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Circulating microRNA testing for the early diagnosis and follow-up of colorectal cancer patients

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Abstract

Early detection of colorectal cancer is key for prevention and the ability to impact long term survival of colorectal cancer patients. However, the compliance rate of recommended colonoscopy for the population aged from 50 to 75 is only 50-75 percent in the USA. A highly sensitive and specific non invasive test is needed to enhance colorectal cancer management. As for late stage patients, a non-invasive prognostic biomarker is also critical for improving patient treatment protocols. The discovery that non-coding microRNAs (miRNAs) are stable in body fluids such as plasma, serum and exosomes, presents the opportunity to develop novel strategies taking advantage of circulating miRNAs as early diagnostic biomarkers of colorectal cancer. The goal of using circulating miRNA based prognostic biomarkers for colorectal cancer patients has been pursued extensively. In this review, we will try to cover the major recent advancements at the frontiers of this research area.

Keywords

cancer biomarker; circulating miRNA; colorectal cancer

Introduction

Colorectal cancer is one of the leading causes of cancer death in the United States with more than 50,000 deaths every year [1]. Colorectal cancer screening using colonoscopy in adults aged 50 years and older has made a significant impact on colorectal cancer early detection, prevention and treatment. However, the compliance rate of colonoscopy is still not high enough. A non invasive, blood based colorectal cancer test would be ideal, and efforts have been devoted to discover protein based, DNA based, or circulating tumor cell based screens. On the treatment end, reliable prognostic biomarkers are also needed to guide clinical management of colorectal cancer.

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While cancer is a genetic disease, it is well understood that epigenetic alternations including alterations in DNA methylation, histone modifications as well as alterations in non-coding RNAs can play an important role in tumorigenesis. These epigenetic changes can alter the characteristics of the tumor cells, allowing them to better adapt to the growth conditions of the tumor microenvironment. While protein coding mRNA are of great importance to the functional characteristics of the cell, only about 2% of transcripts code for mRNA templates for protein synthesis. The remaining large portion of transcribed RNA are non coding and include long non-coding RNA (lncRNA), micro RNA (miRNA) and piwi interacting RNA (piRNA). Many of these non-coding RNAs are highly conserved, reflecting the important functional roles they may play in the cell.

miRNAs are a class of small non-coding RNAs with crucial regulatory functions [2, 3]. miRNAs can regulate protein expression through various mechanisms including promoting RNA degradation through association with the RISC complex. miRNAs can also inhibit mRNA translation to carefully control protein expression and in some cases, play a role in regulating the transcription of certain genes. Most mammalian miRNAs are thought to repress target gene expression at the translational level through imperfect base pairing with the 3' UTR of target mRNA [2, 4, 5]. The importance of miRNAs in cancer was revealed through the discovery of a link between the down regulation of miR-15 and miR-16 and chronic lymphocytic leukemia [6]. This has led to thorough investigations of the impact of miRNAs in cancer.

Coding mRNAs have been the major focus of biomarker research, prior to the discovery of miRNA. However, one of the major disadvantages of mRNA based biomarkers is concerns about RNA stability. The requirement of clinical sample quality in terms of snap frozen time after surgery is crucial for maintaining mRNA stability as a result; it is a big challenge to procure high quality samples for RNA based analysis. miRNAs provide an opportunity to overcome the stability challenges presented by mRNA. We and others have systematically assessed miRNA stability using archival FFPE specimens and to our surprise, miRNAs are highly stable in FFPE samples [7, 8]. This discovery opened the door for future miRNA based biomarker research. Mitchell et al.'s discovery that miRNAs are also stable in body fluids such as plasma and serum, along with their demonstration that blood based miRNA analysis could detect the presence of cancer in both mouse models as well as clinical samples, set the stage for further investigation into the feasibility of utilizing circulating miRNAs as biomarkers for cancer and other diseases [9]. Although it is still not entirely clear where circulating miRNAs originate, there are several reports show evidence that they may arise from lysed tumor cells [10], exosomes [11], or blood cell origin [12]. miRNA have been shown to be actively exported from cells both in vesicles such as exosomes as well as associated with proteins independent of vesicles [13]. Exosomes have also been found to traffic RNA including miRNA between cells [14]. However, more work needs to be done to fully understand the source of circulating miRNA.

Technical issues related to miRNA quantification in serum/plasma samples

There are several issues related to using circulating miRNA as biomarkers. Depending on serum or plasma based miRNA detection, standardized sample processing procedures will

be crucial as any potential contamination of serum or plasma samples with RBC, PBMCs and platelets will contribute to the circulating pool of miRNAs. Any handling step that causes hemolysis or platelet activation will alter the miRNAs present in the sample, contaminating the circulating miRNA with blood cell derived miRNA. It is recommended that whole blood sample needs to be immediately processed to either serum or plasma based on protocols of NCI's Early Detection Research Networks (EDRN). The importance of developing a standard method of isolating and analyzing circulating miRNA, is emphasized by studies demonstrating that serum factors and other contaminants can effect miRNA quantification, and different miRNAs exhibit different stabilities, resulting in altered ratios between miRNAs depending on handling procedures [15, 16]. Another technical issue is that the serum/plasma samples collected have to be free of heparin. It has been well documented that heparin is a potent inhibitor of reverse transcriptase during cDNA synthesis [9]. If serum/plasma samples already contain heparin, then the isolated RNA samples have to be further purified by treating with heparinase prior to RT-PCR analysis. Another concern is that the amount of RNA isolated from serum/plasma is low and RNA carrier has to be included to improve yield. This low yield of RNA can pose a challenge to the efficiency and reproducibility of serum/plasma miRNA analysis. Finding the proper way of normalizing miRNA expression is also a key issue. The prevalence of this issue is made quite clear in scanning the literature, as different studies utilize different methods for normalization.

In some cases, uniform volume of serum/plasma samples or equal amount of RNA has been used for normalization. Another approach is to use spike-in control miRNAs such as synthetic *C. elegans* mir-39 as reference miRNA [17-19]. Other studies utilize endogenous controls, either a single miRNA such as miR-16 or a panel of a few miRNAs [20-22]. Some studies have selected their endogenous control based on a comparison of expression of several candidate endogenous control genes between sample populations [23]. Of particular concern regarding the selection of endogenous controls is the observation in one study that two miRNAs selected in a pilot study as candidate endogenous controls, were found to be differentially expressed in the validation study [24]. Thus, it is quite clear that the determination of a reliable endogenous control is important for the reliability and accuracy of circulating miRNA biomarker research. This is a challenge that is well understood in the field as many studies detail this as a possible limitation of their study and circulating miRNA biomarker studies in general. These issues have to be carefully dealt with in the discovery phase of the project as well as future clinical settings in order to ensure accuracy of miRNA quantification [25, 26].

Another major challenge facing the field of circulating miRNA biomarker research is the issue of the specificity of any given circulating miRNA in indicating the presence of a particular cancer. For example, in a study on circulating miR-21, it was found that mean serum levels of miR-21 were higher for patients with breast cancer, CRC, lung cancer, esophageal cancer, as well as gastric cancer [20]. Therefore in this study, miR-21 did not represent a potential biomarker for any one of these cancer types but rather a broad spectrum cancer marker. A study on circulating miRNAs in breast cancer revealed that of 13 candidate biomarker miRNAs, 10 were found to have significantly altered expression in other cancer types [27]. This data clearly demonstrates that a detailed and through approach must be taken in assessing circulating miRNA biomarkers.

This also suggests that in order to increase confidence in the specificity of a potential biomarker, controls for other cancer types need to be included in the analysis, a concern echoed by Kanaan et al. [28]. Further investigation aimed at understanding the mechanism by which certain circulating miRNAs become differentially expressed, may help to pinpoint those miRNAs that are markers for a specific type of cancer. This type of investigation will likely also provide more insight into understanding the discrepancies that are observed between the levels of a particular miRNA in tumor tissue compared to serum/plasma. [17, 29].

The reproducibility of results is also a major concern facing the field of circulating miRNA biomarker research. In a literature analysis performed by Leidner et al., they found that of 158 candidate miRNA that were found to be differentially expressed in breast cancer by 5 genome wide profiling studies, only 16 overlapped in 2 studies, and none overlapped in 3 studies [27]. Furthermore, they demonstrated by comparing the data they generated using to an Illumina Human v2 Microarray, to that generated by another group using the same platform, there was no correlation between the data sets, and in some cases, the expression of top candidates generated in one study were altered in the opposite direction in the other data set [27]. This points to some drastic difference in findings between studies and suggests much work needs to be done to improve the reproducibility of these types of studies. There is however some consistently analyzed circulating miRNA, such as miR-29a which has been shown on multiple studies to be increased in the plasma of CRC patients [22, 21, 30]. It is possible that by addressing some of the technical issues associated with these types of studies, which will consist of developing a standardized procedure for sample collection, handling and analysis, as well as determining a standard well defined endogenous control for normalization, some of the inconsistencies in the data may be alleviated. Until some of these issues are addressed, in pushing forward with the use of circulating miRNA as cancer biomarkers, it may be best to focus on those miRNA that have been identified in multiple independent studies, to perhaps increase the probability a particular miRNA is in fact a reliable biomarker.

Despite some of these technical challenges, there remains promise in the use of circulating miRNA as CRC biomarkers. Investigations have revealed miRNAs that may be useful for both early detection as well as prognosis of CRC, some of which are highlighted in Table 1.

miRNA based biomarker in serum/plasma samples of colorectal cancer

Early detection

The key requirements for cancer early detection biomarkers are high sensitivity and specificity. A non-invasive circulating miRNA based test for early detection of colorectal cancer will be attractive as a screening tool before colonoscopy. Dysregulated miRNAs have been reported in colorectal cancer in serum using sequencing based analysis. Ng *et al.* reported miR-17-3p and miR-92 with 89% sensitivity and 70% specificity in discriminating colorectal cancer from normal controls [31]. Pu *et al.* reported an elevated miR-221 in plasma samples of colorectal cancer patients and it is also correlated with p53 status [32]. This miRNA expression was normalized against a standard curve of serial dilutions of the cDNA samples. The AUC value of miR-221 is 0.606 (86% sensitivity and 41% specificity).

Another report has investigated 12 selected miRNAs using 157 plasma samples from advanced adenomas and carcinomas of colorectal neoplasia as well as 59 healthy control samples. Based on the study, miR-29a and miR-92a have significant cancer diagnostic values with a combined AUC of 0.883 with 83% sensitivity and 84.7% specificity. For detecting advanced adenomas, the power of AUC is 0.773 with 73% sensitivity and 79.7% specificity. miR-16 was used as a reference control for normalization [21]. Another study has shown that both miR-29a and miR-18a are upregulated in the serum of stage 3 CRC patients compared to controls, as determined by microarray analysis followed qRT-PCR validation and normalization to a panel of 3 endogenous controls [22]. Recent studies show consistent results for miR-92a based on 200 serum samples of colorectal cancer and 50 advanced adenomas as both a diagnostic and prognostic biomarker. In addition, serum miR-21 also has potential with comparable AUC values of 0.709. These two miRNAs could be potentially helpful in distinguishing precancerous adenoma patients from controls, and exhibited a combined sensitivity and specificity of 70% [33]. Being that early detection of CRC cancer is key, the identification of biomarkers of precancerous adenoma is of great advantage. Kanaan et al. have determined that a panel of 8 plasma miRNAs including miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652, could identify adenoma patients with a sensitivity of 88% and a specificity of 64% [28]. In another study involving miRNA profiling on pooled plasma samples from 10 CRC patients (5 stage II and 5 stage III) and 10 normal controls, followed by validation via qRT-PCR on 191 independent samples with 90 CRC patients, 43 advanced adenoma patients and 58 healthy controls, miR-601, and miR-760 were found to be decreased in CRC as well as advanced adenomas compared to controls. Combined, these miRNAs showed an AUC of 0.683, with 72.1% sensitivity and 62.1% specificity in distinguishing advanced adenoma patients from controls. In discriminating CRC from control they demonstrated an AUC of 0.943 with 83.3% sensitivity and 93.1% specificity [17]. In addition, consistent with other findings, this study also revealed both miR-29a and miR-92a as upregulated in CRC. In a study that attempted to focus on differentially expressed plasma miRNA that correlated with miRNA that were differentially expressed at the tissue level, Yong et al. found that a combination of miR-193a-3p, miR-23a and miR-338-5p, demonstrated an AUC of 0.887 with 80.0% sensitivity and 84.4% specificity. These three miRNAs all demonstrated increased expression in CRC patients, both in tissue as well as in circulation [34].

Prognosis

In addition to their importance for early detection, circulating miRNA may also provide prognostic insight into CRC, hopefully aiding in the goal of developing personalized cancer care after years of translational research. Serum miR-92a has been reported to be associated with prognosis in colorectal cancer [35]. miR-141 has been reported to be significantly associated with patient survival of colorectal cancer based on Kaplan-Meier survival analysis ($P = 0.004$) [36]. In addition to miR-92a, miR-21 is one of the most investigated miRNAs as a circulating prognostic biomarker for colorectal cancer. miR-21 was highly expressed in many cancer types including colorectal cancer [37, 38]. In addition to the elevated miR-21 in tumor tissues, the expression of miR-21 was also elevated in serum/plasma samples of colorectal cancer patients based on Taq-Man analysis [39]. The most recent studies on expression of serum miR-21 of colorectal cancer was conducted using 186

preoperative serum samples, 60 postoperative patients, 43 advanced adenoma patients and 53 control samples. miR-21 expression was found to be significantly elevated in preoperative serum of adenoma patients ($P < 0.001$) and colorectal cancer patients ($P < 0.001$) [29]. The miR-21 levels distinguish adenoma from controls with AUC of 0.813 and colorectal cancer with AUC of 0.919. In addition, they also report that miR-21 expression was significantly ($P = 0.03$) associated with patient survival based on multi-variant analysis. Other circulating miRNAs have also been found to associate with CRC prognosis. miR-200c was found to be increased in patients with liver metastasis as well as distant lymph node metastasis as opposed to regional lymph nodes. Multivariate analysis revealed that miR-200c was an independent predictor of lymph node metastasis of CRC. Kaplan-Meier analysis also revealed that higher serum miR-200c levels were associated with decreased survival ($P=0.0064$) [19]. Lymph node metastasis has also been associated with increased plasma expression of miR-181b ($P=0.024$) [40]. The more widely studied miR-29a, has also been shown to be associated with liver metastasis. In a study involving 58 metastatic patients and 56 non metastatic patients, serum miR-29a expression was significantly higher in metastatic patients. miR-29a appeared to be a biomarker for metastasis, with a AUC of 0.803 with a 75% sensitivity and specificity [30].

In addition to metastasis, another significant prognostic parameter is responsiveness to treatment. In a study analyzing differential plasma miRNA expression in responders vs. non responders to oxaliplatin based treatment, three miRNAs (miR-106a, miR-484 and miR-130) were all upregulated in non-responders. Increased expression of three other miRNA (miR-27b, miR-148a, miR-326) were associated with decreased progression-free survival, with miR-326 also associating with decreased overall survival ($P=0.003$) [24].

An important complication of cancer treatment is relapse following treatment. A study investigating the role of miR-29c in CRC found that serum levels of this miRNA are increased in patients that experienced early relapse (local recurrence or distant metastasis within 1 year following resection) compared to non-early relapse patients ($P=0.012$) [18]. This along with the other data presented above indicates that there is hope that miRNAs could prove incredibly useful for prognosis of CRC, helping to guide the appropriate course of action for treating the disease in different patients.

Conclusion

In conclusion, a number of studies have provided compelling evidence that circulating miRNAs have great potential as colorectal cancer biomarkers for early detection and prognosis. The attractive characteristics of miRNAs as biomarkers are high stability and multiple targets/pathways. It will be relatively easy to integrate such assay into clinical diagnostic settings for managing colorectal cancer with traditional pathological and histological diagnosis. However, the circulating miRNA based test has to have high sensitivity and specificity. It also requires a new clinical management strategy to monitor and follow up with these high risk patients using regular colonoscopy. A highly effective chemoprevention treatment strategy will also be a key for these high risk patients. There are multiple technical challenges facing this field that remain to be overcome. In addition, a deeper understanding of the mechanism involved in the regulation of miRNA in circulation

will improve the understanding of what makes particular miRNAs useful biomarkers for a given type of cancer. As these challenges are overcome, it will be a matter of time until these promising circulating miRNA biomarkers will benefit patients, once they are fully validated by collaborative and multi-center large patient cohort retrospective and prospective studies using the most robust and uniformed methodology including sample handling, RNA isolation, RT-PCR protocol, reference standard, and statistical analysis.

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Table 1

Potential candidate miRNAs as biomarkers in circulation for colorectal cancer

Plasma miRNA	Circulating Expression in CRC	Potential of Plasma Biomarker	Reference
miR-92	Elevated	-Distinguish both CRC and advanced adenomas from controls	[31, 21, 33]
miR-29	Elevated	-Distinguish both CRC and advanced adenomas from controls -Distinguish patients with liver metastasis from those without liver metastasis	[21, 30, 22]
miR-21	Elevated	-Distinguish both CRC and advanced adenomas from controls -Correlates with decreased overall survival	[29, 33]
miR-141	Elevated	-Distinguishes Stage IV CRC patients from other stages -Correlates with poorer survival	[36]
miR-601	Decreased	-Distinguishes CRC patients from controls -Lowest expression in Stage IV	[17]
miR-760	Decreased	-Distinguishes CRC patients from controls -Lowest expression in Stage IV	[17]
miR-200c	Elevated	-Increased expression in CRC vs. control -Highest in Stage IV compared to Stage I-III -Associated with distant metastasis -Associates with poorer survival	[19]