Review

MicroRNAs as Regulators, Biomarkers and Therapeutic Targets in the Drug Resistance of Colorectal Cancer

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Key Words

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Abstract

Chemotherapy and targeted therapy are the main options for advanced colorectal cancer (CRC). However, resistance to these therapies is a major challenge in the clinic. Understanding molecular mechanisms and developing effective strategies against the drug resistance are highly desired. Increasing evidence has revealed that microRNAs (miRNAs) are closely linked to drug resistance in CRC. The explosion of knowledge in this field has brought forward new predictive and therapeutic opportunities. In this review, we systemically summarize the roles of miRNAs as regulators, tissue or circulating biomarkers, and therapeutics in the CRC resistance to 5-fluorouracil (5-FU), oxaliplatin and anti-EGFR therapy. We also discuss the potential unsettled issues and future directions concerning these processes.

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Introduction

Colorectal cancer (CRC) is the third most prevalent malignancy and the second leading cause of cancer-related death worldwide [1]. Early diagnosis and efficient therapeutic strategies involving surgery, radiotherapy and drug therapy have improved the prognosis of CRC patients. Systemic chemotherapy is the first-line therapy for patients with unresectable tumors, aiming to reduce tumor burden to an extent where resection becomes possible [2]. The most common chemotherapies for CRC are FOLFOX (folinic acid, fluorouracil and oxaliplatin) and FOLFIRI (folinic acid, fluorouracil and irinotecan) [3]. With the improved comprehension of CRC pathogenesis, epidermal growth factor receptor (EGFR) targeted therapy, such as cetuximab or panitumumab, is introduced. During the last few years, the combination of chemotherapy with EGFR-targeted therapy has been established in the clinic and benefited the survival rates of CRC patients [4]. However, quite a lot of CRC patients are inherently

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refractory to the chemotherapy or targeted therapy, leading to unsatisfactory therapeutic efficacy. Accumulating studies have demonstrated the molecules in pharmacodynamic and pharmacokinetic pathways as the mechanisms for chemoresistance [5-7], while KRAS mutation has been associated with a lack of response to anti-EGFR therapy [8]. The elucidation of potential mechanisms underlying drug resistance and the discovery of reliable biomarkers to predict drug response are the key to improving treatment efficacy.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally inhibit gene expression via binding to the 3' untranslated region (3'UTR) of target mRNAs [9]. MiRNAs can act as oncogenes or tumor suppressors and are involved in various biological processes of cancer, including tumor initiation, tumor progression [10-12] and drug resistance etc [13, 14]. A large amount of miRNAs are reported to be dysregulated in CRCs, and some have been linked to the response to anti-cancer therapies via the regulation of drug metabolism, drug transport, DNA damage response or cell apoptosis [15]. Importantly, a subset of miRNAs could serve as potential predictive biomarkers for drug response, especially as non-invasive biomarkers in the circulation. Furthermore, with development of RNA delivery technology, miRNA-based interventions may act as novel therapeutic means to overcome drug resistance of CRC [16].

In our review, we summarize the roles of miRNAs in the regulation of resistance to 5-FU, oxaliplatin, VEGF/VEGFR-targeted and EGFR-targeted therapy, the main therapeutic regimens in CRC. Moreover, we highlight the clinical implications of miRNAs as predictive biomarkers for drug response in both tissue and circulation, and as therapeutic means for drug resistance in CRC.

Current drug therapies in CRC

5-FU

5-FU is one of the main component of adjuvant and palliative therapy for CRC treatment and has a proven effect on survival in CRC patients [3]. It elicits cytotoxicity by inhibiting the nucleotide synthetic enzyme thymidylate synthase (TYMS or TS), which is essential for converting intracellular dUMP into dTMP. CRCs that are refractory to 5-FU-based chemotherapy are shown to have higher TYMS enzymatic activity than those that are sensitive [17]. It also functions to incorporate fluoronucleotides into RNA and DNA. Capecitabine is a prodrug of 5-FU and possesses improved tolerability and higher intratumour concentrations [18]. In clinical practice, the use of 5-FU as a monotherapy is moderately effective in CRC, but its combination with other chemotherapeutic agents, such as oxaliplatin and irinotecan, significantly improved the therapeutic outcome.

Oxaliplatin

Oxaliplatin (1,2-diaminocyclohexane-oxalate platinum) is a member of platinum family that contains cisplatin and carboplatin as well [19]. It is the only platinum analogue that has cytotoxic activity in CRC. Oxaliplatin mainly forms intrastrand adducts between two adjacent guanine residues or between guanine and adenine, thus disrupting DNA replication and transcription [19, 20]. Oxaliplatin-induced DNA damage has been reported to be repaired by nucleotide excision repair pathway [21]. Oxaliplatin has also been reported to involved in the activation of p38 kinase, phosphatidylinositol 3-kinase/AKT pathway and intrinsic apoptotic pathway [22-24]. Nevertheless, the downstream molecular events underlying the resistance to oxaliplatin have not been well characterized.

EGFR-targeted therapy

EGFR is expressed in approximately 85% of patients with metastatic CRC (mCRC) [25]. The anti-EGFR monoclonal antibodies (mAb), such as cetuximab and panitumumab, have been shown to achieve modest but clinically meaningful objective response rates (approximately 10%) in mCRC patients with chemotherapy-refractory and EGFR expression [26, 27].

It is well established that KRAS mutation status is a key biomarker for anti-EGFR therapy, i.e. EGFR-targeted antibodies are only effective in KRAS wild-type mCRC [28, 29]. The mutation of BRAF, which is downstream of KRAS, has also been associated with nonresponse to cetuximab and panitumumab [30]. However, after an initial response, most patients end up with secondary resistance, thereby limiting the clinical benefit of the targeted therapy. The amplification of proto-oncogene MET is known to be associated with the acquired resistance in KRAS wild-type CRCs during anti-EGFR therapy [31].

VEGF/VEGFR-targeted therapy

Angiogenesis plays a pivotal role in tumor growth and metastasis, providing an available therapeutic alternative for cancer treatment [32]. The vascular endothelial growth factor (VEGF) is a well known pro-angiogenic factor, and blocking the VEGF pathway has been widely applied in the clinical practice for cancer treatment, including mCRC [33, 34]. Bevacizumab, a humanized monoclonal antibody for VEGF-A, is most extensively approved for the treatment of CRC [35]. Another VEGF-targeted drug for CRC is Aflibercept, which is a recombinant fusion protein, and acts as a “trap” for VEGF-A, VEGF-B and PlGF [36]. In addition, drugs targeting VEGFR have also been developed, such as Ramucirumab, a monoclonal antibody for VEGFR2 [37]. The VEGF/VEGFR-targeted drugs have been used in combination with common chemotherapeutic agents to improve the therapeutic efficacy.

Apart from the above mentioned drugs, there are some novel therapeutics in CRC treatment, such as regorafenib, trifluridine and tipiracil. Regorafenib is a multitarget receptor tyrosine kinases (RTK) inhibitor, which has potent anti-angiogenic effects by targeting VEGFR and direct anti-tumor activities by inhibiting platelet-derived growth factor receptor and stem cell growth factor receptor [38]. Trifluridine is a thymidine-based nucleotide analog and tipiracil hydrochloride is a thymidine phosphorylase inhibitor that improves the stability of trifluridine. These two drugs comprise the anti-cancer drug TAS-102, which is approved for the treatment of mCRC [39].

MiRNAs as regulators of drug resistance in CRC

Drug resistance is often multifactorial and may rise from reduced intracellular accumulation of drugs, increased DNA damage repair, reduced apoptosis and altered expression of oncogenes and tumor suppressors etc. MiRNAs are intimately involved in these processes and therefore modulate the development of drug resistance, including 5-FU, oxaliplatin and EGFR-targeted therapy, among which 5-FU is the most widely studied (Table 1, 2).

Table 1. MiRNAs involved in 5-FU resistance of CRC. ↑: upregulated; ↓: downregulated

miRNAs	Expression in resistant cells	Gene targets	ref
miR-203	↓	TYMS	41
miR-218	↓	TYMS, BIRC5	42
miR-494	↓	DPYD	3
miR-27a, miR-27b	↓	DPYD	43-45
miR-21	↑	MSH2	40
miR-10b	↑	BIM	2
miR-23a	↑	APAF-1, ABCF1	46-48
miR-425-5p	↑	PDCD10	49
miR-139-5p	↓	BCL2	50
miR-302, miR-369, miR-200c	↑	MRP8	51
miR-519c	↓	ABCG2, HuR	54
miR-22	↓	BTG1	56
miR-204	↓	HMGA2	57

Table 2. MiRNAs involved in oxaliplatin and EGFR-targeted resistance of CRC

miRNAs	Therapy	Expression in resistant cell	Gene targets	ref
miR-203	oxaliplatin	↑	ATM	58
miR-20a	oxaliplatin	↑	BNIP2	59
miR-520g	oxaliplatin	↑	P21	60
miR-153	oxaliplatin	↑	FOXO3a	62
miR-409-3p	oxaliplatin	↓	Beclin-1	63
miR-143	oxaliplatin	↓	IGF-1R	64
miR-133b	EGFR-targeted	↓	EGFR	65
miR-7	EGFR-targeted	↓	EGFR, RAF-1	66
miR-199a-5p, miR-375	EGFR-targeted	↑	PHLPP1	67

Roles of miRNAs in 5-FU resistance

Deregulation of 5-FU related enzymes. The sensitivity to 5-FU is closely related to 5-FU-metabolizing enzymes, including TYMS, thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPYD or DPD) [40]. As the key therapeutic target of 5-FU, the high activity of TYMS is linked to poor clinical response to 5-FU treatment. MiR-203 has been reported to target TYMS and suppress TYMS levels in colon cancer cells. Inhibition of miR-203 expression enhanced 5-FU resistance, while overexpression of miR-203 promoted 5-FU sensitivity via the downregulation of TYMS in colorectal cancer [41]. Similarly, miR-218 also enhanced 5-FU cytotoxicity by suppressing TYMS in CRC cells [42].

The DPYD enzyme catalyzes the catabolism of 5-FU. Low expression of DPYD was associated with longer survival, indicating that high DPYD level may result in 5-FU chemoresistance [3]. MiR-494 was downregulated in 5-FU-resistant SW480 cells compared to parental cells. Ectopic expression of miR-494 increased the sensitivity of chemoresistant CRC cells and xenografts to 5-FU by targeting DPYD expression [3]. Similarly, overexpression of miR-27a and miR-27b sensitized CRC cells to 5-FU via targeting DPYD [43]. On the other hand, patients with low DPYD activity are at an increased risk of toxicity, due to the increased half-life of 5-FU [44]. Amstutz *et al.* showed that a common variant of miR-27a (rs895819) was associated with low DPYD expression and predicted early-onset fluoropyrimidine toxicity in patient, leading to an unsatisfactory therapy response [45]. Additionally, contradicted to the chemoresistant role of DPYD mentioned above, Deng *et al.* found that miR-21 increased the 5-FU resistance of CRC cells HT-29 by targeting MSH2 and indirectly downregulating the expression of TP and DPYD [40].

Impairment of 5-FU-induced apoptosis. Inhibition of pro-apoptotic protein or overexpression of anti-apoptotic protein are generally known to confer drug resistance. In CRC cells, miR-10b and miRNA-23a have been reported to target pro-apoptotic molecule BIM [2] and apoptosis-activating factor-1 (APAF-1) [46-48] respectively, protecting cells from 5-FU-induced apoptosis and conferring resistance to 5-FU. MiR-425-5p was significantly upregulated in resistant HCT-116 cells. Programmed cell death 10 (PDCD10) is the direct target of miR-425-5p, mediating the role of miR-425-5p in chemoresistance to both 5-FU and oxaliplatin [49]. B-cell lymphoma 2 (BCL2), a key anti-apoptotic protein, has been shown to be targeted by several miRNAs, including miR-143, miR-365, miR-1915, miR-129 and miR-139-5p in CRC. The inhibition of BCL2 by those miRNAs triggered the activation of intrinsic apoptotic pathway, and enhanced chemosensitivity to 5-FU [50]. MiR-218 was previously stated to target TYMS, and it could also promote apoptosis in CRC cells by suppressing BIRC5, a member of the inhibitor of apoptosis (IAP) gene family [42].

Increased efflux of 5-FU. Increased export of drugs ATP-binding cassette transporters (ABC transporters) on cell membrane is a common resistance mechanism [17]. Downregulation of stemness-related miRNAs (miR-302, miR-369, miR-200c) decreased the sensitivity of CRC cells to 5-FU probably through relieving the suppression of MRP8 (ABCC11) [51], which mediates cellular efflux of the cytotoxic metabolite of 5-FU [52, 53]. Overexpression of ABCG2 has been shown to cause resistance to 5-FU and irinotecan. MiR-519c could in-

hibit ABCG2 expression by binding to its 3'UTR and a mRNA binding protein HuR. Inhibition of miR-519c led to chemoresistance to 5-FU via regulating the miR-519c-HuR-ABCG2 pathway in CRC cells [54]. In contrary to ABCG2, ABCF1 was reported to be increased in 5-FU responders in CRC patients [55]. Consistently, Li *et al.* found that miR-23a promoted 5-FU resistance via targeting ABCF1 in microsatellite instability (MSI) CRC cells, providing implications for therapeutic approaches to overcome 5-FU resistance in MSI CRC [46].

Others. Autophagy is considered as an important mechanism of cancer cell chemoresistance and thought to promote chemoresistance by promoting cellular energy production. Autophagy has become one of the most important mechanisms of chemotherapy resistance by supporting the survival of tumor cells under metabolic and therapeutic stress. We showed that miR-22 inhibited autophagy and promoted apoptosis to increase the sensitivity of CRC cells to 5-FU treatment both *in vitro* and *in vivo*. B-cell translocation gene 1 (BTG1) was identified as a new target of miR-22, which could reverse the inhibition of autophagy induced by miR-22. Thus, miR-22 may function as an important switch between autophagy and apoptosis to regulate 5-FU sensitivity through post-transcriptional silencing of BTG1 [56]. The miR-204/HMGA2 axis modulated the resistance of tumor cells to 5-FU in HCT-116 and SW480 colon cancer cells via activation of the PI3K/AKT pathway. These results demonstrate that the miR-204/HMGA2 axis could play a vital role in the 5-FU resistance of colon cancer cells [57].

Roles of miRNAs in oxaliplatin resistance

Most of the previous studies of platinum have been focused on resistance to cisplatin. The mechanisms involved in oxaliplatin resistance remain poorly understood. As oxaliplatin functions to induce DNA damage through adduct formation, modulation of DNA damage repair would affect the response to this drug. Zhou *et al.* found that miR-203 is upregulated in three oxaliplatin-resistant CRC cell lines, and knockdown of miR-203 sensitized resistant cells to oxaliplatin by targeting ataxia telangiectasi mutated (ATM), a primary mediator of DNA damage response [58].

From the aspect of cell apoptosis regulation, several miRNAs have been identified. By miRNA expression array and qPCR, Chai *et al.* found the elevation of miR-20a in chemoresistant SW620 cells. Overexpression of miR-20a conferred resistance to oxaliplatin by directly targeting BNIP2, a Bcl-2-interacting partner [59]. MiR-520g, a miRNA negatively regulated by p53, also conferred resistance to oxaliplatin. Further studies indicated that miR-520g mediated drug resistance through downregulation of p21 expression [60]. Similar to p53, transcription factor FOXO3a is critical in initiating apoptotic programs through upregulating pro-apoptotic genes or downregulating anti-apoptotic genes. Loss-of-function of FOXO3a frequently occurs by post-translational modifications in human cancer [61]. As the direct target of miR-153, upregulation of miR-153 increased CRC resistance to oxaliplatin both *in vitro* and *in vivo* [62].

Recently, autophagy is considered as an important mechanism of chemoresistance by promoting cellular energy production. A study found that oxaliplatin-resistant colon cancer cells exhibited downregulated miR-409-3p levels and higher autophagic activity than sensitive cells. Beclin-1, a key autophagy gene, was predicted as the target of miR-409-3p. Overexpression of miR-409-3p inhibited cell autophagic activity and enhanced the sensitivity to oxaliplatin, which were abrogated by restoration of beclin-1, suggesting that miR-409-3p sensitized CRC to oxaliplatin by inhibiting beclin-1-mediated autophagy [63].

In addition, insulin-like growth factor-1 receptor (IGF-1R), a well-known RTK for survival, was found as a novel direct target of miR-143, whose expression levels were inversely correlated in human CRC specimens. Overexpression of miR-143 inhibited cell proliferation and increased sensitivity to oxaliplatin treatment in an IGF-1R-dependent manner [64].

Roles of miRNAs in EGFR-targeted therapy resistance

MiR-133b has been reported as a tumor suppressor in several malignancies by regulating EGFR. A study characterized the downregulation of miR-133b in CRC tissues. The com-

bination of miR-133b mimics with anti-EGFR mAb, exhibited stronger inhibitory effects on CRC cells than treatment with either alone. Thus, the study suggested that miR-133b enhanced the sensitivity to cetuximab, and the combination of miR-133b and cetuximab may be a potential treatment for CRC patients [65]. Similarly, an alike role was found on miR-7. Through the transfection of miR-7 precursor, Yokobori *et al.* found that EGFR and RAF-1 are direct targets of miR-7. Furthermore, miR-7 in combination with cetuximab potently suppressed the proliferation of CRC cells. These data indicated that miR-7 regulated cetuximab sensitivity, and miR-7 precursor in combination with cetuximab may be useful in therapy against CRC [66]. Apart from the direct regulation of EGFR, Mussnich *et al.* found another target of miRNAs in cetuximab response [67]. They utilized a miRNA array to investigate the miRNA expression profile of cetuximab sensitive CRC cells and their resistant counterpart. MiR-199a-5p and miR-375 were found to be upregulated in resistant cells and their enforced expression promoted cetuximab resistance. Mechanistically, the ability of miR-199a-5p and miR-375 to target PHLPP1 (PH domain and leucine-rich repeat protein phosphatase 1), a tumor suppressor that inhibits AKT pathway, was responsible for the resistance. The study proposes miR-199a-5p and miR-375 as contributors to cetuximab resistance in CRC.

MiRNAs as biomarkers for therapeutic response in CRC

Drug therapies have been shown to improve the outcomes of CRC patients. However, the response rate is unsatisfactory in the clinic. Selecting out the patients, who would benefit from drug treatment, will help to promote therapy efficacy and avoid a waste of resources. Due to the high tissue specificity and stability of miRNAs and their altered expression in drug resistance, miRNAs have been suggested as predictive biomarkers for therapeutic response. Here, we summarized miRNA predictors of response to 5-FU/oxaliplatin chemotherapy and EGFR-targeted treatment in tissue and in blood (Table 3).

MiRNA biomarkers of clinical 5-FU/oxaliplatin response

Through miRNA expression profiling of colon adenocarcinoma and paired noncancerous tissues, Schetter *et al.* found that high miR-21 expression was associated with a poor therapeutic outcome in 5-FU-based adjuvant chemotherapy [68]. Consistently, miR-21 was also shown to have predictive significance of pathological response to 5-FU-based neoadjuvant chemotherapy in locally advanced rectal cancer patients, yielding a 86.6% sensitivity and 60.0% specificity [69]. However, another study using deep sequencing in rectal tumor biopsies prior to neoadjuvant chemotherapy drew an opposite conclusion. In the study, miR-21-5p was found to be overexpressed in complete responders, suggesting miR-21-5p as a predictive biomarker to select patients with response to neoadjuvant chemoradiotherapy [70]. In addition, low miR-150, low miR-148a or high miR-320e expression in colorectal tumors have been demonstrated to be associated with poor response to adjuvant 5-FU plus oxaliplatin (FOLFOX) chemotherapy by several studies [71-73].

Although single miRNA may predict chemotherapy response, the use of a panel of miRNAs may be more specific and accurate. A set of 13 miRNAs (up-regulated: miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909*, miR-630, miR-765; down-regulated: miR-1274b, miR-720) have been shown to strongly associate with complete pathological response in rectal cancer patients receiving neoadjuvant capecitabine and oxaliplatin (XELOX) therapy, representing specific predictor of therapy response in rectal cancer [74]. By using tumors from 20 rectal cancer patients who were classified as most sensitive or resistant to neoadjuvant chemotherapy, Svoboda *et al.* reported that a set of miRNAs (miR-215, miR-190b and miR-29b-2*) was overexpressed in non-responders, while another set (let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a*) had higher expression levels in responders to fluoropyrimidine-based treatment [75].

Apart from the application in neoadjuvant chemotherapy, 5-FU and oxaliplatin are also used as the first-line treatment for patients with mCRC. Molina-Pinelo *et al.* showed that

Table 3. Tissue and blood microRNA biomarkers for therapeutic response in CRC. ISH: in situ hybridization; —: not mentioned

miRNAs	therapy	sample type	detection time	Patient number (Training /validation cohort)	method	Expression in non-responders	ref
miR-21	fluorouracil-based	Tissue	pre-therapy	84/113	qRT-PCR	high	68
miR-21	fluorouracil-based	Tissue	post-therapy	67/—	qRT-PCR	high	69
miR-21-5p	fluorouracil-based radiation	Tissue	pre-therapy	27/16	microarray assay	low	70
miR-150	fluorouracil-based	Tissue	pre-therapy	239/—	qRT-PCR, ISH	low	71
miR-148a	fluorouracil-based	Tissue	pre-therapy	273/—	qRT-PCR, ISH	low	72
miR-320e	fluorouracil, oxaliplatin	Tissue	pre-therapy	100/237	Microarray assay, qRT-PCR	high	73
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*	capecitabine, oxaliplatin, radiation	Tissue	pre-therapy	38/—	microarray assay, qRT-PCR	low	74
miR-1274b, miR-720	capecitabine, oxaliplatin, radiation	Tissue	pre-therapy	38/—	microarray assay, qRT-PCR	high	74
miR-215, miR-190b and miR-29b-2'	fluorouracil-based, radiotherapy	Tissue	post-therapy	20/—	microarray assay, qRT-PCR	high	75
let-7e, miR-196b, miR-450a, miR-450b-5p, miR-99a*	fluorouracil-based, radiotherapy	Tissue	post-therapy	20/—	microarray assay, qRT-PCR	low	75
miR-107 and miR-99a-3p	fluoropyrimidine-based	Tissue	pre-therapy	39/39	microarray assay, qRT-PCR	low	76
miR-126	capecitabine, oxaliplatin	Tissue	pre-therapy	89/—	qRT-PCR, ISH	low	77
miR-664-3p	capecitabine, oxaliplatin, bevacizumab	Tissue	pre-therapy	212/121	qRT-PCR, ISH	low	78
miR-455-5p	capecitabine, oxaliplatin, bevacizumab	Tissue	pre-therapy	212/121	qRT-PCR, ISH	high	78
miR-196b-5p, miR-592	capecitabine, oxaliplatin,	Tissue	pre-therapy	212/127	qRT-PCR, ISH	low	78
miR-625-3p	capecitabine, oxaliplatin	Tissue	pre-therapy	94/—	microarray assay, qRT-PCR	high	79
miR-31-3p	anti-EGFR	Tissue	pre-therapy	33/—	microarray assay, qRT-PCR	high	80
miR-592	anti-EGFR	Tissue	pre-therapy	33/—	microarray assay, qRT-PCR	low	80
miR-31-5p	anti-EGFR	Tissue	pre-therapy	102/20	qRT-PCR	high	81
miR-181a	anti-EGFR	Tissue	pre-therapy	80/—	qRT-PCR	low	82
Let-7c, miR-99a, miR-125b	anti-EGFR	Tissue	pre-therapy	74/109	microarray assay, qRT-PCR	low	83
Let-7	irinotecan, cetuximab	Tissue	pre-therapy	59/—	qRT-PCR	low	84
miR-200b	capecitabine, oxaliplatin, anti-EGFR	Tissue	pre-therapy	34/—	microarray assay, qRT-PCR	low	8
miR-143	capecitabine, oxaliplatin, anti-EGFR	Tissue	pre-therapy	34/—	microarray assay, qRT-PCR	high	8
miR-106a, miR-484, and miR-130b	fluorouracil, oxaliplatin	blood	post-therapy	150/—	qRT-PCR	high	89
miR-19a	FOLFOX	blood	post-therapy	72/—	microarray assay, qRT-PCR	high	90
miR-20a, miR-130, miR-145, miR-216, miR-372	FOLFOX	blood	post-therapy	40/173	microarray assay, qRT-PCR	high	91
miR-155	leucovorin, cetuximab, 5-FU	blood	pre-therapy	15/—	qRT-PCR	high	92
miR-345	cetuximab, irinotecan	blood	pre-therapy	138/—	microarray assay, qRT-PCR	high	93

mCRC patients with high expression of miR-107 and miR-99a-3p achieved an objective response to fluoropyrimidine-based chemotherapy regimens [76]. Similarly, miR-126 could serve as a valuable predictor for mCRC patients who were sensitive to first-line XELOX treat-

ment [77]. Boisen et al. reported that high expression of miR-196b-5p and miR-592 predicted improved prognosis of mCRC patients receiving XELOX therapy, while high miR-664-3p and low miR-455-5p levels were correlated with improved outcome under XELOX plus bevacizumab treatment [78]. By using laser capture microdissected cancer cells from responsive and non-responsive patients receiving XELOX/FOLFOX, Rasmussen *et al.* found that high level of miR-625-3p was associated with poor response but was not associated with disease recurrence, indicating that miR-625-3p is solely a response biomarker for XELOX/FOLFOX treatment in mCRC patients [79].

MiRNA biomarkers of EGFR-targeted therapy response

In order to find miRNAs predicting the efficacy of anti-EGFR mAb, Mosakhani et al. performed miRNA array analysis in mCRC patients with wild-type KRAS/BRAF. They found that miRNA profiling could efficiently predict the benefits of anti-EGFR mAb (cetuximab or panitumab) treatment. For instance, upregulation of miR-31* (miR-31-3p) or downregulation of miR-592 was observed in disease progression versus disease control [80]. Consistently, another group of researchers reported that high level of miR-31-5p, which had the same pre-miRNA with miR-31-3p, was associated with shorter progression-free survival (PFS) in patients with CRC treated with anti-EGFR therapeutics. Indeed, miR-31-5p has been reported to regulate BRAF activation and regulate signaling downstream of EGFR in CRC. These results suggested that miR-31 may be a useful prognostic biomarker for anti-EGFR therapy [81]. Another miRNA that seems to correlate with anti-EGFR therapy response is miR-181a, which is correlated to β -catenin expression. Low miR-181a expression level was associated with decreased PFS in wild-type KRAS patients treated with anti-EGFR mAb (cetuximab or panitumumab) [82]. In addition, Cappuzzo *et al.* investigated the miRNA signature of mCRC patients receiving EGFR-targeting monoclonal antibodies (cetuximab or panitumumab). They identified that the cluster Let-7c/miR-99a/miR-125b was linked to treatment response. Patients with high-intensity signature had a longer PFS and overall survival than low-signature individuals in KRAS wild-type but not KRAS mutated patients, suggesting the Let-7c/miR-99a/miR-125b signature may help select patients with KRAS wild-type mCRC for anti-EGFR therapy [83].

Mutations in genes downstream of EGFR, such as KRAS, are considered to induce resistance to anti-EGFR therapy. Let-7 is the most widely studied miRNA as biomarker for anti-EGFR therapy in patients with KRAS mutations. Let-7 downregulates KRAS by binding to its mRNA, and let-7 might also provide a survival advantage by inhibiting other genes such as cell cycle regulators, Myc, Bcl-2 and so on. In patients with KRAS mutations, high levels of let-7 were found to be significantly associated with a better survival under anti-EGFR (cetuximab) therapy by several studies [84], indicating that let-7 serves to identify subgroup of patients with KRAS mutations who might still benefit from EGFR inhibition. However, a let-7 complementary site (LCS6) polymorphism in KRAS 3'UTR, which exhibited disrupted let-7 binding capacity, failed to correlate with clinical outcome in stage III CRC patients treated with FOLFOX alone or combined with cetuximab [85]. Additionally, in KRAS-mutated tumors, high miR-200b and low miR-143 expression were found to be associated with better PFS in patients treated with XELOX in combination with cetuximab and bevacizumab [8].

MicroRNAs as non-invasive biomarkers of drug response in CRC

Circulating nucleic acids (CNAs) offer a non-invasive opportunity for early diagnosis, prognosis evaluation and drug response prediction in cancer. The circulating miRNAs are stable and reproducible in blood, making them promising candidates [86, 87]. Recently, the occurrence of miRNAs in the serum or plasma of humans has been repeatedly observed and become the focus of biomarker discovery [88]. Here, we listed the blood miRNA biomarkers for response to 5-FU/oxaliplatin chemotherapy and EGFR-targeted treatment in CRC patients.

For chemotherapy, Kjersem *et al.* [89] examined the expression of miRNAs in the plasma from 24 mCRC patients before and after four cycles of 5-FU/oxaliplatin treatment. The ele-

vated miRNAs in non-responder group was confirmed with a validation cohort. They found three miRNAs (miR-106a, miR-484 and miR-130b) significantly upregulated in non-responders before treatment. The study demonstrated that pre-therapy plasma miRNAs may serve as non-invasive markers to predict outcome in mCRC patients treated with 5-FU/oxaliplatin chemotherapy [89]. Aberrant elevation of serum miR-19a was also found to predict non-responders to FOLFOX chemotherapy in advanced CRC [90]. Another study identified a distinct serum miRNA expression signature in response to chemotherapy. The expression of miRNAs were investigated by TaqMan low-density array from pooled serum of 253 CRC patients. Unsupervised cluster analysis showed a differential expression of serum miRNA between sensitive and resistant patients. Moreover, they identified a profile of five serum miRNAs (miR-20a, miR-130, miR-145, miR-216 and miR-372) as biomarker for predicting the chemosensitivity of CRC, facilitating chemotherapy regimen selection [91].

As for anti-EGFR therapy, elevation of serum miR-155 levels after surgery and chemotherapy foreshadowed chemoresistance in CRC patients treated with 5-FU and leucovorin plus cetuximab [92]. A recent study further reported that high levels of circulating miR-345 were associated with lack of response to treatment with cetuximab and irinotecan in metastatic CRC [93].

MiRNA-based therapeutics to improve the therapy efficacy in CRC

Due to the small size and potential to target multiple molecules, miRNAs and anti-miRNA constructs are now under investigation as therapeutic agents for cancer. Technics capable of targeting or delivering miRNAs to tumors holds great promise to control disease progression, and also to enhance therapies, such as re-sensitizing the drug resistant tumors [94]. The strategy of inhibiting pro-chemoresistant miRNAs or restoring pro-chemosensitive miRNAs might maximize therapeutic effect and improve clinical outcomes in patients with CRC [88].

Targeting miRNA

MiRNAs can be silenced by anti-miRs, antagomiRs, locked nucleic acids (LNAs), or miRNA sponges. Anti-miRNA oligonucleotides (AMOs) are single-stranded oligonucleotides that are complementary to the target miRNA [95]. Due to its low stability and cleavage by RISC nuclease, chemical modifications have been developed. For instance, antagomiRs are cholesterol-conjugated and 2'-O-methylmodified single-stranded RNA analogues. Another modification resulting in enhanced stability and binding affinity is LNAs [96], whose right is owned by Danish pharmaceutical company Santaris Pharma A/S [97]. The use of anti-miR-122 (miravirsin) for hepatitis C treatment is based on LNA technology and is now in clinical trial [8]. In colon adenocarcinoma, LNA-anti-miR-21 was found to inhibit cell growth and invasiveness in LS174T cells, suggesting the therapeutic potential of LNA-anti-miR-21 in colon adenocarcinoma [98]. In addition to anti-miR based strategy, miRNAs sponges also suppress miRNA. Synthesized miRNA sponge is a single-stranded RNA [99] with multiple complementary 3'UTR mRNA sites for target miRNA. Thus, sponges interfere with miRNA function by competitively binding the miRNA [100, 101]. A miR-34a sponge construct, which drives the transcription of a decoy mRNA containing multiple tandem miR-34a binding sites, has been applied in colon cancer cells [102].

MiRNA replacement

Overexpression of miRNAs can be induced either by virus-mediated miRNA replacement or using synthetic miRNA mimics. Viral-based delivery system usually employs adenovirus-associated viral vector or lentiviral vector to generate the pre-miRNA hairpin structure, ensuring long-time and stable expression of mature miRNA. Non-viral system delivers miRNA mimics, small synthesized double-stranded RNAs, by using liposomes or nanoparticles polymers. The advantage of non-viral approach is safety and avoiding induction of toxic im-

mune response compared with viral delivery. A study reported the use of polyethylenimine (PEI) for delivery of unmodified miRNAs, miR-145 and miR-33a, to validate the method in a mouse model of colon carcinoma. After local or systemic application, the intact miRNA molecules were delivered into xenograft tumors, leading to profound antitumor effects [103].

Currently, only a limited number of studies have been conducted *in vivo*, thus there may be a long way for the first miRNA-based therapy for CRC in the future. Nevertheless, miRNA inhibition or miRNA replacement therapy could be expected to be a promising strategy when combined with drug therapy.

Conclusions and Outlooks

One of the major challenges in CRC is the frequent drug resistance, which is a complicated process involving multiple mechanisms. MiRNAs offer an attractive option as critical regulators for drug resistance and stable biomarkers for response assessment in both tumor tissue and circulation. Furthermore, the combination of miRNA-based strategy with chemotherapy may overcome chemoresistance and improve clinical outcomes in CRC patients.

However, despite the extensive investigation and distinct progress in this field, there are still many challenges. Currently, most of the studies focused on the role of miRNA in just one kind of therapy, such as either 5-FU or oxaliplatin. Actually, CRC patients commonly receive combined chemotherapy, like FOLFOX or FOLFORI, in the clinic. Thus, it is rational to evaluate the role of miRNA in the regulation of the combined therapy resistance, under which multiple molecules may be responsible.

Although many miRNA biomarkers have been reported to predict drug response in CRC patients, there is rare overlap between miRNA signature identified in different studies, and some are even opposite [104]. The reasons may involve the selection of patients, collection of samples, sample processing and detection method etc. As miRNAs are easy to degrade, measures to ensure the stability during sample processing should be employed. Moreover, in the quantification of circulating miRNAs, endogenous control is under debate, because many mRNA and rRNA are absent in blood due to circulating RNase [105]. A proposed solution to this problem may be the introduction of exogenous control, such as λ polyA⁺ RNA [106].

With regard to miRNA-based therapeutic agent, the field will benefit from the development of RNA delivery technology and chemical modifications of miRNAs. The former may protect miRNA from degradation in the blood and enable miRNA to reach tumors efficiently and specifically. The latter could enhance the stability of miRNA and reduce toxicity, minimizing side effects [88].

In summary, a better understanding of the roles of miRNAs in drug resistance of CRC could help to identify biomarkers for drug response, and provide better strategies to enhance sensitivity to existing therapies.

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Disclosure Statement

No potential conflicts of interest relevant to this article were reported.

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