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miRNAs as biomarkers in colorectal cancer diagnosis and prognosis

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Abstract

Since the discovery of noncoding small miRNAs and their function in controlling mRNA translational rate, the small non-coding miRNA world has become a research wonderland for cancer and other human diseases. Due to the critical regulatory function, miRNA can act as an oncogene or a tumor-suppressor gene. This review will cover some of the recent discoveries of the potential of miRNAs as cancer biomarkers in colorectal cancer, future challenges and solutions.

Noncoding RNAs (siRNAs, **miRNAs**, tncRNAs, rasiRNAs and piRNAs) are a class of small RNAs with critical regulatory function, as evidenced by the recent Nobel Price recognition [1]. Ambros *et al.* made a landmark discovery in 1993 that a small noncoding RNA, lin-4 impacted the development of *Caenorhabditis elegans* [2]. The potential roles of miRNAs in cancer were reported almost a decade later, opening up a new paradigm for discoveries in cancer research. Currently, over 1000 mammalian miRNAs have been identified by cloning and sequencing approaches [3]. miRNA is a class of small, 20–22-nucleotide-long noncoding RNA processed from larger pre-miRNAs by the RNase III enzyme, Dicer, into miRNA duplexes [4]. One strand of this duplex can associate with the RNA-induced silencing complex (RISC), with the other strand generally degraded by cellular nucleases [4]. The miRNA–RISC complex has been shown to bind to specific mRNA targets, leading to translational repression or cleavage of these mRNAs. Thus, miRNAs modulate protein expression by promoting RNA degradation, inhibiting mRNA translation and, in some cases, affecting transcription. Currently, hundreds of human miRNAs have been identified [5]. Although miRNA-mediated mRNA degradation occurs in mammals, most miRNAs are thought to use a secondary mechanism of gene regulation via imperfect base pairing to the 3′-untranslated regions (3′-UTRs) of their target mRNAs. This leads to the repression of target gene expression post-transcriptionally, likely at the translational level [2,6,7]. Such translational regulation provides the cell with a more precise, immediate and energy efficient way of controlling the expression of a given protein [8]. Such regulation can induce rapid changes in protein synthesis without the need for transcriptional activation and subsequent steps in mRNA processing. Additionally, translational control of gene expression has the advantage of being readily reversible, providing the cell with great flexibility in responding to various cytotoxic stresses. Clearly, it is essential to not only know the levels of individual mRNAs, but also the extent to which mRNAs are

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being translated into their corresponding proteins and the miRNAs that regulate these processes. Thus, post-transcriptional and translational control mediated by miRNAs has become a new frontier for cancer research.

Technologies to investigate miRNA expression

Numerous approaches have been developed to analyze and quantify the expression of miRNAs. These technologies include microarray-based miRNA expression platforms [9-14], real-time qRT-PCR-based approaches [15], solution-based bead-based approaches [16], *in situ* detection [17,18], single-molecule detection [19] and, most recently, sequencing-based approaches [20]. Table 1 summarizes the various technologies for miRNA detection and quantification. Most of these approaches are developed against the so-called 'gold standard' Northern blot. Each has its unique advantages and disadvantages, such as throughput, sensitivity, ease of use and cost. The bead-based hybridization approach is in-solution hybridization and may have advantages compared with the surface hybridization of array-based approaches. However, it requires a relatively large quantity of RNA and some of the probes are not able to distinguish pre-miRNA from mature miRNA. It is important to distinguish between signals from pre-miRNA and mature miRNA as miRNA processing is quite important [21]. Most of the capture probe designs will not be able to distinguish such differences. Real-time qRT-PCR has the advantage of using a small quantity of RNA with greater sensitivity and specificity. It will also be able to distinguish between pre-miRNAs and mature miRNAs [22]. This could be one of the reasons for the lack of correlation among some of these platforms. The other issue relates to normalization. Due to the small number of miRNAs compared with mRNAs, some of the widely used normalization approaches (e.g., average median) originally developed for mRNA gene expression arrays may not be appropriate. This may also contribute to the lack of correlation between different platforms [23]. In fact, this was one of the issues when different types of mRNA array-based high-throughput expression platforms were introduced in the early days. Therefore, this problem is likely to be resolved over time. All of these approaches are useful in research and discovery settings. However, they may face different challenges in the clinical settings.

miRNA stability in clinical specimens

There are many issues associated with finding reliable and clinically useful **biomarkers**, ranging from sample size and quality of the specimen to availability of the clinical follow-up information. The stability of the miRNAs in archival specimens is a key factor in large-scale retrospective biomarker discovery studies. We have systematically investigated the stability of miRNAs by comparing fresh frozen and paired formalin-fixed, paraffin-embedded (FFPE) samples and found that miRNAs are rather stable. The different length of formalin fixation time and paraffin embedding has little impact on miRNA stability [24]. This ensures that miRNA biomarker discovery using archival FFPE specimens with a large sample size is feasible. miRNA was also found to be stable in blood and body fluids, which has expanded the biomarker discovery sample types [25,26]. The stability of miRNAs is one of the most important factors in using archival clinical FFPE specimens for biomarker discovery to ensure the reliability of the biomarker and to facilitate cross-validation.

miRNAs in colorectal cancer

Colon cancer is the second leading cause of death from cancer in the Western world. It has been estimated that **colorectal cancer** is responsible for 50,000 deaths in the USA and 500,000 deaths worldwide [27]. Epigenetic alterations play key roles in colorectal cancer [28,29], and recent studies suggest that noncoding RNAs play important roles in cancer development and metastasis [3,30]. There is mounting evidence indicating that post-transcriptional and translational controls mediated by various regulatory molecules, such as RNA-binding proteins

and noncoding miRNAs, are critically important in the development of various diseases, including cancer [31-33]. Tremendous efforts were made towards the discovery of predictive and prognostic biomarkers in colorectal cancer before the discovery of miRNA. Fluoropyrimidine-based chemotherapy is one of the main treatment options for colorectal cancer; however, due to the low response rate, many patients go through chemotherapy without any benefit. It is crucial to have a set of reliable predictive biomarkers that can guide personalized chemotherapy.

Expression profiling of miRNAs in colorectal cancer

The efforts of discovering miRNAs in colorectal cancer began with expression profiling. The first report of alterations of miRNA expression was in 2003 by Michael *et al.*, who found that miR-143 and miR-145 were reduced in colorectal specimens [34]. Recent studies discovered that one of the key targets of miR-143 is the *KRAS* oncogene [35]. The first large-scale miRNA expression profiling was reported in 2006 by Cummins *et al.* [5]. These studies suggest that some miRNAs are closely associated with the development of colorectal cancer [5].

miRNAs & p53

Over 50% of colorectal cancer patients carry p53 mutations and deletions. We took a rationalized approach to discovering miRNAs potentially mediated by p53. We first revealed the impact of p53 on miRNA expression using high-through-put expression analysis using paired HCT116 (wt-p53) and HCT-116 (null-p53) colon cancer cell lines and discovered a number of miRNAs that are potentially regulated by p53 [36]. Our bioinformatic analysis further revealed that over half of the 328 miRNA putative promoter regions (e.g., miR-34, miR-192/215 and miR-26a) contain p53 binding sites. This regulatory mechanism has been shown to be important in colon cancer, as miR-34 was subsequently demonstrated to be regulated directly by p53 [37-39]. We and two other groups have provided further direct evidence that miR-192 is another miRNA regulated by p53 [40-42]. The positive-feedback mechanism between p53 and some of these miRNAs has proved to be an important part of the regulatory function and networks of p53 mediated through miRNAs. Due to the broad impact of miRNA on regulating translation rate, miRNAs exert finer tuning of the tumor-suppressor function of p53. We have reasons to believe that modulation of miRNA will have a broad impact in colorectal and other cancer types.

Some of the important targets have also been identified. We have identified miR-192 and miR-215-mediated mRNA targets, such as thymidylate synthase (TYMS, TS) and dihydrofolate reductase (DHFR), two of the most important chemotherapeutic targets in cancer treatment [40,43]. Elevated miR-192 reduces cell proliferation and impacts chemosensitivity to thymidylate synthase inhibitors. Mishra *et al.* reported that another miR, miR-24, regulates DHFR expression, and polymorphisms at the 3'-UTR regions of DHFR are the reason for elevated DHFR expression in colorectal tumors [44]. It was demonstrated that 829C→T, a naturally occurring SNP near the miR-24 binding site in the 3'-UTR of DHFR, affects DHFR expression by interfering with miR-24 function, resulting in DHFR overexpression and methotrexate resistance. Further studies reveal that miR-24 is a potential tumor suppressor as it reduces tumor cell proliferation in a p53-independent fashion and mediates several key cell cycle-related genes, such as *p21*, *E2F*, *Myc* and other cell cycle-control genes [45,46].

Another potential p53-mediated miRNA is miR-26a. We have demonstrated that miR-26a was downregulated due to the loss of p53 and miR-26a may be directly regulated by the tumor suppressor p53 in colon cancer [36]. A recent report showed that miR-26a not only impacts colorectal cancer, it also plays a key role in liver cancer both as a potential therapeutic agent and a prognostic biomarker [47,48].

miRNA & chemoresistance in colon cancer stem cells

Chemoresistance is one of the major factors for the failure of chemotherapy. Chemotherapeutic agents are ineffective to the slow proliferating cancer stem cells. It is important to discover genes and pathways that are responsible for the resistance mechanism. Owing to the broad influence of miRNA in gene expression, we believe that miRNAs may offer new insights to the resistance mechanism. We have recently reported that miR-140 is overexpressed in CD133⁺CD44⁺ colon cancer stem-like cells and, by reducing its expression, we can sensitize chemoresistant CD133⁺CD44⁺ colon cancer stem-like cells to 5-fluorouracil (5-FU) treatment [49]. This study offers a new angle and strategy for overcoming chemoresistance. Figure 1 summarizes the relationships between p53, miRNA, cell cycle control and cell death after genotoxic stress signal. p53 and certain miRNAs (e.g., miR-140, miR-192, miR-215, miR-26a, miR-34s) form a positive feedback loop during genotoxic stress triggered by chemotherapeutic agents to arrest proliferating cancer cells at the G1 and/or G2 phases of the cell cycle. This is achieved by upregulating some of the cell cycle-control genes such as p21 and E2F family proteins. However, the life or death decision is different among these miRNAs. miR-34 triggers apoptosis in a p53-dependent manner. By contrast, miR-140, miR-192 and miR-215 trigger cell cycle arrest without cell death. This can be explained by the fact that different miRNAs target unique sets of genes and the global impact and outcome will be more important than a single gene target. In the case of miR-140, the upregulation of miR-140 has contributed to chemoresistance, in part due to the reduced proliferation rate. A more comprehensive systems biology approach is needed to fully understand the impact of miRNA in terms of signaling pathways and targets associated with each pathway.

miRNAs as a colorectal cancer biomarker

Several recent reports have demonstrated the potential of miRNAs as diagnostic biomarkers in colorectal cancer [26,50]. Ng *et al.* revealed that the expression of miR-92 is significantly elevated in the plasma of patients with colorectal cancer and can be a potentially noninvasive molecular marker for colorectal cancer screening [50]. Huang *et al.* also reported that miR-29a and miR-92a have significant diagnostic values in advanced colorectal cancer [26]. Table 2 summarizes some important miRNAs that show promise as potential biomarkers in colorectal cancer.

miRNAs also hold great potential as both predictive and prognostic biomarkers in colorectal cancer. We have investigated the potential of miRNAs as prognostic and predictive biomarkers in colorectal cancer patients treated with 5-FU-based chemotherapy. We also investigated miRNAs as prognostic and predictive biomarker in colorectal cancer patients treated with the next-generation oral 5-FU compound S-1 [51]. We demonstrated that the expression of miR-181b and let-7g was significantly associated with chemoresponse to S-1.

Several studies have demonstrated the potential of miRNAs as prognostic indicators in colorectal cancer. A recent microarray-based miRNA expression profiling study of ten normal mucosa samples and 49 stage-II colon cancers revealed several differentially expressed miRNAs between normal tissue and tumor microsatellite subtypes, with miR-145 showing the lowest expression in cancer relative to normal tissue. Microsatellite status for the majority of cancers could be correctly predicted based on miRNA expression profiles. They further demonstrated that a biomarker based on miRNA expression profiles could predict recurrence of disease with an overall performance accuracy of 81%, indicating a potential role for miRNAs in determining tumor aggressiveness. The expression levels of miR-320 and miR-498, both included in the predictive biomarker, correlated with the probability of recurrence-free survival by multivariate analysis [52].

We have provided experimental evidence that high levels of miR-200c were associated with poor prognosis in colorectal cancer [53]. The close association of let-7g and miR-200c with clinical outcomes of colorectal cancer is no accident. It is now known that miR-200c plays key roles in epithelial–mesenchymal transition (EMT) by regulating the expression of ZEB1 and ZEB2 [54]. EMT is a key event in cancer progression and both let-7g and miR-200c are important miRNAs in regulating EMT process [55].

Another important miR, miR-21, has been shown to be closely associated with colorectal patients' clinical outcome in colorectal cancer [56,57]. Shetter *et al.* found several miRNAs, including highly elevated miR-21, to be associated with poor survival and lack of response to chemotherapy. Overexpression of miR-21 is associated with low sensitivity and poor response to chemotherapy.

Conclusion & future perspective

Improved detection and quantification technology for miRNA expression analysis should make it possible to use various types of clinical specimens for cancer biomarker discovery. There is no doubt that miRNAs will be important biomarkers in cancer diagnosis and prognosis, based on some of these promising studies. Owing to their critical functions, these miRNAs also have significant impacts on other major cancer types. Collaborative efforts are essential to fully realize the potential clinical utilities of these miRNA biomarkers using large patient cohorts. miRNAs have the potential to be among the novel therapeutic targets for treating cancer as modulating the expression of one particular miRNA can mediate many targets and pathways in a reversible and fine tuning manner. With clinically friendly and cost-effective miRNA expression technologies, miRNAs may soon directly impact cancer treatment.

Executive summary

- The relatively small number of miRNAs facilitates biomarker discovery.
- Better stability in archival tumor tissues makes miRNAs ideal for cancer biomarker discovery.
- Increased sensitivity of miRNA expression technology will ultimately realize miRNA-based colorectal cancer diagnosis and prognosis.
- miRNAs provide important opportunities for future cancer diagnosis and therapy.

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Key Terms

miRNA	Class of small noncoding RNAs that interact with their target mRNAs, mainly at the 3'-UTR region to suppress translation
Biomarker	Indicator (e.g., DNA mutation, mRNA expression, protein expression and/or modification) to distinguish disease from healthy state, different disease stage/progression, response or resistance to chemotherapy
Colorectal cancer	Fifth most common cause of cancer in the USA and the third leading cause of cancer-related deaths worldwide

Chemoresistance

Tumor cells are highly heterogenous and certain populations of tumor cells are resistant to chemotherapeutic treatment (e.g., resistant to 5-fluorouracil in colorectal cancer)

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Figure 1. Feedback regulatory mechanism between p53 and miRNAs in regulating the cell cycle and cell proliferation

Table 1
Technology platforms for miRNA expression analysis

Technology	Advantages	Limitations	Ref.
Microarray	High throughput	Sensitivity	[9-14]
Real-time PCR	Both low and high throughput Distinguish pre- and mature miRNA	Cost	[15]
Bead-based	High throughput Solution hybridization	Large amount of RNA	[16]
Northern blot	'Gold standard'	Low throughput Large amount of RNA	[20]
Next-Gen Sequencing-based	High throughput Detecting rare miRNAs	Cost	[20]
<i>In situ</i> hybridization	Localization informaton	Sensitivity	[17,18]

Table 2
miRNAs as biomarker in colorectal cancer

miRNAs	Ref.
<i>Diagnostic marker</i>	
miR-29a	[26]
miR-92a	[50]
<i>Key targets</i>	
TYMS, TS (miR-192, miR-215)	[43]
DHFR (miR-192, miR-215, miR-24)	[40,44]
KRAS	[35]
Bcl-2 (miR-34)	[37-39]
HDAC4 (miR-140)	[49]
ZEB1, ZEB2 (miR-200)	[54,55]
<i>Chemoresponse</i>	
miR-181b	[51]
let-7g	[51]
<i>Outcome</i>	
miR-145	[52]
miR-21	[56,57]
miR-200c	[53]
miR-320	[52]
miR-498	[52]
let-7g	[52]