

## MicroRNAs in the prognosis and therapy of colorectal cancer: From bench to bedside

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**Author contributions:** All authors contributed equally to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the submitted version.

**Supported by the Direct Grant for Research, Faculty of Medicine, the Chinese University of Hong Kong, No. 4054371.**

**Conflict-of-interest statement:** No potential conflicts of interest. No financial support.

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**Manuscript source:** Invited manuscript

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Received: May 2, 2018  
Peer-review started: May 4, 2018  
First decision: June 6, 2018

Revised: June 28, 2018

Accepted: June 30, 2018

Article in press: June 30, 2018

Published online: July 21, 2018

### Abstract

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs that can post-transcriptionally regulate the expression of various oncogenes and tumor suppressor genes. Dysregulated expression of many miRNAs have been shown to mediate the signaling pathways critical in the multistep carcinogenesis of colorectal cancer (CRC). MiRNAs are stable and protected from RNase-mediated degradation, thereby enabling its detection in biological fluids and archival tissues for biomarker studies. This review focuses on the role and application of miRNAs in the prognosis and therapy of CRC. While stage II CRC is potentially curable by surgical resection, a significant percentage of stage II CRC patients do develop recurrence. MiRNA biomarkers may be used to stratify such high-risk population for adjuvant chemotherapy to provide better prognoses. Growing evidence also suggests that miRNAs are involved in the metastatic process of CRC. Certain of these miRNAs may thus be used as prognostic biomarkers to identify patients more likely to have micro-metastasis, who could be monitored more closely after surgery and/or given more aggressive adjuvant chemotherapy. Intrinsic and acquired resistance to chemotherapy severely hinders successful chemotherapy in CRC treatment. Predictive miRNA biomarkers for response to chemotherapy may identify patients who will benefit the most from a particular regimen and also spare the patients from unnecessary side effects. Selection of patients to receive the new targeted therapy is becoming possible with the use of predictive miRNA biomarkers. Lastly, forced expression of tumor suppressor miRNA or silencing of oncogenic miRNA in tumors by gene therapy can also be adopted to treat CRC alone or in combination with other

chemotherapeutic drugs.

**Key words:** MicroRNA; Colorectal cancer; Multidrug resistance; Prognosis; Therapeutic target; Apoptosis; Metastasis; Recurrence; Risk stratification

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**Core tip:** MicroRNAs (miRNAs) are important mediators regulating the initiation, progression, metastasis and recurrence of colorectal cancer (CRC). Numerous studies have reported dysregulation of miRNAs in tumor specimens and body fluids, including serum, plasma and feces. Furthermore, the miRNAs were more recently found in circulating exosomes from CRC patients. Fortunately, this finding suggests potential diagnostic, prognostic and therapeutic applications of miRNAs at different stages of CRC. In this review, we aim to outline the current body of knowledge pertaining to the critical roles played by miRNAs in the molecular pathogenesis of CRC. Our focus is to delineate practical applications of miRNAs as prognostic biomarkers and therapeutic targets in the treatment of CRC.

To KK, Tong CW, Wu M, Cho WC. MicroRNAs in the prognosis and therapy of colorectal cancer: From bench to bedside. *World J Gastroenterol* 2018; 24(27): 2949-2973 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i27/2949.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i27.2949>

## INTRODUCTION

Colorectal cancer (CRC) is the 3<sup>rd</sup> most common cancer and the 3<sup>rd</sup> leading cause of cancer-related deaths worldwide<sup>[1]</sup>. For 2018, there is an estimated incidence of over 140000 new cases and mortality of over 50000 annually in the United States<sup>[2]</sup>. It is widely believed that CRC develops in multi-step process, from aberrant crypt foci, through benign precancerous lesions (adenomas), to malignant tumors (adenocarcinomas) over an extended period of time<sup>[3]</sup>. The majority of CRC is sporadic, though approximately 20%-30% of CRC patients carry inherited mutations<sup>[4,5]</sup>. Accumulation of numerous genetic mutations and/or epigenetic changes is required to drive the carcinogenic progression through progressive functional disruption of tumor suppressor genes and oncogenes. While the prognosis for advanced CRC remains dismal, the disease is curable in its early stages, thereby highlighting the importance of prevention and early detection.

Treatment of CRC usually involves surgical resection of the primary tumor(s) followed by chemotherapy and/or targeted therapy for the advanced (stage III and IV) disease<sup>[6]</sup>. New drugs approved for CRC in recent years mainly fall into the class of molecular targeted drugs, including the vascular endothelial growth factor receptor

(VEGFR) and epidermal growth factor receptor (EGFR) inhibitors<sup>[7]</sup>.

MicroRNAs (miRNAs) are short (~22 nucleotides in length) endogenous non-coding RNAs that repress gene expression in eukaryotic organisms. There are numerous studies reporting the correlation between dysregulated miRNAs and the aberrant regulation of signaling pathways involved in CRC initiation and progression. Michael *et al.*<sup>[8]</sup> was the first to report a dramatic downregulation of miR-143 and miR-145 in CRC relative to normal colon epithelial cells, thereby suggesting a role of miRNAs in CRC pathogenesis. Other studies have demonstrated the tumor suppressive or oncogenic functions of miRNAs in CRC. Therefore, miRNAs might have diagnostic and prognostic value for CRC patients. MiRNAs may also represent novel therapeutic targets for gene therapy in the treatment of CRC.

In this review, the dysregulation of miRNAs leading to the initiation, progression and metastasis of CRC will be discussed. We also describe the translation of miRNA research to potential prognostic and therapeutic applications in the management of CRC in clinical settings.

## MIRNAS AND DYSREGULATION OF SIGNALING PATHWAYS IN CRC

Gene regulation by miRNAs is mediated by the formation of hybrids with the 3'-untranslated region (3'UTR) sequences of the target mRNAs, leading to mRNA degradation and/or translational inhibition<sup>[9]</sup>. MiRNAs play key roles in numerous cellular processes, including cell proliferation, differentiation, apoptosis, and development. These miRNAs are observed to regulate the expression of approximately one-third of human protein-coding genes<sup>[10]</sup>.

### *miRNAs and WNT/ $\beta$ -catenin pathway*

The dysregulation of the Wnt/ $\beta$ -catenin pathway is one of the earliest events driving CRC carcinogenesis. Constitutively active  $\beta$ -catenin upregulates the expression of Wnt target genes transcriptionally to initiate CRC formation<sup>[11]</sup>. There is mounting evidence suggesting there is crosstalk between miRNAs and the Wnt/ $\beta$ -catenin pathway in CRC development. While miRNAs were found to activate or inhibit the canonical Wnt pathway, Wnt activation was also shown to increase expression of miRNAs by directly binding to their gene promoters.

MiR-224 was recently shown to activate the Wnt/ $\beta$ -catenin signaling and direct the nuclear translocation of  $\beta$ -catenin in CRC by downregulating GSK3 $\beta$  and SFRP2<sup>[12]</sup>. Knockdown of miR-224 was found to restore the expression of GSK3 $\beta$  and SFRP2 and inhibit Wnt/ $\beta$ -catenin-mediated cell metastasis and cell proliferation.

Cancer stem cells (CSCs) are widely believed to be the key driving force for the initiation of cancer. CSC

subpopulations, capable of self-renewal and having the ability to initiate and sustain tumor growth, metastasis and resistance to chemotherapy, have been identified in CRC<sup>[13]</sup>. The Wnt pathway plays a key role in the induction of symmetrical cell division of CSCs, which disrupts the homeostasis of normal stem cells and leads to cancer formation<sup>[14,15]</sup>. To this end, miR-146a was shown to activate the Wnt pathway in colorectal cancer CSCs to stabilize  $\beta$ -catenin, thereby regulating the symmetrical cell division to promote CRC initiation and progression<sup>[16]</sup>. Interestingly, miR-146a was itself activated transcriptionally by Snail *via* a  $\beta$ -catenin-TCF4 complex<sup>[16]</sup>. Therefore, a feedback loop consisting of Snail-miR-146a- $\beta$ -catenin is operating in CSC of colorectal cancer to maintain Wnt activity.

### miRNAs and EGFR pathways

In recent years, anti-EGFR targeted drugs have been approved for treating metastatic CRC. However, treatment response is hindered by dysregulation of the PI3K/AKT and KRAS/RAF/ERK pathways downstream of EGFR.

In the PI3K/AKT pathway, mutation of the *PIK3CA* gene has been demonstrated in 15%-20% of CRC cases<sup>[17]</sup>. Interestingly, mutations in the miR-520a and miR-525a binding sites at the 3'UTR of *PIK3CA* were found to enhance the sensitivity of CRC cell lines to saracatinib, which inhibits the activation of Akt-dependent signaling<sup>[18]</sup>. Conversely, reduced expression of miR-126 has been shown to mediate amplification of the PI3K/AKT signal in CRC<sup>[19]</sup>.

Activating KRAS mutations constitutes up to 30%-60% of all CRC cases<sup>[20]</sup>, which mediates primary resistance to anti-EGFR targeted therapy. Moreover, even in CRC bearing wild-type KRAS, the response to anti-EGFR targeted therapy is less than 40%<sup>[21]</sup>. Additional molecular signature(s) are needed for the selection of patients who respond well to anti-EGFR targeted therapy. To this end, miRNAs such as let-7<sup>[22]</sup>, miR-18a\*<sup>[23]</sup>, miR-30b<sup>[24]</sup>, miR-143<sup>[25]</sup>, and miR-145<sup>[26]</sup> have been identified as tumor suppressors that inhibit KRAS expression. These miRNAs may be used as biomarkers to predict favorable response in patients to anti-EGFR therapy.

### miRNAs and TGF- $\beta$ signaling pathway

Transforming growth factor-beta (TGF- $\beta$ ) plays a key role in inhibiting cell proliferation, and it also modulates tumor invasion and tumor microenvironment modification. It has been estimated that 30% of CRC cases are due to mutations in TGF- $\beta$  type II receptor (TGF $\beta$ R2)<sup>[27,28]</sup>. TGF- $\beta$  binds to its receptors (TGF- $\beta$ R) and mediates the activation of its downstream pathway through phosphorylation of Smad. The complex is subsequently translocated to the nucleus, and it regulates the expression of transcriptional factors, including Snail, ZEB and Twist. Several miRNAs, including miR-21<sup>[29]</sup>, miR-106a<sup>[30]</sup>, and miR-301a<sup>[31]</sup>, have been reported to induce

stemness or promote cancer migration and invasion in CRC by targeting the TGF- $\beta$ /Smad signaling pathway. Conversely, decreased expression of miR-25 in CRC cell lines was also found to activate SMAD7, which is a negative regulator of TGF- $\beta$  signaling pathway, to promote cancer proliferation and metastasis<sup>[32]</sup>. Interestingly, miR-187, a validated downstream effector of the TGF $\beta$  pathway, was shown to suppress Smad-mediated epithelial-mesenchymal transition (EMT) in CRC cells<sup>[33]</sup>.

### miRNAs and epithelial-to-mesenchymal transition (EMT)

The activation of EMT, a cellular process of converting polarized epithelial cells to mesenchymal cells, enables cancer cells to migrate and invade into metastatic sites<sup>[34]</sup>. A number of miRNAs have been identified as key regulators of this EMT process in CRC<sup>[35]</sup>. MiR-29c has been shown to be remarkably downregulated in primary CRC with distant metastasis and it was associated with significantly shorter patient survival<sup>[36]</sup>. Importantly, forced expression of miR-29c was demonstrated to inhibit cell migration and invasion *in vitro* and metastasis *in vivo*<sup>[36]</sup>, which is related to its inhibition of the ERK/GSK3 $\beta$ / $\beta$ -catenin and AKT/GSK3 $\beta$ / $\beta$ -catenin pathways. Conversely, liver metastatic tissues were found to express higher level of miR-200c than the primary CRC tumor<sup>[37]</sup>. This increased expression has been specifically associated with hypomethylation of the promoter of the miRNA gene. The Wnt/ $\beta$ -catenin pathway was also shown to transactivate miR-150, which subsequently promoted EMT of CRC cells by suppressing CREB signaling<sup>[38]</sup>.

## MIRNAS AS POTENTIAL CLINICAL BIOMARKERS IN CRC

### miRNAs as diagnostic biomarkers for CRC

It is widely believed that a series of sequential genetic changes are needed to drive the conversion of normal colonic epithelium to malignant CRC. If the precancerous adenoma can be detected and treated prior to the development of advanced carcinoma, then patient mortality would be reduced<sup>[39]</sup>. While colonoscopy remains the gold standard screening test for the diagnosis of CRC<sup>[39,40]</sup>, the application of miRNA as diagnostic markers for CRC has attracted a lot of attention in recent years. Two of the first few miRNAs identified in CRC tumor specimens, miR-143 and miR-145, were, relative to normal colonic mucosa, found to be consistently downregulated in adenoma and other stages of CRC<sup>[8,41]</sup>. More recently, several other miRNAs (miR-21, miR-29a, miR-92a and miR-135b) have also been shown to, when compared to normal tissues, be upregulated in patients with high-risk adenomas<sup>[42]</sup>. A few excellent review articles about the development of miRNA-based biomarkers for the diagnosis of CRC can be found in recent literature<sup>[43-47]</sup>. Our review will focus more on the prognostic application and prediction of

therapeutic response by miRNAs in CRC.

### **miRNA as prognostic biomarkers for CRC**

Although tissue-specific signatures of miRNAs have been reported<sup>[48,49]</sup>, more work is still needed to define a set of differentially expressed miRNAs suitable for screening CRC in the clinical setting. Besides using miRNAs as a tool to screen for early adenomas and CRC cases, it may be more useful to use miRNAs as a prognostic tool to guide treatment for CRC patients.

### **miRNAs in tumor tissues**

Since surgical resection of the primary tumor is the treatment of choice in early stages of CRC, tumor tissue can be available as an important source for the identification of CRC-related miRNAs. Numerous miRNAs have been reported to be upregulated or downregulated in CRC cell lines in tumor specimens from patients, suggesting their association with patients' prognosis and response to anticancer drugs. Table 1 summarizes representative miRNAs of prognostic value in CRC tissues.

It is known that oncogenic miRNAs (also known as oncomiRs) and tumor suppressor miRNAs are differentially expressed during the development and progression of CRC. Moreover, miRNA expression in CRC is regulated in stage-specific manner (Figure 1). Therefore, tracing the order of miRNA regulation during CRC progression may indicate disease prognosis and predict treatment response. To this end, let-7, miR-21, miR-29a and miR-17-92 cluster were of particular importance.

Let-7 was known to regulate the expression of RAS and MYC genes, both of which are key factors mediating CRC progression and metastasis. Interestingly, the let-7 miRNA single nucleotide polymorphism (SNP) in the K-Ras 3'UTR has been reported to be prognostic in early-stage CRC (see below for more discussion about miRNA binding site SNP)<sup>[50]</sup>. MiR-17-92 is a highly conserved cluster that gives rise to six mature miRNAs, including miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92-1. This cluster is frequently amplified in CRC, suggesting an oncogenic role for these miRNAs<sup>[51]</sup>. Moreover, miR-17 and Tumor, Node, and Metastasis (TNM) staging were shown to be significant but independent prognostic biomarkers in CRC patients<sup>[51]</sup>. MiR-92a, targeting the anti-apoptotic protein BIM, was found to be expressed at higher level in both adenomas and carcinoma<sup>[52]</sup>. The other miRNAs within this cluster were shown to regulate multiple targets to drive CRC progression (PTEN – miR-19a and miR-19b-1; BCL2L11 – miR-19a, miR-19b-1, miR-20a, miR-92; E2F1 – miR-17 and miR-20a; TGF- $\beta$  receptor II – miR-17 and miR-20a<sup>[53-56]</sup>).

The remarkable upregulation of miR-21 in CRC has advocated its function as a prognostic marker<sup>[57]</sup>. In a small cohort of 29 CRC patients, high level of miR-21 in the tumor specimens was found to be associated with lymph node metastasis and also distal metastasis<sup>[58]</sup>.

In a bigger cohort of 156 CRC cases, elevated level of miR-21 was also found to be correlated with venous invasion, liver metastasis and advanced Dukes' stage<sup>[59]</sup>. A differentially expressed microRNA signature was recently reported in American CRC patients with liver metastasis<sup>[60]</sup>. miR-21, together with miR-93 and miR-103, were found to be increased in advanced CRC with liver metastasis.

The risk of CRC recurrence was found to be associated with high expression of miR-29a in tumor tissues in patients with stage II CRC<sup>[61]</sup>. miR-29a was later identified as a novel metastasis-promoting factor through upregulation of matrix metalloproteinase 2 and downregulation of E-cadherin *via* targeting the tumor suppressor gene KLF4<sup>[62]</sup>.

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## **LIMITATION WITH MEASURING MIRNAS IN ARCHIVAL TUMOR TISSUES**

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The major limitation of detecting miRNAs in archival tumor tissues is heterogeneity (from within the same primary tumor and also between different metastatic sites). Therefore, evaluating miRNA expression from serum/plasma (*i.e.* circulating miRNAs) may be potentially preferable for predicting prognosis in the clinical setting.

### **Circulating miRNAs**

For CRC, the traditionally used blood-based biomarkers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), suffer from low sensitivity, particularly for early stage CRC<sup>[63]</sup>. In recent years, miRNA-based biomarkers have shown promise as CRC-specific prognostic tests due to their high sensitivity and specificity. MiRNAs are remarkably stable in blood despite the high level of ribonuclease that rapidly degrade RNA<sup>[64]</sup>. MiRNAs are protected from enzyme degradation because they are stored in exosomes or vesicles, or bound to lipoproteins<sup>[65,66]</sup>.

Several miRNAs isolated from serum have been evaluated for their use as prognostic markers in CRC (Table 2). A recent report revealed a panel of miRNAs (miR-21, let-7g, miR-31, miR-92a, miR-181b, and miR-203) that can be reliably used as prognostic marker for CRC, with 93% sensitivity and 91% specificity in comparison with the traditional markers (CEA and CA19-9)<sup>[67]</sup>. Furthermore, miR-21 has also been shown to be able to differentiate both adenoma and CRC from healthy controls<sup>[68,69]</sup>. The detection of miR-141 in plasma samples has been used as a supplementary prognostic marker besides CEA for the detection of CRC patients with distant metastasis<sup>[70]</sup>. Moreover, the changes in plasma levels of miR-24, miR-320a and miR423-5p can be evaluated to predict the risk of post-surgery metastasis in CRC patients<sup>[71]</sup>.

### **Fecal-based miRNAs**

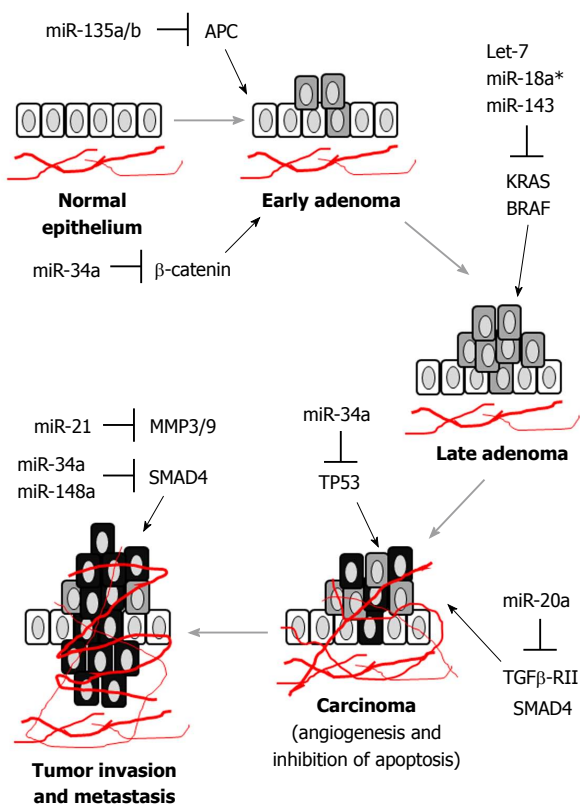
MiRNAs are known to be sufficiently stable for



**Table 1** A list of representative miRNAs identified in tumor tissues that are of prognostic value in colorectal cancer patients

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-15a/miR-16	qRT-PCR	126	miR-15a/miR-16 downregulation were significantly associated with advanced TNM staging, poorly histological grade, positive lymph node metastasis. miR-15a/miR-16 combination were identified as independent predictors of unfavorable OS and DFS	[183]
miR-17-5p	qRT-PCR	110	High expressions were associated with pathological tumor features of poor prognosis. miR-17-5p correlated with DFS only at early stages	[184]
miR-21	<i>In situ</i> hybridization	84	High miR-21 expressions were strongly associated with poor survival, more advanced TNM staging and poor therapeutic outcome	[90]
miR-29a	miRNA microarray, qRT-PCR	110	High expressions were associated with a longer DFS in CRC patients with stage II but not in stage I tumor	[61]
miR-34a-5p	qRT-PCR	205	The tissue expressions of miR-34a-5p was positively correlated with DFS. Moreover, expression of miR-34a-5p was an independent prognostic factor for CRC recurrence	[185]
miR-106a	qRT-PCR	110	Downregulation of miR-106a predicted shortened DFS and OS, independent of tumor stage	[184]
miR-132	miRNA microarray, qRT-PCR	28 (testing); 151 (validation)	Low expressions were associated with poor OS and occurrence of liver metastasis	[186]
miR-150	qRT-PCR, <i>in situ</i> hybridization	239	High expressions were associated with longer OS. Low expressions were associated with poor therapeutic outcome in patients treated with 5-FU-based chemotherapy with or without leucovorin, levamisole or cisplatin	[91]
miR-181a	qRT-PCR	162	High expressions were correlated with poor patient prognosis. Overexpression of miR-181a repressed the expression of the tumor suppressor (PTEN) at mRNA level	[187]
miR-181b	qRT-PCR	345	High expressions were correlated with poor survival in black patients with stage II CRC	[188]
miR-188-3p	Level 3 Illumina miRNASeq data were analyzed from TCGA database <sup>c</sup>	228	High expressions were associated with lower OS, higher tumor stage and indirectly with BRAF status	[99]
miR-195	qRT-PCR	85	Reduced expressions of miR-195 were correlated with occurrence of lymph node metastasis and advanced tumor stage	[189]
miR-199b	miRNA microarray and qRT-PCR	60	Higher level in metastatic CRC tissue compared with non-metastatic CRC tissue; low expressions were associated with longer OS	[190]
miR-203	microRNA microarray, qRT-PCR	197	High expressions were associated with more advanced TNM staging and poor survival	[90]
miR-215	qRT-PCR	34	High expressions were closely associated with poor OS	[191]
miR-218	qRT-PCR	63	High expressions were significantly associated with higher PFS, OS and response to 5-FU based chemotherapy	[192]
miR-320e	miRNA microarray and qRT-PCR	100	Elevated expressions were associated with poorer DFS and OS in stage III CRC patients	[93]
miR-429	qRT-PCR	116	High levels were correlated with OS; low levels were associated with favorable response to 5-FU-based chemotherapy	[193]
miR-494	qRT-PCR	104	High expressions were significantly associated with shorter DFS and OS. When used as a panel with 5 other miRNAs, the signature can distinguish early relapsed from non-early relapsed CRC.	[194]
miR-625-3p	qRT-PCR	94	High expressions were associated with higher OS, PFS and better response to treatment	[94]
3-miRNA signature (let-7i, miR-10b, miR-30b)	qRT-PCR	232	The addition of miR-30b to the 2-miRNA signature allowed the prediction of both distant metastasis and hepatic recurrence in patients with stage I-II CRC who did not receive adjuvant chemotherapy	[195]
A multi-RNA-based classifier (consisting of 12 mRNAs, 1 miRNA (miR-27a) and 1 lnc RNA)	mRNA, miRNA and lncRNA data were retrieved from the TCGA data portal	663	The classifier can divide patients into high and low risk groups with significantly different OS. Moreover, the classifier is not only independent of clinical features but also with a similar prognostic ability to the well-established TNM stage	[196]

OS: Overall survival ; DFS: Disease free survival; lnc RNA: Long non-coding RNA; TCGA : The Cancer Genome Atlas Project (<http://tcga-data.nci.nih.gov/>).



**Figure 1 MicroRNA dysregulation and colorectal cancer progression.** Altered expression of representative miRNAs are shown in different stages of CRC (in bold: Normal epithelium → early adenoma → late adenoma → carcinoma → metastasis). Upregulation or downregulation of miRNAs can affect signaling pathways and propel progression of CRC, leading to angiogenesis, cell invasion, metastasis and inhibition of apoptosis. APC: adenomatous polyposis coli; CRC: Colorectal cancer; KRAS: Kirsten rat sarcoma viral oncogene homolog; TP53: tumor protein p53.

detection in stool samples because they are protected in exosomes<sup>[72]</sup>. Moreover, fecal matter comes into direct contact with the lumen of colon and may include cells exfoliated from cancerous colonocytes. Molecular changes in CRC are more readily detected in the stool rather than in the blood<sup>[73]</sup>. Although fecal miRNAs were less extensively studied than circulatory miRNAs, the expression of numerous fecal miRNAs have been reported to be dysregulated in patients with CRC or advanced adenomas (Table 3). Recently, a panel of miRNAs isolated from the stool of CRC patients were found to differentiate not only CRC cases from healthy subjects but also differentiate TNM stages with high sensitivity and specificity<sup>[74]</sup>. Additionally, miR-135b was found to differentiate among different stages of CRC<sup>[75]</sup>.

**miRNAs in CRC-derived exosomes**

Exosomes are a unique forms of extracellular vesicles, ranging from 30 nm to 100 nm in diameter, which are released into the extracellular space upon the fusion of multivesicular bodies with plasma membranes from diverse cell types. Tumor-derived exosomes are emerging as local and systemic intercellular mediators of oncogenic information through the horizontal transfer of

mRNAs, miRNAs, and protein during tumorigenesis<sup>[76]</sup>. In a recent proteome profiling study of exosomes derived from human primary and metastatic CRC cells, a selective enrichment of metastatic factors and signaling pathway components was observed<sup>[77]</sup>. High expression of exosomal miR-17-92a cluster was found to be associated with recurrence in late stage CRC patients<sup>[78]</sup>. Moreover, an elevated exosomal level of miR-19a was reported in the sera samples of CRC patients with poor prognosis<sup>[78]</sup>. Interestingly, a recent report has demonstrated an enrichment of miR-328 from CRC patients' plasma samples collected from colonic veins (*i.e.*, mesenteric veins) when compared to peripheral vein plasma samples and that it was significantly correlated with the development of liver metastasis<sup>[79]</sup>. Table 4 summarizes a short list of miRNAs of prognostic value identified in exosomes released from CRC cells.

**MIRNAS FOR STRATIFYING RISK OF RECURRENCE IN STAGE II CRC PATIENTS**

Adjuvant chemotherapy following surgery is adopted as the standard treatment to improve survival in stage III CRC patients<sup>[80]</sup>. Conversely, in stage II CRC patients, surgical resection of the primary tumor is highly effective without other concomitant treatment. Therefore, the use of adjuvant chemotherapy in all stage II CRC patients is debatable<sup>[81-83]</sup>. However, a notable sub-group of stage II CRC patients (~30%) develop tumor recurrence and have poor prognoses. At present, there are a few clinical parameters, including poorly differentiated histology, extramural venous invasion, intestinal obstruction and perforation, that are used as risk factors to identify high-risk patients for adjuvant chemotherapy. Since these clinical parameters are not specific, more reliable biomarkers are needed to identify the high-risk stage II CRC patients and initiate adjuvant chemotherapy to improve their survival. Likewise, other Stage II CRC patients with lower risk can be spared from the toxicity of conventional cytotoxic chemotherapeutic drugs. To this end, clinical trials have been initiated to investigate the use of miRNA to identify stage II CRC patients who may benefit from chemotherapy (including an on-going study being conducted in China (NCT02635087)).

Schetter *et al.*<sup>[84]</sup> showed that the combination of miR-21 expression in primary tumor tissues and inflammatory risk score was a better predictor of prognosis than either biomarker alone. Data from a large population-based study conducted on 520 stage II CRC patients showed that elevated miR-21 expression in tumor specimens correlated significantly with poor cancer-free survival<sup>[85]</sup>. Another six-miRNAs based classifiers (miR-20a-5p, miR-21-5p, miR-103a-3p, miR-106a-5p, miR-143-5p, and miR-215) were reported to be a promising predictive markers for tumor recurrence

**Table 2** A list of representative circulating miRNAs of prognostic value in colorectal cancer patients

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-21	qRT-PCR	102	Lower serum expressions were associated with higher local recurrence and mortality	[197]
miR-23b	qRT-PCR	96	CRC patients with low miR-23b expression in plasma exhibited a shorter recurrence-free survival and poorer overall survival rate	[198]
miR-96	TaqMan miRNA microarray	50 (screening); 234 (validation)	Elevated plasma levels were strongly correlated with shorter OS, especially in stage II and III CRC patients	[199]
miR-124-5p	qRT-PCR	71	Higher plasma levels were correlated with longer OS	[200]
miR-141	qRT-PCR	102	High plasma levels were significantly associated with stage IV colon cancer and poor prognosis	[70]
miR-148a	qRT-PCR	55	Lower levels were associated significantly with shorter DFS and poorer OS rates	[201]
miR-155	qRT-PCR	146	High serum levels correlated with poor PFS and OS	[202]
miR-183	qRT-PCR	118	High plasma levels were significantly associated with lymph node metastasis, distant metastasis, higher TNM staging, and tumor recurrence	[203]
miR-200b	TaqMan miRNA microarray	50 (screening); 234 (validation)	Elevated plasma levels were correlated with shorter OS, especially in stage II and III CRC patients	[199]
miR-200c	qRT-PCR	182 CRC patients and 24 controls	High serum expressions were strongly correlated with lymph node, distant metastasis, tumor recurrence and poor prognosis.	[204]
miR-203	qRT-PCR	144 (validation)	High serum levels were associated with poor survival and metastasis;	[205]
miR-218	qRT-PCR	189	Serum levels were significantly associated with TNM stage, lymph node metastasis and differentiation. Patients with low miR-218 serum level had shorter survival.	[206]
miR-221	qRT-PCR	103	Elevated plasma level is a significant prognostic factor for poor overall survival in CRC patients	[207]
miR-345	TaqMan miRNA microarray	138	High plasma levels were significantly associated with shorter PFS and lack of response to treatment with cetuximab and irinotecan	[108]
miR-885-5p	miRNA microarray, qRT-PCR	169	High serum expressions were highly correlated with poor prognosis, lymph node metastasis and distant metastasis	[208]
miR-1290	miRNA microarray, qRT-PCR	324	High serum levels were associated with lower OS, lower DFS and more advanced tumor stage	[209]
2-miRNA prognostic panel (miR-23a-3p & miR-376c-3p)	miRNA microarray	427	A prognostic panel consisting of miR-23a-3p and miR-376c-3p, independent of TNM stage, was established	[210]
miR-200 & miR-141	qRT-PCR	380	High serum levels of miR-200 and miR-141 were associated with higher propensity of CRC patients to develop liver metastasis	[211]
miR-122 & miR-200 family	miRNA microarray	543	Increased plasma miR-122 levels were associated with a "bad" prognostic subtype in metastatic CRC and a shorter relapse-free survival and overall survival for non-metastatic and metastatic CRC patients. Several members of the miR-200 family were associated with patients' prognosis and clinicopathological characteristics	[212]

OS : Overall survival; DFS: Disease free survival; PFS : Progression free survival; CRC: Colorectal cancer; TNM: Tumor, node, and metastasis.

in patients with stage II CRC<sup>[86]</sup>. More recently, the up-regulation of miR-181c in CRC specimens was also found to be associated with higher recurrence<sup>[87]</sup>.

#### **miRNAs for predicting response to chemotherapy**

There has been intense research interest in the development of miRNA signature to predict response to anticancer treatment, thus allowing a more personalized approach for treating CRC with better efficacy and fewer adverse effects. To this end, a genome-wide miRNA profiling in twelve CRC cell lines has been performed

to derive an *in vitro* signature of chemosensitivity<sup>[88]</sup>. Numerous studies have also reported the potential use of miRNAs as biomarkers in tissues and blood to predict response to the most common drug therapies (5-fluorouracil (5-FU)/oxaliplatin cytotoxic chemotherapy and EGFR targeted therapy) in CRC patients (Table 5).

#### **Response to classical cytotoxic chemotherapy (5-FU/oxaliplatin) overexpression or downregulation of single miRNA in CRC tumor specimens**

In 5-FU-based neoadjuvant chemotherapy setting

**Table 3** A list of representative miRNAs identified in fecal samples from colorectal cancer patients that are of prognostic value

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-135b	qRT-PCR	424	miR-135b showed a significant increasing trend across the adenoma to cancer sequence. miR-135b level may be used to differentiate between different stages of CRC. Stool miR-135b level dropped significantly upon removal of CRC or advanced adenoma.	[75]
miR-19-b-3p, miR-20a-5p, miR-21-3p, miR-92a-3p, miR-141	qRT-PCR	20	Expression levels of three out of the five overexpressing miRNAs returned to values comparable to normal controls after curative surgery; this was correlated with the	[213]
12 upregulated and 8 downregulated miRNA panel	miRNA microarray, qRT-PCR	60	A panel of 12 upregulated miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p, miR-214) and 8 downregulated miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222, miR-938) were found to differentiate not only CRC cases from healthy subjects but also differentiate TNM stages with high sensitivity and specificity.	[74]

**Table 4** A list of representative miRNAs isolated from exosomes in biological samples from colorectal cancer patients or cell lines that are of prognostic value

miRNA	Method of detection	Type of samples	Tumor stage	Ref.
miR-17-92a	miRNA microarray	Tumor specimens	IV	[78]
miR-19a	miRNA microarray	Tumor specimens	IV	[78]
let-7a	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-21	qRT-PCR and miRNA microarray	Serum; plasma	I, II, IIIa, IIIb, IV	[214]
miR-23a	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-150	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-203	qRT-PCR	Serum	I, II, III, IV	[215]
miR-223	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-1246	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-1229	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-548c-5p	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-638	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-5787	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-8075	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-6869-5p	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-486-5p	miRNA microarray and qRT-PCR	Serum (upregulated)	I, II, IIIa, IIIb, IV	[216]
miR-3180-5p	miRNA microarray and qRT-PCR	Serum (upregulated)	I, II, IIIa, IIIb, IV	[216]
miR-96-5p and miR-149	qRT-PCR	Plasma	III	[217]
miR-100	qRT-PCR	Cell lines (DKO-1, Dks-8, DLD-1)		[218]
miR-192	qRT-PCR	Cell lines (HCT-15, SW480, WiDr)		[219]
miR-210	qRT-PCR	Cell line (HCT-8)		[220]
miR-221	qRT-PCR	Cell lines (HCT-15, SW480, WiDr)		[219]
miR-379	qRT-PCR	Cell lines (HCT-116, HT-29)		[221]

to shrink the tumor size before surgery, low tumoral expression of miR-21 was found to be predictive of pathological response to the drug in locally advanced CRC patients<sup>[89]</sup>. In another study performing miRNA expression profiling of CRC, along with their paired non-cancerous tissues, high miR-21 expression in the tumors were found to associate with poor therapeutic response to 5-FU-based adjuvant chemotherapy as an add-on treatment<sup>[90]</sup>. Also predictively, low miR-148a, low miR-150 or high miR-320e level in tumor specimens were found to be associated with poor response to adjuvant 5-FU/oxaliplatin (FOLFOX) regimen in several other studies<sup>[91-93]</sup>. Interestingly, in a recent study on

responsive and non-responsive metastatic CRC patients receiving XELOX/FOLFOX regimen, high expression of miR-625-3p in the tumor was found to be associated with poor response to the drug but was not related to disease recurrence<sup>[94]</sup>.

**Signature of miRNAs panel in tumor specimens**

While the increased/decreased expression of a single miRNA may predict response to chemotherapy as described above, the use of a panel of miRNAs demonstrating a signature may be more reliable and specific. In a recent study on fluoropyrimidine-based chemotherapy, the responders were found to exhibit high tumoral levels



**Table 5** MiRNAs in tumor specimens and plasma/serum samples reported to predict therapeutic response in colorectal cancer patients

miRNA	Treatment regimen	Detection method	Expression that suggests inadequate response	Patient number	Ref.
Tumor specimens					
Let-7	Irinotecan, cetuximab	qRT-PCR	Low	59	[102]
miR-7	Cetuximab	qRT-PCR	Low	105	[97]
miR-21	5-FU	qRT-PCR	High	84	[90]
miR-21	5-FU	qRT-PCR	High	67	[89]
miR-21-5p	5-FU + radiation	Microarray	Low	27	[222]
miR-31-3p	Anti-EGFR	Microarray, qRT-PCR	High	33	[98]
miR-31-5p	Anti-EGFR	qRT-PCR	High	102	[100]
miR-126	Capecitabine, oxaliplatin	qRT-PCR, ISH	Low	89	[104]
miR-143	Capecitabine, oxaliplatin, anti-EGFR	Microarray, qRT-PCR	High	34	[103]
miR-146b-3p	Cetuximab	qRT-PCR	High	25	[223]
miR-148a	5-FU	qRT-PCR, ISH	Low	273	[92]
miR-150	5-FU	qRT-PCR, ISH	Low	239	[91]
miR-181a	Anti-EGFR	qRT-PCR	Low	80	[99]
miR-200 family	Fluoropyrimidine	qRT-PCR	Low	127	[103]
miR-200b	Capecitabine, oxaliplatin, anti-EGFR	Microarray, qRT-PCR	Low	34	[103]
miR-320e	5-FU, oxaliplatin	Microarray, qRT-PCR	High	100	[93]
miR-455-5p	Capecitabine, oxaliplatin, bevacizumab	qRT-PCR, ISH	High	212	[224]
miR-486-5p	Cetuximab	qRT-PCR	High	25	[223]
miR-519c	5-FU, irinotecan	qRT-PCR	Low	26	[153]
miR-592	Anti-EGFR	Microarray	Low	33	[98]
miR-625-3p	Capecitabine, oxaliplatin	Microarray, qRT-PCR	High	94	[94]
miR-664-3p	Capecitabine, oxaliplatin, bevacizumab	qRT-PCR, ISH	Low	212	[224]
Let-7c, miR-99a, miR-125b	Anti-EGFR	Microarray, qRT-PCR	Low	74	[101]
miR-1274b, miR-720	Capecitabine, oxaliplatin, radiation	Microarray, qRT-PCR	High	38	[96]
miR-107, miR-99a-3p	Fluoropyrimidine	Microarray, qRT-PCR	Low	39	[225]
miR-215, miR-190b, miR-29b-2	5-FU, radiotherapy	Microarray, qRT-PCR	High	20	[95]
Let-7e, miR-196, miR-450a, miR-450b-5p, miR-99a	5-FU, radiotherapy	Microarray, qRT-PCR	Low	20	[95]
miR-17-3p, miR-193b-5p, miR-204-5p, miR-501-5p, miR-545-3p, miR-592, miR-644-3p, miR-15a-5p, miR-196b-5p, miR-552	First-line capecitabine and oxaliplatin with or without bevacizumab	qRT-PCR, ISH	Low	212 (screening); 121 (validation)	[224]
miR-1183, miR-622, miR-765, miR-1471, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-483-5p, miR-671-5p, miR-1909	Capectabine, oxaliplatin, radiation	Microarray, qRT-PCR	Low	38	[96]
Blood					
miR-19a	FOLFOXa	Microarray, qRT-PCR	High	72	[226]
miR-126	Bevacizumab		High		[104]
miR-143	Oxaliplatin	qRT-PCR	Low	62	[227]
miR-155	Leucovorin, cetuximab, 5-FU	qRT-PCR	High	15	[107]
miR-345	Cetuximab, irinotecan	Microarray, qRT-PCR	High	138	[108]
miR-1914*	Capecitabine + oxaliplatin	qRT-PCR	Low	49	[228]
miR-106a, miR-130b, miR-484	5-FU, oxaliplatin	qRT-PCR	High	150	[105]
miR-20a, miR-130, miR-145, miR-216, miR-372	FOLFOX	Microarray, qRT-PCR	High	40	[106]

FOLFOX: 5-FU + oxaliplatin + leucovorin; 5-FU: 5-fluorouracil; ISH: *In situ* hybridization; Anti-EGFR: Anti-EGFR targeted monoclonal antibody therapy; EGFR: Epidermal growth factor receptor.

of let-7e, miR-99a\*, miR196b, miR450a and miR450b-5p whereas the non-responders were found to have high expression of miR-29b-2\*, miR-196b and miR-215<sup>[95]</sup>. Another set of thirteen miRNAs, exhibiting a unique signature with 11 upregulated miRNAs (miR-125a-3p,

miR-188-5p, miR-483-5p, miR-622, miR-630, miR-671-5p, miR-765; miR-1183, miR-1224-5p, miR-1471, and miR-1909\*) and 2 downregulated miRNAs (miR-720 and miR-1274b) were shown to be strongly associated with complete pathological response to neoadjuvant

capecitabine and oxaliplatin (XELOX) regimen in CRC patients<sup>[96]</sup>.

### **Response to anti-EGFR/anti-VEGF targeted therapy**

There has been intense research effort to identify miRNAs predictive of a favorable response to anti-EGFR monoclonal antibody. Consistent with the fact that miR-7 targets EGFR, reduced miR-7 expression was linked to poor prognosis of anti-EGFR therapy<sup>[97]</sup>. The efficacy of anti-EGFR therapy is related to the mutation status of KRAS/BRAF. Patients bearing wild-type KRAS/BRAF are normally considered candidates for anti-EGFR therapy. Therefore, numerous studies have been carried out with an aim to identify miRNAs that predict success with EGFR inhibition with wild-type KRAS. In metastatic CRC patients bearing wild-type KRAS/BRAF, high expression of miR-31<sup>[98]</sup>, low expression of miR-592<sup>[98]</sup>, or low expression of miR-181a<sup>[99]</sup> were found to be strongly associated with disease progression and short progression-free survival after treatment with anti-EGFR therapy (cetuximab or panitumab). To this end, miR-31-5p has been reported to regulate BRAF activation and thus the downstream signaling pathway of EGFR<sup>[100]</sup>. Conversely, miR-181a has been reported to regulate  $\beta$ -catenin expression<sup>[99]</sup>. Apart from individual miRNAs, the high-intensity signature of the cluster let-7c/miR-99a/miR-125b was found to be associated with favorable response (longer progression-free survival and overall survival) to cetuximab or panitumumab in metastatic CRC patients bearing wild-type KRAS<sup>[101]</sup>.

Drug resistance mediated by KRAS mutations is severely hindering the clinical efficacy of anti-EGFR therapy in CRC because KRAS mutations drive the downstream MAPK and Akt signaling independent of EGFR. However, treatment with anti-EGFR therapy is still feasible, and a few miRNAs have been reported to predict favorable responses in metastatic CRC patients bearing KRAS mutations. To this end, let-7 is the most extensively studied miRNA as predictive biomarker for anti-EGFR therapy in patients with KRAS mutations<sup>[102]</sup>. Let-7 downregulates KRAS by directly binding to its mRNA 3'UTR. Interestingly, in patients with KRAS mutations, high levels of let-7 were shown to be significantly associated with better survival following cetuximab treatment<sup>[102]</sup>. Thus, let-7 level may be used to select subgroup of CRC patients with KRAS mutations to benefit from anti-EGFR therapy. In another study investigating KRAS-mutated metastatic CRC tumors, low miR-143 and high miR-200b expression in the tumor were found to be associated with better progression-free survival after treatment with combination of XELOX, cetuximab and bevacizumab<sup>[103]</sup>.

The combination of anti-VEGF monoclonal antibody (bevacizumab) and cytotoxic chemotherapy has been approved for treating metastatic CRC. The predictive value of circulating miR-126 in treatment response to first-line chemotherapy combined with bevacizumab has

been demonstrated in patients with metastatic CRC<sup>[104]</sup>. While non-responding patients had a remarkable increase in circulating miR-126 level, responding patients exhibited a mild decrease in circulating miR-126.

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## **CIRCULATING MIRNAS AS NON-INVASIVE BIOMARKERS OF DRUG RESPONSE IN CRC**

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Besides serving as prognostic markers, circulating miRNAs can also be used as non-invasive and convenient biomarkers of drug response to classical cytotoxic drugs and EGFR-targeted therapy in CRC patients.

Pre-treatment level of plasma miRNAs have been shown to predict treatment response to 5-FU/oxaliplatin in metastatic colorectal cancer patients<sup>[105]</sup>. By measuring the expression profile of plasma miRNAs in metastatic CRC patients before and after four cycles of 5-FU/oxaliplatin chemotherapy, a set of three miRNAs (miR-106a, miR-130b, and miR-484) were found to be significantly upregulated in non-responders<sup>[105]</sup>. In another study using Taqman low-density array to evaluate the expression profile of serum miRNAs in chemotherapy responsive versus resistant CRC patients by unsupervised cluster analysis, a set of five serum miRNAs (miR-20a, miR-130, miR-145, miR-216, and miR-372) was found to predict response to classical chemotherapy and it may guide the selection of the chemotherapy regimen<sup>[106]</sup>. Recent studies have also shown that elevated levels of serum miR-155<sup>[107]</sup> and miR-345<sup>[108]</sup>, respectively, were associated with inadequate response to 5-FU/leucovorin/cetuximab and irinotecan/cetuximab regimens in metastatic CRC patients.

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## **POLYMORPHISMS IN MIRNA BINDING SITES PREDICT CRC PROGNOSIS AND TREATMENT RESPONSE**

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Single nucleotide polymorphisms (SNPs) located in the 3' UTR of miRNA target genes have been shown to affect miRNA-mRNA interaction and thus expression of target genes<sup>[109,110]</sup>. While several studies have reported the correlation between miRNA SNPs and risk of developing CRC<sup>[110,111]</sup>, only a few studies have been conducted to investigate the use of miRNA SNPs to predict clinical outcomes of cancer therapy.

Using genome-wide approach, two SNPs rs1051424 and rs11704 were identified in the putative miRNA target sites of the 3'UTR of RPS6KB1 and ZNF839<sup>[112]</sup>. Importantly, overall survival was found to be inversely proportional to the occurrence of the two SNPs in CRC patients<sup>[112]</sup>. The SNPs resulted in the loss of the miRNA binding sites on both RPS6KB1 and ZNF839, thereby enabling the upregulation of the two genes. Activated RPS6KB1 is known to phosphorylate ribosomal protein

S6 to increase protein synthesis and to promote cancer cell survival<sup>[113]</sup>. On the other hand, ZNF839 is not well characterized but reportedly involved in humoral immune response to cancer<sup>[114]</sup>.

Similar miRNA SNP-mRNA interaction has also been shown to predict therapeutic response to cancer treatment in CRC. While it is well known that KRAS mutations predict resistance to anti-EGFR monoclonal antibodies treatment in CRC, the predictive marker for therapeutic response in CRC patients bearing wild-type KRAS is less studied. In a phase II study, the occurrence of a SNP in the let-7 miRNA complementary site of wild-type KRAS 3'UTR was found to correlate with the objective response rate to cetuximab monotherapy in metastatic CRC patients<sup>[115]</sup>.

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## MIRNAS DEMONSTRATED TO BE THERAPEUTIC TAGETS IN CRC

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The development of miRNA-based therapy for cancer treatment is based on the premise that aberrantly expressed miRNAs play crucial role in the development of cancer and therapeutic response to anticancer drugs. Therefore, correcting the miRNA deficiency or restoring the miRNA function could be used as novel strategy in cancer treatment<sup>[116,117]</sup>. Key miRNAs that have been studied are described below.

### **miR-21 and tumor invasion**

Overexpression of miR-21 was associated with tumor invasion of CRC by promoting nuclear translocation of  $\beta$ -catenin<sup>[118]</sup>. Interestingly, CRC patients bearing *adenomatous polyposis coli* (APC) mutated tumor who have high levels of serum miR-21 were found to have poor prognosis, but this correlation was not observed in CRC patients bearing APC-wild type tumor. Mechanistically, miR-21 targets PDCD4, which inhibits cancer transformation and invasion<sup>[119]</sup>.

Furthermore, miR-21 was also shown to confer resistance to 5-FU chemotherapy by downregulation of human MutS homolog 2 (*MSH2*)<sup>[120]</sup>. Overexpression of miR-21 was found to suppress the G2/M arrest and apoptosis induced by 5-FU. To this end, a miR-21 antisense inhibitor against hepatocellular carcinoma is under development by Regulus Therapeutics<sup>[121]</sup>.

### **miR-34a and tumor suppressor p53**

miR-34a is one of the most important downstream molecules of p53 that is transcriptionally activated to exert the tumor suppressive effect through cell cycle arrest and induction of apoptosis<sup>[122,123]</sup>. However, p53 mutation is observed in 50%-75% of CRC<sup>[3]</sup>. Downregulation of miR-34a is commonly observed in CRC. It has been reported that miR-34a expression was significantly decreased in CRC patients versus healthy individuals, suggesting that miR-34a could be a useful biomarker and therapeutic target in CRC<sup>[124,125]</sup>. Recently, it has been shown that p53-dependent expression of

miR-34a blocks the IL-6R/STAT3/miR-34 feedback loop and subsequently inhibit tumor progression in CRC<sup>[126]</sup>. Since activation of IL-6R and STAT3 promote cancer proliferation, the overexpression/restoration of miR-34a may develop into a new treatment strategy for CRC.

### **miR-101 and WNT/ $\beta$ -catenin pathway**

More than 60% of CRC cases are known to carry inactivating APC gene mutations, which lead to stimulation of the Wnt/ $\beta$ -catenin pathway to drive tumor initiation and recurrence<sup>[3]</sup>. Besides this extensively studied APC gene-mediated mechanism, miRNAs have also been shown to regulate the Wnt pathway in CRC. Downregulation of miR-101 was associated with activation of Wnt/ $\beta$ -catenin pathway<sup>[127]</sup>. Interestingly, overexpression of miR-101 in CRC was found to impair  $\beta$ -catenin nuclear localization and inhibit cancer stem cells-related genes<sup>[127]</sup>. Therefore, pharmacological restoration of miR-101 may represent a novel strategy to prevent recurrence of CRC.

### **miR-135B and downregulation of APC in CRC**

miR-135a/b has been shown to be upregulated in colorectal adenomas and carcinomas, which correlates well with low APC expression in the tumor<sup>[128]</sup>. MiR-135a/b was found to target the 3'UTR of APC to suppress the expression of APC gene, and thus activating the downstream Wnt pathway to promote tumor transformation and progression<sup>[129]</sup>. To this end, miR-135b up-regulation was also found to be common in sporadic and inflammatory bowel disease-associated human CRCs; similarly, increased miR-135b expression was correlated with advanced tumor stages and poor clinical prognosis<sup>[129]</sup>. Importantly, inhibition of miR-135b, in CRC mouse xenograft models, was able to inhibit tumor growth by regulating genes involved in proliferation, invasion and apoptosis.

### **miR-143/145 as tumor suppressors in CRC**

miR-143 and miR-145 were first identified as potential tumor suppressors in an analysis investigating the alteration of microRNAs expression from adenomatous to carcinoma stages in CRC<sup>[8]</sup>. Consistent with this finding, the upregulation of miR143/145 was observed in tumor tissues from advanced rectal cancer patients demonstrating favorable response to neoadjuvant chemoradiotherapy<sup>[130]</sup>. Mechanistically, miR-143 was found to bind directly to and suppress KRAS, DNMT3A and ERK5 whereas miR-145 was shown to target IRS-1, c-Myc, FLI1, IRS-1, STAT1 and YES1 to suppress tumor growth<sup>[131]</sup>. Forced expression of miR-143 and miR-145 have been shown to inhibit proliferation of CRC cell lines *in vitro* and *in vivo*<sup>[132]</sup>.

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## MIRNAS REGULATING THE EGFR SIGNALING PATHWAY

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The crucial role played by EGFR signaling in the survival

of numerous cancer types, including CRC, has led to the approval of anti-EGFR targeted therapy, including tyrosine kinase inhibitors and monoclonal antibodies for treating CRC. However, drug resistance to these targeted agents is severely hindering their clinical efficacy. To this end, the activation of PI3K and mutation of KRAS, two key signaling pathways downstream of EGFR, have been extensively studied as mechanisms leading to the drug resistance to anti-EGFR therapy<sup>[133]</sup>. In recent years, increasing evidence has suggested that aberrant expression of miRNAs are affecting EGFR signaling and are causing drug resistance to anti-EGFR therapy.

Low expression of miR-30b was observed in CRC tumor specimens, which was significantly associated with advanced TNM staging and poor prognosis<sup>[24]</sup>. Forced expression of miR-30b was found to promote G1 arrest and induce apoptosis by targeting PIK3CD and BCL2, thereby inhibiting CRC cell growth *in vitro* and *in vivo*. On the other hand, miR-126 has been shown to inhibit PI3K signaling by targeting the p85b regulatory subunit responsible for PI3K stabilization<sup>[19]</sup>.

Activating mutation of KRAS is known to drive resistance to anti-EGFR therapy by bypassing the inhibited EGFR. To this end, let-7 has been shown to post-transcriptionally downregulate KRAS and administration of let-7 to CRC animal models expressing activating KRAS mutations was shown to inhibit tumor growth<sup>[134]</sup>. More importantly, in CRC patients bearing mutant KRAS tumor who received salvage cetuximab plus irinotecan, higher let-7 level was found to be significantly associated with better survival outcomes<sup>[102]</sup>. miR-4689 was found to directly target KRAS and AKT1, therefore activation of the KRAS pathway may thus be mediated by downregulation of miR-4689. Forced expression of miR-4689 has been reported to inhibit CRC cancer growth and induce apoptosis both *in vitro* and *in vivo*<sup>[135]</sup>.

The tumor suppressor PTEN (phosphatase and tensin homolog) plays an important role in regulating the PI3K/Akt pathway. Overexpression of miR-17-5p has been shown to promote drug resistance and tumor metastasis of CRC by downregulating PTEN<sup>[136]</sup>. Similarly, miR-32 was found to directly repress PTEN to regulate tumorigenesis of CRC<sup>[137]</sup>.

## MIRNAS REGULATING EMT

EMT is a complex process controlled by a number of transcriptional regulators and signaling pathways. In CRC, EMT is associated with a more malignant phenotype and metastasis. Recently, miRNAs have been suggested to be involved in the acquisition of cancer stem cell-like properties by regulating EMT. To this end, TGF- $\beta$  is a major inducer of EMT by activating Smad signaling pathway. The miR-200 family have been reported to target TGF- $\beta$ 2. In particular, miR-

200c was found to be significantly upregulated in tumor samples from patients with metastatic colon cancer and also colon cancer cell lines<sup>[37]</sup>, which has been correlated with reduced expression of its direct target (ZEB1) to inhibit EMT<sup>[37,138,139]</sup>.

miR-130a/301a/454 family of microRNAs have also been shown to regulate TGF- $\beta$  signaling by inhibiting SMAD4<sup>[140]</sup>. Overexpression of these miRNAs was reported to increase cell proliferation and migration in colon cancer cell lines (HCT116 and SW480)<sup>[140]</sup>. Similarly, miR-1269a was also found to promote EMT and metastasis in CRC cells *in vitro* and *in vivo*<sup>[141]</sup>. Importantly, the overexpression of miR-1269a in tumor specimens from patients with late-stage CRC was associated with relapse and metastasis<sup>[141]</sup>.

## MIRNAS AS REGULATORS OF DRUG RESISTANCE IN CRC

While surgical resection of the primary tumor is the treatment of choice for most CRC patients, chemotherapy is commonly used as both neoadjuvant and adjuvant therapy to reduce the tumor load and to prevent recurrence and metastasis, respectively. Commonly used chemotherapeutic agents include 5-FU, oxaliplatin, irinotecan, and the anti-EGFR/VEGF monoclonal antibodies<sup>[142]</sup>. Drug resistance to these anticancer drugs pose a major obstacle for effective chemotherapy<sup>[143]</sup>. To this end, miRNAs have been found to play an important role in the induction of chemoresistance and they also represent emerging prognostic markers to indicate treatment success<sup>[144-146]</sup> (Table 6).

### miRNAs mediating resistance to 5-FU

5-FU is the most commonly used first-line chemotherapeutic drug for CRC. Hypoxia inside CRC tumors has been shown to reduce drug sensitivity to 5-FU by upregulating two hypoxia-responsive miR-21 and miR-30d, which altered amino acid metabolism. Importantly, treatment with miR-21 and miR-30d antagonists were able to sensitize hypoxic and resistant CRC cells to 5-FU<sup>[147]</sup>. Reduced miR-34a expression in CRC cell line was also shown to mediate resistance to 5-FU. Ectopic expression of miR-34a was able to reverse the resistance by downregulating Sirt1 and E2F3<sup>[148]</sup>. It has been demonstrated that high expression of miR-23a could trigger 5-FU resistance by inhibiting the APAF-1/caspase-9 apoptotic pathways in CRC cell lines<sup>[149]</sup>. While miR-494 expression was found to be reduced in drug-resistant cell lines, forced expression of miR-494 was shown to potentiate the anticancer effect of 5-FU by targeting DPYD<sup>[150]</sup>. On the other hand, overexpression of miR-587 has been observed in CRC cells resistant to 5-FU *in vitro* and *in vivo*<sup>[151]</sup>, which is mediated by downregulation of PPP2R1B and thus increased Akt phosphorylation and elevated XIAP



**Table 6** A list of representative miRNAs involved in drug resistance to colorectal cancer therapy

miRNA	Drug(s) affected	Known molecular target(s) in CRC	Ref.
Overexpression of miRNA causing drug resistance to conventional chemotherapeutic drugs			
Let-7g	S-1 (Tegafur/gimeracil/oteracil)	Cyclin D, c-myc, E2F, RAS	[229]
miR-10b	5-FU	BIM	[230]
miR-19b	5-FU	MYBL2	[231]
miR-20a	5-FU, oxaliplatin	BNIP2	[170]
miR-21	5-FU	hMSH2 and hMSH6	[120]
miR-23a	5-FU	APAF-1	[149]
miR-31	5-FU	-	[232]
miR-140	5-FU	HDAC4	[233]
miR-148a	5-FU, oxaliplatin	-	[92]
miR-181b	S-1, 5-FU	Cyclin D, c-myc, E2F, RAS	[229]
miR-192/miR-215	5-FU	DHFR	[234,235]
miR-195	5-FU	WEE1	[236]
miR-203	Oxaliplatin	ATM	[154]
miR-224	5-FU	-	[237]
miR-520g	5-FU, oxaliplatin	p21	[238]
miR-587	5-FU	PPP2R1B	[151]
miR-625-3p	Oxaliplatin	-	[94]
Downregulation of miRNA causing drug resistance to conventional chemotherapeutic drugs			
miR-34a	5-FU, oxaliplatin	SIRT1, KIT, LDHA, TGF- $\beta$ , SMAD4	[239-241]
miR-139-5p	5-FU	NOTCH-1	[242]
miR-153	Oxaliplatin	FOXO3	[243]
miR-194	Oxaliplatin, irinotecan	HMGA2	[244]
miR-200 family	5-FU	EMT-related genes	[245]
miR-200b-3p	Oxaliplatin	PRDX2	[246]
miR-203	5-FU	TYMS	[247]
miR-218	FOLFOX	EZH2	[248]
miR-761	5-FU	FOXO1	[249]
miR-1915	Oxaliplatin	BCL2	[155]
miR-141/miR-200c	Oxaliplatin	ZEB1	[250]
miR-18a*/miR-4802	5-FU, capecitabine	ATG7, ULK1	[251]
Overexpression of miRNA causing drug resistance to molecular targeted drugs			
miR-31	Cetuximab	-	[252]
miR-126	Bevacizumab	VEGF	[104]
miR-100/miR-125b	Cetuximab	Wnt/ $\beta$ -catenin negative regulators	[253]
Downregulation of miRNA causing drug resistance to molecular targeted drugs			
Let-7 family	Cetuximab, panitumumab	KRAS	[115,223]
miR-7	Cetuximab	EGFR, ERK1/2, AKT	[97]

EGFR: Epidermal growth factor receptor; CRC: Colorectal cancer.

expression to inhibit apoptosis.

Overexpression of the multidrug resistance transporter ABCG2 has been shown to cause resistance to 5-FU. Our research team has previously demonstrated the specific targeting of ABCG2 by miR-519c<sup>[152]</sup>. Interestingly, we revealed the shortening of the ABCG2 3'UTR in several ABCG2-overexpressing resistant cell lines, which removes the miR-519c binding site and has a repressive effect on mRNA stability and translocation blockade allowing ABCG2 overexpression without a change in miR-519c expression to mediate drug resistance<sup>[152]</sup>. We further validated the effect of ABCG2 dysregulation on CRC sensitivity to 5-FU-based chemotherapy using pairs of archival tissue blocks of CRC tumors and their matched non-cancerous colon epithelial cells from CRC patients<sup>[153]</sup>. In the unresponsive tumor, high ABCG2 expression was found to be associated with the concomitant overexpression of a mRNA binding protein HuR but a low expression of

miR-519c because miR-519c was known to target both HuR and ABCG2<sup>[153]</sup>.

#### **miRNAs mediating resistance to oxaliplatin**

Oxaliplatin is a key platinum-based anticancer drug in combination regimens for CRC treatment. High expression of miR-203 has been shown to mediate oxaliplatin resistance by suppressing the key DNA double-strand break sensor ATM<sup>[154]</sup>. Likewise, high expression of miR-625-3p was associated with poor response to first-line treatment with oxaliplatin in metastatic CRC<sup>[94]</sup>. Moreover, forced expression of miR-1915 in an oxaliplatin-selected multidrug resistant cell line HCT116/L-OHP was found to induce apoptosis by inhibiting Bcl-2<sup>[155]</sup>.

#### **miRNAs mediating resistance to irinotecan**

Irinotecan is another key cytotoxic chemotherapeutic drug in the FOLFIRI regimen (5-FU + leucovorin +

**Table 7** miRNA therapeutics investigated in animal models of colorectal cancer

miRNA	Animal model	Restoring or suppressing miRNA function	Therapeutic outcome	Ref.
miR-34a	Transgenic mice	Restoring	Anti-tumor	[126]
miR-135b	Xenotransplantation of tumor-derived organoids to mice	Suppression (antisense)	Anti-tumor	[129]
miR-143	Mice xenograft	Restoring	Anti-tumor	[132]
miR-145	Mice xenograft	Restoring	Negative effect	[132]
miR-4689	Mice xenograft	Restoring (mimic)	Anti-tumor	[135]

irinotecan) for metastatic CRC. In colonospheres exhibiting cancer stem cell properties and chemoresistance from CRC cell lines, miR-451 was found to be remarkably downregulated<sup>[156]</sup>. Restoration of miR-451 in the colonospheres was found to specifically reverse drug resistance to irinotecan by decreasing the expression of the drug efflux ATP-binding cassette drug transporter ABCB1<sup>[156]</sup>.

### **miRNAs mediating resistance to targeted drugs for CRC**

Clinical efficacy with the anti-EGFR or anti-VEGF monoclonal antibodies in CRC is known to be severely hindered by drug resistance due to numerous mechanisms. Downregulation of miR-216b has been reported to mediate adapted autophagy (through upregulation of Beclin-1) in CRC cell lines to mediate resistance to anti-EGFR therapy<sup>[157]</sup>. Strategies to restore miR-216b or inhibit autophagy are thus proposed to improve the efficacy of anti-EGFR therapy in CRC. Conversely, the reduced expression of a tumor suppressor miR-7, which targets EGFR and RAF-1, was observed in CRC tumor specimens from patients unresponsive to cetuximab<sup>[97]</sup>. Reduced expression of miR-181a in tumor specimens from CRC patients was also found to correlate with poor response to EGFR antibodies<sup>[99]</sup>.

## **MODULATING MIRNAS FOR TREATMENT OF CRC**

Modulation of miRNA expressions and/or miRNAs-regulated pathways may be used as a therapeutic strategy to potentiate the cytotoxic effect from other anticancer drugs in CRC<sup>[158]</sup>. Guidelines have been proposed for the investigation and evaluation of potential microRNA-based therapeutics for cancer therapy<sup>[159]</sup>. Briefly, the up- or downregulation of miRNAs have to be established in appropriate cancer models. Gain/loss of function studies are then performed *in vitro* and in animal models. After the potential miRNA targets have been identified, an efficient delivery system has to be available to administer the miRNA modulating modalities for pharmacological, pharmacokinetic and pharmacodynamic studies. Finally, clinical trials will be conducted to evaluate the efficacy and safety of the potential miRNA therapeutics in patients. Only a few miRNA modulating systems have been investigated *in*

*vivo*, which are summarized in Table 7.

## **RESTORING TUMOR SUPPRESSIVE MIRNAS**

Downregulation of tumor-suppressive miRNAs are known to contribute to cancer development. Strategies have been devised to effectively restore the function of these "lost" miRNAs using synthetic miRNA-like molecules, including miRNA mimics and miRNAs encoded in expression vectors. MiRNA mimics are short and chemically modified (2'-O-methoxy) RNA duplexes, which are processed into single-strand form intracellularly and loaded into RNA-induced silencing complex (RISC) to mediate the target mRNA degradation and/or post-translational blockade<sup>[160,161]</sup>. This approach has been investigated in cell models and orthotopic mice models of colorectal and hepatocellular carcinomas and hepatic metastasis from primary CRC to restore the expression of downregulated miR-26a, miR-33a, miR-34a and miR-145<sup>[159,162-164]</sup>. The restoration of these tumor suppressor miRNAs was found to inhibit cancer cell proliferation, induce apoptosis, shrink tumor size and prolong animal survival.

miR-34 is remarkably downregulated in a number of different cancer types including CRC<sup>[165]</sup>. As discussed above, miR-34 has been shown to regulate multiple oncogenic pathways. Thus, the restoration of miR-34 may represent a novel therapeutic approach to treat cancer. To this end, a first-in-human phase I clinical trial (NCT01829971) has been conducted to investigate the safety, pharmacokinetics and clinical activity of a liposomal formulation of miR-34 mimic (known as MRX34) in patients with advanced solid tumors including CRC<sup>[166]</sup>. While MRX34 was tolerated and displayed antitumor activity in a subset of patients with refractory advanced solid tumors<sup>[166]</sup>, the sponsoring company (Mirna Therapeutic, Inc.) has voluntarily halted the enrollment and dosing of MRX34 in clinical study in September 2016 following multiple immune-related severe adverse events reported in patients dosed with MRX34.

## **INHIBITING ONCOGENIC MIRNAS**

Different approaches have been developed to inhibit mature miRNAs, which include antisense oligonucleotides (also known as ASOs, anti-miRs, or antagomirs), miRNA

masking, miRNA sponges, and anti-miR peptides. ASOs are single-stranded oligonucleotides that are complementary to the target miRNA<sup>[167]</sup>. The miRNAs bind to the RISC complexes, which subsequently block the interaction of miRNAs with their endogenous mRNA targets. This strategy has been evaluated in cell culture and mice models of CRC by targeting overexpressed oncogenic miRNAs. Specific inhibition of miR-135b has been achieved in human colon cancer SW480 cells to inhibit cell proliferation and to induce apoptosis<sup>[129]</sup> and in HCT116 cells to suppress cell migration<sup>[168]</sup>. Other oncogenic miRNAs, including miR-20a, miR-21, miR-95, and miR-675, have also been inhibited by similar approaches in human CRC cell lines<sup>[120,169-171]</sup>. ASOs are often chemically modified by conjugation to cholesterol to avoid cleavage by RISC nuclease and to increase their stability. Locked nucleic acids (LNAs) are nucleic acid analogs in which the ribose ring is "locked" with a methylene bridge connecting the 2'-O atom with the 4'-C atom<sup>[172]</sup>. Modification by LNAs is another approach used to enhance stability of ASOs, which also increases the binding affinity to the target miRNA<sup>[173]</sup>. LNA-containing oligonucleotides also have excellent mismatch discrimination capabilities<sup>[174]</sup>, thus avoiding potential off-target secondary effects. LNA-anti-miR-21 was an ASO developed by the LNA technology<sup>[175]</sup>. It has been shown to inhibit cancer growth and invasion in a colon cancer cell line LS174T<sup>[175]</sup>. On the other hand, miRNA masking refers to the use of single-stranded 2'-O-methyl modified oligonucleotides complementary to miRNA binding sites in the 3'UTR of the target mRNAs to interfere with specific miRNA-mRNA interaction<sup>[176]</sup>. A miR-34a sponge construct, encoding a decoy mRNA containing multiple tandem miR-34a binding sites, has been shown to inhibit self-renewal of colon cancer stem cells *in vitro*<sup>[177]</sup>. Anti-miR peptides are peptide nucleic acids (PNAs), modified from DNA or RNA, with the sugar backbone replaced by a peptide-like backbone consisting of N-(2-aminoethyl)glycine units. Aside from being more stable and able to be administered systematically with low toxicity, PNAs have been shown to bind to target miRNA more tightly than equivalent oligonucleotides<sup>[178]</sup>.

## USE OF SMALL MOLECULE MODULATORS OF MIRNA EXPRESSION

Besides making use of pharmaceutical carriers to deliver the miRNA mimics/inhibitors to the tumor, attempts have also been made in preclinical studies to screen for small molecule drugs that modulate miRNA expression and function by luciferase-based reporter assay<sup>[179]</sup>. These compounds do not inhibit target mRNA recognition by the miRNAs, but they instead modulate the expression of the targeted miRNAs. With potential application in treating CRC, diazobenzene and its derivatives were identified to specifically inhibit miR-21 expression<sup>[179]</sup>.

Most recently, aberrant expression of the lipid metabolic enzymes, acyl-CoA synthetase/stearoyl-CoA desaturase (ACSL/SCD) have been shown in CRC patients to promote cancer migration and invasion<sup>[180]</sup>. miR-19b-1 was found to a major regulator of ACSL/SCD expression. Importantly, lower miR-19b-1 expression and higher ACSL/SCD levels in tumor specimens from CRC patients were strongly associated with shorter disease-free survival<sup>[180]</sup>. Therefore, miRNA replacement therapy was proposed to increase the expression level of miR-19b-1 in colorectal tissue to improve the prognosis. Interestingly, since induction of miR-19b-1 expression has been achieved by a low protein diet in piglets<sup>[181]</sup>, dietary modulation of this particular miRNA may also be feasible. More *in vivo* investigation is needed to test the efficacy and safety before we might benefit from miR-19b-1 replacement therapy in the management of CRC.

## HURDLES IN MODULATING MIRNAS TO TREAT CRC

Since miRNAs only require partial sequence complementarity to target mRNA, non-specific targeting of bystander mRNAs may lead to off-target effects, toxicity and/or unfavorable immune response. Moreover, rapid degradation of miRNAs or anti-miRNAs by cellular nucleases and poor cellular uptake are also preventing the development of successful miRNA-based gene therapies. Furthermore, the administration of anti-miRs *in vivo* with an aim to block oncogenic miRNAs may adversely affect physiological functions normally regulated by these miRNAs. Thus, specific site or cell targeting will be needed to avoid the potential adverse effects. The lowest optimum concentration of miRNAs and appropriate delivery systems, such as viral and/or non-viral vectors, are critical factors required to achieve the desired therapeutic goal while minimizing adverse effects.

## CONCLUSION

Accumulating evidence suggests that miRNAs play a crucial role in the regulation of genes driving CRC initiation, progression and metastasis. Differential expression of miRNAs in CRC has provided opportunities for their applications in diagnosis, prognostic prediction and stratification of risk groups. While numerous miRNA biomarkers have been reported to predict prognosis and drug response in CRC patients, it is interesting to note that the miRNA signature identified in different studies usually do not overlap and, in certain cases, they were even found to be contradictory<sup>[182]</sup>. The different selection criteria for patients, collection method and processing of biological samples, and detection methods may contribute to the different miRNA signatures obtained. To obtain reliable results, precaution should be taken to prevent the degradation of miRNAs during sample

processing. It may be helpful to use these novel miRNA signatures together with the conventional biomarkers, such as CEA, to increase the sensitivity and specificity. As novel therapeutic targets, underexpressed miRNAs can be restored, whereas overexpressed miRNAs can be inhibited. Different delivery systems of these miRNA modulating moieties have been extensively studied in cell culture and animal models. Despite adverse effects having yielded a discouraging initial clinical experience with miR-34 replacement therapy, the correction of miRNA dysregulation is a promising therapeutic approach for the treatment of CRC; still, it is necessary to resolve the bystander effects and address toxicity concerns prior to large-scale clinical use.

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**P- Reviewer:** Kato J, Nakayama Y, Tomazic A **S- Editor:** Gong ZM  
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ISSN 1007-9327

