Title: Roles of microRNAs as non-invasive biomarker and therapeutic target in colorectal cancer

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Abstract

MicroRNAs are endogenous, short non-coding RNA molecules that function as critical regulators of various biological processes. There is a strong functional evidence linking the involvement of dysregulated miRNAs to the occurrence, development and progression of colorectal cancer. Studies indicate that while overexpression of oncomiRs, and repression of tumor suppressor miRNAs tends to drive the overall tumorigenic process, the global picture of aberrant miRNA expression in colorectal cancer can classify the disease into multiple molecular phenotypes. Moreover, the expression pattern of miRNAs in colorectal cancer make them viable disease determinants as well as potential therapeutic targets. Through this review, we will summarize the importance of miRNAs in the etiology and progression of colorectal cancer. Specifically, we will explore the key role played by these RNA molecules as likely therapeutic avenues and the strategies presently available to target them. Finally, we will investigate the role of miRNAs as potential non-invasive diagnostic and prognostic biomarkers in colorectal cancer.

1. Introduction

MicroRNAs (miRNAs) represent a novel class of endogenous, evolutionarily conserved small non–coding ribonucleic acids (ncRNAs) of ~19-24 nucleotides that function as a strong cellular workhorse regulating a myriad of physiological and developmental processes. Discovered serendipitously in *Caenorhabditis elegans*, the progenitor of the miRNA superfamily was a product of the lin-4 gene that was found to repress the expression of *lin-14* and *lin-28*, thereby allowing the developmental progression of the nematode through the early larval stages (Lee *et al.*, 1993). The importance of timed release of *lin-4* encoded small antisense miRNA can be reiterated in mutant worms with reduced *lin-4* that show a prolonged expression of *lin-14* and *lin-28* causing developmental defects such as absence of egg-laying structures, long body shape, and uncoordinated movement in adults (Feinbaum & Ambros, 1999, Esquela-Kerscher, 2014). While such small ncRNAs were thought to be indigenous to the nematodes, further research demonstrated the existence of a large pool of miRNAs within the animal kingdom, including humans. In fact the online public miRNA repository miRBase, in its latest release (v22) has identified 1917 hairpin precursor miRNAs, and 2654 mature sequences in the human genome (Kozomara *et al.*, 2019). Such a large group of miRNAs, although form a small percentage of the genome, have been found to exhibit multiple roles in virtually every cellular process, from embryonic development to neoplasmic progression. Not surprisingly, the number of miRNA related publications in the field of disease biology, specifically cancer, have risen tremendously over the past decade. Dysregulated miRNA expression has been identified in most forms of cancer, including but not limited to, breast cancer, lung cancer, colorectal cancer, pancreatic cancer and hepatocellular carcinoma (Iorio *et al.*, 2005, Zhu *et al.*, 2014, Castro *et al.*, 2017, Strubberg & Madison, 2017, Yonemori *et al.*, 2017). While these small ncRNAs can function as oncogenes or tumor suppressors assisting in cancer development and progression, studying their signatures could provide vital clues for the classification, diagnosis as well as for determining the prognosis of the developing tumor. Moreover, this also provides for an opportunity to seek newer targets for cancer research as well as the therapeutic targeting of the disease.

Colorectal cancer (CRC) represents the third most commonly diagnosed cancer, while being the second leading cause of cancer related deaths worldwide (Bray *et al.*, 2018). While majority of CRC cases are sporadic, roughly a quarter of the affected cases occur due to a genetic predisposition. At the molecular level, the disease represents a very slow, multistep process involving several events that are responsible from the initiation to the progression and spread of the disease (Vogelstein *et al.*, 1988, Vogelstein & Kinzler, 1993). In a nutshell, the pathophysiology of development of CRC begins with an aberrant crypt hyperproliferation that progresses to benign adenoma, subsequently leading to carcinoma *in situ* and finally to metastatic carcinoma. While mutations in the adenomatous polyposis coli (APC) gene and kirsten rat sarcoma viral oncogene homolog (KRAS) have been commonly associated with the initial events, malignant transformation of the CRC tumor is primarily driven by allelic losses and additional mutations in tumor suppressor genes such as, tumor protein p53 (*TP53),* phosphoinositide 3-kinase (*PI3K),* Mothers against decapentaplegic homolog 4 *(SMAD4),* and mitogen-activated protein kinases (*MAPK*), (Vogelstein *et al.*, 1988, Jen *et al.*, 1994, Smith *et al.*, 1994). More recently, the role of miRNAs as vital contributors to the process of CRC pathogenesis has been explored in a detail. One of the earliest studies identifying the involvement of miRNAs in the development of CRC demonstrated the role of miR-143 and miR-145 as potential tumor suppressors owing to their frequent downregulation in tumor samples (Michael *et al.*, 2003). In the subsequent years, miRNA profiling and deep sequencing efforts have resulted in the identification of numerous such miRNAs that are not only dysregulated in CRC, rather also provide vital clues as potential diagnostic or prognostic biomarkers, as well as therapeutic targets.

Through this review, we aim to provide a brief overview of the current body of knowledge of miRNAs in health and disease, specifically cancer, with a keen focus on CRC. While we summarize the known miRNAs in CRC, we shall also discuss their clinical relevance as potential therapeutic targets and biomarkers.

2. MiRNA biogenesis and its dysregulation in CRC

Following the discovery of the first miRNA, a global interest arose towards understanding the mechanisms associated with the biogenesis and the molecular regulation of these small ncRNAs. We now know that nearly 50% of miRNAs have been identified as intragenic, originating primarily from protein-coding gene introns and a limited number of exons; while the rest have an intergenic origin which are transcribed and regulated independently (Lee *et al.,* 2004, Borchert *et al.,* 2006, Kim & Kim, 2007, de Rie *et al.,* 2017). The miRNA biogenesis pathway traditionally begins with gene transcription by RNA polymerase II or III, resulting in 70-120-nucleotide primary miRNA (pri-miRNA) transcripts. These transcripts are cleaved by a microprocessor complex consisting of the ribonuclease (RNase) III family enzyme, Drosha and its co-factor, DiGeorge syndrome critical region gene 8 (DGCR8) or Pasha into a 60-70 nucleotide long, double-stranded hairpin precursor (pre-miRNA) (Lee *et al.,* 2003, Bartel, 2004). The active transport of pre-miRNA from the nucleus to cytoplasm is mediated by Exportin 5 (XPO5), a Ran guanosine triphosphate (RanGTP)-dependent nuclear transport receptor protein (Yi *et al.*, 2003, Lund *et al.*, 2004). Finally, in the cytosol the pre-miRNA is processed by a RNAse III enzyme, Dicer into ~18-23 nucleotide long, mature duplex miRNA sequences (Lee *et al.*, 2002, Yi *et al.,* 2003, Chendrimada *et al.*, 2005, Lee *et al.*, 2006, Bhaskaran & Mohan, 2014). Subsequently, the transactivation response RNA binding protein (TRBP), along with the Dicer-miRNA complex, enters the RNA-induced silencing complex (RISC) that contains the Argonaute (AGO) family of proteins (In humans, AGO1-4), trinucleotide repeat-containing gene 6A (TNRC6A), protein kinase RNA activator (PACT), and other RNA binding proteins. Within the RISC, one of the strands of the miRNA duplex is commonly degraded (passenger strand), while the other strand (guide strand) associates with the complementary sequence (generally located on the 3'untranslated region) on target messenger RNA (mRNA) causing translational repression or degradation by deadenylation and decapping (Hutvagner & Zamore, 2002, Guo *et al.*, 2010, Huntzinger & Izaurralde, 2011, Bhaskaran & Mohan, 2014, Ipsaro & Joshua-Tor, 2015). Indeed, the high level of molecular regulation exerted within the biogenesis pathway makes the miRNA, a crucial member of several key biological processes including cell proliferation, differentiation and

death and an overall cellular homeostasis. Not surprisingly, mutations within key players of this pathway such as Dicer1, Drosha, Exportin1 and AGO2 that results in abnormal miRNA expression, are frequently associated with the development of multiple cancer types. In fact, just within a decade of discovery of the first miRNA, the earliest record of miRNA dysregulation in cancer was identified in B cell chronic lymphocytic leukaemia (B-CLL) where *miR*-15a and *miR*-16-1 loci was deleted or downregulated in 68% of clinical cases (Calin *et al.*, 2002). Subsequent research by the group deciphered the role of miR-15 and miR-16 as potential tumor suppressors which are known to induce apoptosis by negatively regulating the expression of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein commonly overexpressed in several cancers (Cimmino *et al.*, 2005, Calin *et al.*, 2008). In just a few years, a study by the Hammond lab demonstrated that forced overexpression of the mir-17-92 polycistronic cluster collaborated with c-myc to promote the development of B-cell lymphoma in mice; thus identifying the first oncogenic miRNA, oncomiR-1 (He *et al.*, 2005). Through several similar overexpression as well as loss-of-expression experiments on cancer cell systems, along with animal models, it is now clear the miRNAs play critical roles in tumor initiation, development and progression (Di Leva & Croce, 2010, Lin & Gregory, 2015). While miRNAs have been identified as "cancer-causing" (oncomiRs) or "cancer-inhibiting" (tumor-suppressors), majority studies indicate a global repression of these small ncRNAs in cancer, not only pointing towards the dominant tumor suppressive role of miRNAs, rather also hinting towards the frequent dysregulation of the miRNA biogenesis pathway components which may strongly contribute towards cancer development (Lu *et al.*, 2005, Thomson *et al.*, 2006, Martello *et al.*, 2010). One of the earliest studies supporting this hypothesis demonstrated that impaired miRNA processing machinery can promote tumor development in a mouse model of lung cancer (Kumar *et al.*, 2007). Several subsequent researches demonstrated the pathophysiological relevance of mutations and dysregulations within the components of the miRNA biogenesis pathway, and the occurrence and progression of human tumors (Thomson *et al.*, 2006, Hill *et al.*, 2009, Heravi-Moussavi *et al.*, 2012, Anglesio *et al.*, 2013, Walz *et al.*, 2015).

Contrastingly, in CRC, while several studies have reported that miRNA expression patterns are consistently and reproducibly altered in the disease (Cummins *et al.*, 2006, Volinia *et al.*, 2006, Schetter *et al.*, 2008, Luo *et al.*, 2011), the global profile of miRNAs in CRC shows a higher number of upregulated miRNAs compared to the ones with reduced expression (Schetter *et al.*, 2012). A comprehensive literature review of 23 studies that investigated miRNA expression in CRC identified two thirds of the total number of dysregulated miRNAs to be overexpressed in CRC/adenoma as compared to the adjacent normal tissue/plasma, while only one third of the miRNAs were found to have a reduced expression (Luo *et al.*, 2011). While this is an indicator that miRNAs in CRC may probably have a higher "cancer-causing" function, the data also suggests that in contrast to other cancers, the miRNA processing machinery in CRC may be largely intact (Schetter *et al.*, 2012). Nevertheless, whereas the earliest study showing altered miRNA expression in CRC reported reduced expressions of the tumor suppressors miR-143 and miR-145 (Michael *et al.*, 2003), several subsequent researches identified multiple oncomirs contributing to the initiation, development and progression of CRC, including miR-21, miR-17-92 cluster, miR-155, miR-499, and several more (Slaby *et al.*, 2007, Schetter *et al.*, 2008, Diosdado *et al.*, 2009, Motoyama *et al.*, 2009, Valeri, Gasparini, Fabbri, *et al.*, 2010, Liu, Zhang, *et al.*, 2011). Likewise, the roles of miR-143, miR-145, miR-29, miR-34a, and similar such tumor suppressors has been investigated in CRC (Michael *et al.*, 2003, Fujita *et al.*, 2009, Ding *et al.*, 2011). Furthermore, with the advancement of technology and the consequent ease of studying these small ncRNAs by means of next generation sequencing or microarrays, we can now make use of miRNA profiling in CRC as a valuable clinical tool to classify the disease into phenotypic subgroups. Several studies have identified miRNA profile patterns that can differentiate between premalignant adenomatous lesions, adenocarcinoma as well as carcinoma (Bartley *et al.*, 2011, Oberg *et al.*, 2011, Zhu *et al.*, 2015, Slattery *et al.*, 2016). Moreover, by affecting the expression of molecular drivers of CRC, including microsatellite instability status, mutations in KRAS, BRAF, TP53, etc., the dysregulated miRNA pool in CRC has also been shown to differentiate between patients showing good or poor prognosis. As a proof of principle, Lanza et al. published the earliest study that identified 27 genes dysregulated in CRC, including 8 miRNAs, that could clearly classify microsatellite instability high (MSI-H) and microsatellite stable (MSS) samples

(Lanza *et al.*, 2007). The overexpression of miR-21 in CRC has been shown to downregulate the mismatch repair (MMR) recognition protein complex, human mutS homolog 2 (hMSH2) and 6 (hMSH6), and contribute to an increase in resistance towards 5-fluorouracil (5-FU) therapy (Valeri, Gasparini, Braconi, *et al.*, 2010). Furthermore, the Knuutila group made use of Agilent's miRNA microarrays to demonstrate that distinct miRNA signatures exist in CRC with mutant or wild-type KRAS (Mosakhani *et al.*, 2012). Another potent oncomiR in CRC is miR-31 which has been shown to activate the RAS pathway, by inhibiting the expression of RAS p21 GTPase activating protein 1 (RASA1), a negative regulator of KRAS (Sun *et al.*, 2013, Kent *et al.*, 2016). Moreover, high expression of miR-31 in CRC has also been shown to be frequently associated with *BRAF* mutations and an overall aggressive cancer (Nosho *et al.*, 2014, Choi *et al.*, 2016).

Taken together, the miRNAs strongly influence the occurrence, development and progression of CRC.

3. Strategies to therapeautically target dysregulated miRNAs in CRC

Although CRC is a highly researched disease, with an underlying molecular network thoroughly defined several years ago (Fearon & Vogelstein, 1990), it still remains one of the leading causes of mortality worldwide. One of the primary causative factors includes therapeutic resistance and lack of effective therapeutic targets. There is hence a strong emphasis to determine potential targets for improved therapies in CRC to achieve a better disease prognosis. Considering that miRNAs have a strong hold on the overall process of CRC occurrence and tumorigenesis, these small ncRNAs have been considered as potent theapeutic targets in CRC. Since dysregulated miRNAs in CRC or other cancers can be grouped as "cancer causing" or "cancer preventing", potential therapeutic approaches involve the inhibition of oncomirs and/or restoring the tumor suppressor miRNAs (Schetter *et al.*, 2012).

One of the examples is miR-143. Following the initial discovery of the dysregulated expression of potential tumor suppressor miR-143 and miR-145 in colon tumors (Michael *et al.*, 2003), subsequent

studies have shown that these miRNAs are able to regulate cell proliferation *in vitro* by targeting different oncogenes (Akao *et al.*, 2010). Specifically, miR-143 was shown to reduce cell growth using xenograft mouse models in CRC by translationally repressing the expression of KRAS, Extracellular-signal-regulated kinase 5 (ERK5) and DNA (cytosine-5)-methyltransferase 3A (DNTM3A) indicating the role of miR-143 as a tumor suppressor in CRC (Chen *et al.*, 2009, Zhang *et al.*, 2011, Ng *et al.*, 2014). MiR-143 replacement is hence suggested as an effective strategy for the management of CRC and attenuating its invasive features (Karimi *et al.*, 2019).

Another critical miRNA is miR-21 that has been found consistently upregulated in more than 18 different types of cancer inferring its functional relevance to most malignancies (Feng & Tsao, 2016). Increased miR-21 expression has been demonstrated to increase cell proliferation, decrease apoptosis, and increase angiogenesis as well as the overall metastatic potential, through the repression of multiple tumor suppressor genes such as, Phosphatase and Tensin Homologue (PTEN), Reversion Inducing Cysteine Rich Protein With Kazal Motifs (RECK), Ras Homolog Family Member B (RHOB), Programmed Cell Death 4 (PDCD4), Tropomyosin 1 (TPM1), Nuclear Factor I B (NFIB), mammary serine protease inhibitor (maspin), Sprouty homolog 2 (SPRY2), T Cell Lymphoma Invasion And Metastasis 1 (TIAM1) and Cell Division cycle 25 A (CDC25A) (Meng *et al.*, 2007, Zhu *et al.*, 2007, Asangani *et al.*, 2008, Gabriely *et al.*, 2008, Sayed *et al.*, 2008, Selcuklu *et al.*, 2009, Wang *et al.*, 2009, Cottonham *et al.*, 2010, Liu, Tang, *et al.*, 2011, Schetter *et al.*, 2012). Inhibition of miR-21 expression is hence a logical therapeutic approach in several cancers, including CRC. By making use of locked nucleic acid (LNA)-modified oligonucleotides and antisense oligonucleotides (ASO) targeting miR-21, several studies have demonstrated an inhibition of growth, invasion and metastasis of *CRC in vitro* or *in vivo* (Nedaeinia *et al.*, 2016, Ding *et al.*, 2018). In addition, experimental findings showed that Curcumol which is a natural major component of *Rhizoma Curcumae* reduced the proliferation of CRC cells by targeting miR-21 (Liu *et al.*, 2019), while Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, down-regulated miR-21 and decreased cell density, inhibited cell viability and induced apoptosis in CRC cells (Martin *et al.*, 2018). Although it is not clear if miR-21 is a direct target of curcumol and sulforaphane. Yet, these studies

demonstrated the potential of targeting miR-21, and similar such oncogenic miRNAs, for the treatment of CRC.

Likewise, there are multiple reports showing restoration of expression of certain tumor suppressor miRNAs by active compounds isolated from natural sources. Notable examples include, the upregulation of miR-34a by Ginkgetin, a natural biflavonoid isolated from the leaves of *Ginkgo biloba* with reported antitumor activities (Lee *et al.*, 2017); restoration of miR-134 by astragaloside IV, a bioactive saponin isolated from the dried plant roots of the genus *Astragalus* (Ye *et al.*, 2017); restoration of miR-3666 by All-Trans Retinoic Acid (ATRA), an active metabolite of vitamin A (Liu, Song, *et al.*, 2018); and upregulation of miR-378 by lauric acid which is found naturally in various plant and animal based oil extracts (Weng *et al.*, 2016).

MiR-328 was reported as a tumor suppressor in CRC. Its low expression was clinically correlated with a higher number of cancer stem cell (CSC)-like side population (SP) cells in CRC, and functionally affected the number, drug resistance, and cell invasion of SP cells (Xu *et al.*, 2012). Later, Li et al developed miR-328-loaded mesoporous silica nanoparticles (MSNs) modified with polymerized dopamine, epithelial cell adhesion molecule aptamer and bevacizumab (MSNs-miR-328@PDA-PEG-Apt-Bev) for the dual-targeting treatment of CRC (Li, Duo, Zhai, *et al.*, 2018). The group showed that MSNs-miR-328@PDA-PEG-Apt-Bev had an increased binding ability and could increase the level of miR-328 significantly in CRC cells and consequently repress the expression of its target gene, Ceramide-1-Phosphate Transfer Protein (CPTP), leading to higher cytotoxicity *in vitro* and in animal tumor models. Additionally, the group used a similar strategy to inhibit the expression of an oncogenic miRNA, miR-155, in CRC. They demonstrated that MSNs-anti-miR-155@PDA-Apt could significantly inhibit the expression of miR-155 in the SW480 CRC cell line and subsequently increase the sensitivity of the cells to 5-FU based therapy (Li, Duo, Bi, *et al.*, 2018). These results demonstrate the significance of nanoparticles as efficient drug delivery systems to target potent dysregulated miRNAs in CRC.

MiR-200c is another prominent tumor suppressor in CRC that inhibits tumor growth and progression *in vitro* and *in vivo* (Hur *et al.*, 2013, Lu *et al.*, 2014, Karimi Dermani *et al.*, 2018, Karimi Mazraehshah *et al.*, 2018, Lei *et al.*, 2019). Hence restoration of its expression is the correct approach to treat CRC. Although clinically validated approaches for upregulating the expression of miR-200c in CRC are lacking, there are several reports that demonstrate that the expression of the tumor suppressor miR-200c can be restored by clinically applicable antitumor substances such as, zerumbone (ZER) (a sesquiterpene isolated from subtropical ginger) (Dermani *et al.*, 2018), short-chain fatty acid sodium butyrate (by-product of bacterial anaerobic fermentation of dietary fibre in the colon) (Xu *et al.*, 2018), resveratrol, (a natural compound found in red wine) (Karimi Dermani *et al.*, 2017), niclosamide (an anthelminthic drug) (Suliman *et al.*, 2016), decitabine (a DNA methyltransferase inhibitor) (Tanaka *et al.*, 2015), epigallocatechin-3-gallate (an active catechin present in green tea) (Toden *et al.*, 2016), and Pien Tze Huang (a widely used traditional Chinese medicine) (Shen *et al.*, 2015).

Although, several miRNA targeting drugs/strategies in CRC are currently under research and have shown remarkable promise *in vitro* and *in vivo*, presently there are no miRNA targeting agents specific to CRC that are available for clinical use. It is hence essential to also look at potential miRNA targeting agents in other diseases. Notable example includes Miravirsen, a miR-122 antagonist originated by Santaris Pharma that has been developed to specifically target hepatitis C virus (HCV) infection (Janssen *et al.*, 2013). MiR-122 is a tumor suppressor, highly expressed in the liver cells that protects the hepatitis C viral RNA (Bandiera *et al.*, 2015, Schult *et al.*, 2018). Specifically, the drug is a 15-nucleotide LNA–modified ASO that binds to complementary sites on miR-122, releasing the viral RNA from protection, for subsequent destruction (Gebert *et al.*, 2014). The drug is currently in Phase II clinical trials (Titze-de-Almeida *et al.*, 2017). A similar agent, RG-101 is a mixed chemistry phosphorothioate oligonucleotide inhibitor of miR-122 that is linked to a multivalent N-acetylgalactosamine carbohydrate structure designed to enhance uptake of the oligonucleotide by liver cells through binding to the asialoglycoprotein receptor (Prakash *et al.*, 2014). Although results from the phase 1B trial suggest that the drug was well tolerated and was also found to be highly efficient in

significantly reducing the viral load in patients (van der Ree *et al.*, 2017), the drug was put on a clinical hold in 2017 due to the development of serious adverse event (SAE) of jaundice in patients treated by the drug (Regulus, 2017). Another interesting miRNA targeting agent is MRX34 which is a liposomal mimic of the tumor suppressor miR-34a (frequently repressed in several cancers including CRC) that showed acceptable safety and efficient antitumor potential in patients with advanced solid tumors (Beg *et al.*, 2017). However, the phase 1 study was closed with the discovery of multiple immune related SAE in patients treated with MRX34 (Therapeutics, 2016). These data clearly demonstrate that although there are several potential miRNA-based therapeutic targets, strategies to efficiently target them are still at infancy. Although the earliest evidence of the role of miRNAs in cancer was identified in early 2000s, we are still unsure of the global role of miRNAs in different physiological states. Multiple studies have shown that while some miRNAs may act as tumor suppressors in some cancers, the same molecules can be overexpressed in other cancers and function as potential oncogenes (Ling H, 2013). Furthermore, dysregulated miRNAs in cancer may bear a different physiological function in other diseases. Examples include, miR-15 and miR-34 which are known tumor suppressors in multiple cancers, including CRC. While restoration of the expression of these miRNAs is a logical therapeutic strategy in CRC, low expression of miR-15 and miR-34 has been found to improve cardiac function and protection against heart disease (Bernardo *et al.*, 2012, Hullinger *et al.*, 2012).

Put together, these therapeutic strategies offer a potential direction towards improving our control over the development and progression of CRC. However, only with deeper understanding of the mechanism of action of miRNAs and its regulation, can improved treatment approaches be designed to specifically target these small ncRNAs.

4. Potential of miRNAs to function as non-invasive predictive biomarkers in CRC

The role of miRNAs as potent molecules that can classify CRC into different phenotypic or molecular subgroups has been established. Moreover, the global profile of miRNAs between the healthy colon,

adenomas, adenocarcinomas and metastatic carcinomas is also remarkably different. By profiling the miRNA pool within each subset, it is hence possible to identify a small group of miRNAs that can function as highly predictive diagnostic and prognostic biomarkers in CRC. Currently, the most reliable early screening method for CRC is colonoscopy, although the technique is invasive and is expensive. An alternative is the fecal occult blood test (FOBT) which is indeed a cheap, less invasive test that detects blood within the stool, but it has a low detection sensitivity, requires a strict diet prior to testing and functions only as a diagnostic screening test (Aslam *et al.*, 2009, Ng *et al.*, 2009). Routine clinical investigations of serum carcinoembryonic antigen (CEA) is commonly used to assess CRC progression, and can hence function as a diagnostic and a prognostic biomarker (Moertel *et al.*, 1986). Although, the metabolite lacks a high sensitivity, particularly for predicting disease prognosis, and can also show elevated levels in other diseased conditions including, pancreatitis, inflammatory bowel disease, or other cancers (Hundt *et al.*, 2007). MiRNAs can hence function as reliable biomarkers in CRC that offer several advantages including: high specificity and sensitivity to different physiological/diseased states, stability in multiple tissue types and body fluids (blood, urine, stool, sweat, etc.), greater ease of detection through simple techniques such as quantitive polymerase chain reaction (QPCR) and lastly, the potential to choose from several miRNA molecules to form a panel that offers the highest sensitivity and specificity to predict the disease.

Several studies have reported a concordance between the dysregulated miRNAs in cancerous tissues and their expression levels in biofluids such as, blood. Moreover, circulating miRNAs have been shown to be highly stable and can be easily detected through minimally invasive techniques; all of which makes them highly suitable as potential biomakers in CRC (Chen *et al.*, 2008, Mitchell *et al.*, 2008, Manne *et al.*, 2010, Bartley *et al.*, 2011, Oberg *et al.*, 2011, Zhu *et al.*, 2015, Slattery *et al.*, 2016). We previously reported a repressed expression of miR-139-3p in CRC tissues when compared to paired adjacent normal mucosa (Ng *et al.*, 2017). In corcodance with this data, serum miR-139-3p level was also found to be significantly lower in CRC patients when compared to healthy controls and showed a high performance for CRC screening within our study. The expression of miR-21 is commonly upregulated in adenoma and adenocarcinoma compared to normal mucosa, and is

associated with CRC initiation and progression (Slaby *et al.*, 2007, Schetter *et al.*, 2008, Yamamichi *et al.*, 2009). Increased level of miR-21 has been frequently reported in the plasma or serum of CRC patients when compared to healthy controls (Kanaan *et al.*, 2012, Wang & Zhang, 2012, Liu *et al.*, 2013, Toiyama *et al.*, 2013, Wang *et al.*, 2014, Bastaminejad *et al.*, 2017, Pan *et al.*, 2017, Zhu *et al.*, 2017, Liu, Liu, *et al.*, 2018, Liu, Yang, *et al.*, 2018, Sabry *et al.*, 2019). Other oncogenic miRNAs in CRC for instance, MiR-29a and miR-92a have also exhibited a higher expression in the blood samples of CRC patients (Huang *et al.*, 2010, Brunet Vega *et al.*, 2013, Ramzy *et al.*, 2015, Liu, Liu, *et al.*, 2018), though findings against those results have also been reported (Faltejskova *et al.*, 2012, Zekri *et al.*, 2016, Yang *et al.*, 2018). Furthermore, several groups have reported that the predictive power of miRNAs can be improved by using a panel, instead of a single miRNA molecule. The combination of miR-21, miR-29a, miR-92a and miR-125b had the highest area under the curve (AUC) at 0.952, with a sensitivity of 84.7% and a specificity of 98.7% (Liu, Liu, *et al.*, 2018). The Zhang group discovered that the plasma level of miR-24, miR-320a and miR-423-5p can be used for early detection of CRC with a high AUC (0.941), sensitivity (90.7%) and specificity (70.8%) (Fang *et al.*, 2015). Another study reported a panel of six serum miRNAs (miR-21, let-7g, miR-31, miR-92a, miR-181b, and miR-203) that showed a high sensitivity and specificity in diagnosing CRC, as compared to the traditional tumor based biomarkers CEA and carbohydrate antigen 19-9 (CA19-9) (Wang *et al.*, 2014).

Furthermore, plasma or serum miRNAs have also been suggested as potential prognostic and predictive biomarkers to determine response to therapy, recurrence, metastatic spread and/or the overall disease outcome. Recent studies have demonstrated that a low expression of circulating miR-23b, miR-497, miR-145, miR-29b, miR-194 and miR-101 and elevated expression of miR-203, miR-139-5p, miR-103, miR-21, miR-1290 and miR-122 strongly correlate with an advanced stage and a shorter disease-free or overall survival in CRC (Basati *et al.*, 2016, Imaoka *et al.*, 2016, Kou *et al.*, 2016, Hur *et al.*, 2017, Maierthaler *et al.*, 2017, Miyoshi *et al.*, 2017, He *et al.*, 2018, Wang *et al.*, 2018, Zou *et al.*, 2019). Recently the Gu lab developed a serum-based four-miRNA signature (miR-652-3p, miR-342-3p, miR-501-3p and miR-328-3p) that could predict disease recurrence and

response to adjuvant therapy in CRC patients (Ji *et al.*, 2018). High pre-operative plasma miRNA levels of miR-200b, miR-203, miR-29a and miR-31 were associated with an increased risk of CRC recurrence, whereas postoperative plasma miR-31, miR-141 and miR-16 levels were found to be highly useful to predict recurrence during disease surveillance (Yuan *et al.*, 2017). Plasma miR-20b-5p, miR-29b-3p and miR-155-5p have been reported to predict the outcome of patients with metastatic CRC treated with Bevacizumab (Ulivi *et al.*, 2018).

In addition to blood, a similar concordance has been observed between the dysregulated tissue miRNAs in CRC patients, and the miRNA expression profile in their corresponding stool samples. Therefore, analysis of miRNA levels in stool samples offers yet another source of potential non-invasive biomarkers in CRC. The earliest study in this direction was published in 2009 that was able to detect 7 upregulated (miR-21, miR-106a, miR-96, miR-203, miR-20a, miR-326 and miR-92) and 7 downregulated miRNAs (miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16 and miR-125b) in stool (as well as tissue) samples of CRC patients (Ahmed *et al.*, 2009). A subsequent study also identified an increased expression of miR-21 and miR-106a in the stool samples of 29 CRC patients compared to their levels in the stool samples from 8 healthy controls (Link *et al.*, 2010). The Matsumura group attempted to profile the exfoliated colonocytes isolated from the feces of 197 CRC patients and 119 healthy controls and identified a higher expression of the miR-17-92 cluster and miR-135 in the colonocytes as well as frozen CRC tissues (Koga *et al.*, 2010). The well-documented oncogenic miR-21 has also been reported to exhibit increased levels in stool samples of CRC patients compared to healthy controls, with a high detection sensitivity (86.05%) and specificity (81.08%) (AUC: 0.829) (Bastaminejad *et al.*, 2017). Furthermore, the study also reported that high levels of miR-21 in stool correlated significantly with several patient clinicopathological parameters including tumor stage, presence of nodes, presence of metastasis, as well as overall cancer stage, with a very high predictive potential (AUC: 0.872). Faecal let-7f expression levels have been reported to have significant sensitivity and specificity in distinguishing between patients with CRC and healthy subjects (Ghanbari, Mosakhani, Sarhadi, *et al.*, 2015). Likewise, several stool based miRNAs have been validated as potential biomarkers in CRC, including miR-29a, miR-223, miR-224, miR-4487

and miR-1295b-3p that show a decreased expression, while miR-21, miR-92a, miR-20, miR-221, miR-135b are overexpressed, in the faeces of CRC patients (Wu *et al.*, 2012, Wu, Ng, *et al.*, 2014, Ghanbari, Mosakhani, Asadi, *et al.*, 2015, Yau *et al.*, 2016, Zhu *et al.*, 2016). Of note, Phua et al demonstrated that while faecal miR-223 and miR-451 represented robust markers in distinguishing CRC patients from healthy subjects with an AUC of 0.939 and 0.971 respectively, presence of blood in stool affected the miRNA expression levels to a varying extent, significantly affecting its clinical interpretation (Phua *et al.*, 2014).

In addition to the miRNA expression levels, DNA isolated from stool has been shown to illustrate miRNA promoter methylation patterns that has a potential to be used as diagnostic tool in CRC. The hypermethylation pattern of miR-34a and miR-34b had a 75% sensitivity and 84 % specificity in distinguishing CRC patients from healthy controls using stool samples (Kalimutho *et al.*, 2011). A subsequent study tested the methylation of miR-34a and miR-34b/c promoter in CRC tissue specimens and stool samples. While the faecal miR-34a methylation test showed a high sensitivity (76.8%) and specificity (93.6%), faecal miR-34b/c methylation test showed a higher sensitivity and specificity of 95% and 100%, respectively, for detecting CRC using stool samples (Wu, Song, *et al.*, 2014).

These findings demonstrate the high potential of miRNAs as potent non-invasive diagnostic biomarkers in CRC and/or predictive, prognostic biomarkers to monitor response to therapy, and/or surveilence biomarkers for early detection of recurrence. Although several shortcomings have been noted, including but not limited to, lack of appropriate miRNA expression normalization controls (usage of small nucleolar RNAs (snoRNA) such as, U6, or one or several miRNAs or other small ncRNAs), lack of a consesus methodology for the isolation of miRNAs from biospecimens (kit based versus non-kit based) and, a lack of a consensus methodology for the detection of miRNAs from biospecimens (qPCR (SYBR green based versus Taqman based) or digital droplet PCR). Further investigations are warranted to extrapolate the role of miRNAs as valid non-invasive CRC biomarkers in the clinical setting.

5. Conclusions

MiRNAs are vital small non-coding RNAs that play key roles in many crucial biological processes. In recent decades, extensive evidence has emerged showing that miRNAs are also involved in cancer development and progression, and aberrant expression of miRNAs is detected in various types of cancer, including CRC. Studying the dysregulation and specific function of miRNAs in CRC will help to identification of new targets for cancer research, diagnosis and treatment. Although miRNAs as valuable therapeutic targets or diagnostic/prognostic biomarkers has been explored largely *in vitro* and *in vivo*, we are still light years away from applying the study of miRNAs to clinical setting. Only by an improved understanding of the global physiological roles of these small ncRNAs, can we progress in making use of miRNAs from bench to bedside in CRC.

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