve as the internal control for a fecal miRNA quantification assay [71]. Another study suggested that other stably expressed small RNAs, such as the 18S rRNA and miR-16, might be used for normalization; however, further studies are needed to confirm their usefulness [75]. A second issue involves clinical validation. Several studies have demonstrated that the expression levels of several miRNAs can distinguish CRC patients from a healthy population. However, the conclusions of these reports are inconsistent and occasionally even controversial, and none of the miRNAs discussed above offers sufficient predictive value to serve as a diagnostic test [66,71]. The feasibility of the currently proposed fecal miRNAs needs to be further validated using well-designed clinical protocols and an adequate sample size. A third issue involves the miRNA marker panel. The diagnostic performance of miRNA can be improved by combing several miRNAs into a marker panel. Until now, few studies have conducted a systemic approach to explore the fecal miRNA expression profiles. To select the proper miRNA markers with sufficient discriminating power to build a miRNA panel, systematic tests of fecal miRNAs are required.

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