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FRONTIER

MicroRNA biomarkers predicting risk, initiation and progression of colorectal cancer

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Abstract

Colorectal cancer is a major global cause of morbidity and mortality. Current strategies employed to increase detection of early, curable stages of this disease are contributing to a reduction of the negative health impact from it. While there is a genetic component to the risk of disease, diet and environment are known to have major effects on the risk of an individual for developing the disease. However, there is the potential to reduce the impact of this disease further by preventing disease development. Biomarkers which can either predict the risk for or early stages of colorectal cancer could allow intervention at a time when prospects could be modified by environmental factors, including lifestyle and diet choices. Thus, such biomarkers could be used to identify high risk individuals who would benefit from lifestyle and dietary interventions to prevent this disease. This review will give an overview on one type of biomarker in the form of microRNAs, which have the potential to predict an individual's risk for colorectal cancer, as well as providing a highly sensitive and non-invasive warning of disease presence and/or progression. MicroRNA biomarkers which have been studied and whose levels look promising for this purpose include MiR-18a, MiR-21, MiR-92a, MiR-135b, MiR-760, MiR-601. Not only have several individual microRNAs appeared promising as biomarkers, but panels of these may be even more useful. Furthermore, understanding dietary sources and ways of dietary modulation of these microRNAs might be fruitful in reducing the incidence and slowing the progression of colorectal cancer.

Key words: Biomarkers; Epigenetics; Risk; Colorectal cancer; Predisposition; MicroRNA

Core tip: The requirements for colonoscopic technologies in order to detect early stages of colorectal cancer are being superseded by highly sensitive microRNA technologies using various body fluids. As well as providing early warnings of the disease, these also potentially provide a highly sensitive marker of dietary efficacy in disease prevention or slowing of disease progression.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men[1]. In the earliest stages of CRC development, the lesion may not cause obvious symptoms, so individuals may not be prompted to go to a health practitioner until disease is advanced. Hence, many CRC patients present late, when their cancer is advanced and their prognosis for a permanently curative resection is less likely. As well as the asymptomatic nature of early CRC, other reasons why people may present with CRC at a late stage may be because of the insensitivity of screening tests to pick up precancerous lesions, or refusal to undergo a screening test for various reasons. In this review, we briefly consider current screening technologies[2], and then develop a rationale for a new highly sensitive screen using a novel biomarker.

The current CRC screening methods are faecal occult blood test, fecal immunochemical test, sigmoidoscopy and colonoscopy. Guaiac faecal occult blood tests are more effective when a repeat test is performed every 1 or 2 years[2]. Both guaiac faecal occult blood test and faecal immunochemical test have more of an important role in detecting early cancer rather than precancerous lesions as the tests only identifies a small proportion of patients with advanced adenomas[2]. Therefore, due to their low sensitivity for advanced adenomas, most advanced adenomas will go undetected and therefore these stool blood tests will not significantly contribute to prevention of cancer by detection and excision of adenomas. The limitation with sigmoidoscopy is that it is less likely to identify colon neoplasia in a proximal location[2]. In addition to this, sigmoidoscopy is a relatively inconvenient test as a bowel preparation and an office visit is needed[2]. Colonoscopy is comparatively a more attractive option than sigmoidoscopy as patients have their whole colon investigated and they can also have sedation[2]. However, colonoscopy is invasive, expensive and it carries a risk for complications[2]. Clearly, there is a need for a new biomarker or panel of biomarkers which are non-invasive, cost-effective and allows the identification of risk for CRC, for example by identifying patients with precancerous lesions. One such group of potentially highly sensitive biomarkers, microRNAs, will be examined in this manuscript.

RISK FACTORS FOR CRC

Certain non-modifiable and modifiable risk factors are known to be associated with CRC risk. Non-modifiable risk factors which increase an individual's risk of the disease include age, a personal history of adenomatous polyps or inflammatory bowel disease, a family history of adenomatous polyps or colorectal cancer and inherited genetic risk[3]. Modifiable risk factors include diet, physical activity, obesity, cigarette smoking and alcohol consumption[3].

There have been many studies relating various dietary factors to colorectal cancer risk. There is considerable evidence that certain types of dietary fibres reduce the risk of CRC[4]. However, there is also opposing evidence that indicates that other dietary fibres may increase the risk of CRC development[5]. Studies have shown an association between high intake of red and processed meats and increased risks of CRC[6,7]. For the relationship between folate intake and the risk of CRC, there have been inconsistent results[8]. The impact of protein intake on CRC risk is also unclear. Different studies have shown high protein intake to associate with both elevated and reduced risks of CRC[9,10]. These differences in results highlight the impact that the characteristics of the population study and the type of protein can have on the results[11]. There is evidence that obesity increases risk of colon cancer[12].

The human colonic microbiota is composed of bacteria, some of which can enhance while others can protect against colorectal carcinogenesis[13]. The burden of CRC may be alle*via*ted by using certain types of dietary fibre to manipulate the metabolic activity of the bowel microbiota or by changing the composition of the microbiota[13]. Low vitamin D status may also be associated with a higher risk of colorectal cancer[14]. High levels of low-density lipoprotein, triglycerides and total cholesterol may also be associated with increased risk[15]. However, further studies producing more convincing results are needed on this relationship. Reports have also investigated the relationship between minerals and risk of CRC. For example, very low or very high selenium concentrations seem to enhance the risk of cancer, while the optimal concentration and form of selenium may be protective[16].

EARLY STAGES IN CARCINOGENESIS

CRC arises following an accumulation of genetic and

epigenetic changes which transform normal colonic epithelial cells into cancerous cells[17-19]. Mutations in proto-oncogenes and tumour suppressor genes also contribute[17]. There are also many genetic polymorphisms which are being identified as increasing an individual's susceptibility to developing colorectal cancer. However, genetic variants such as mutations and polymorphisms are specific sequences of DNA and are therefore fixed and not modifiable. Hence, although genetic markers may be able to identify individuals at risk for colorectal cancer, they cannot be changed by lifestyle or dietary interventions.

Unlike genetic alterations, epigenetic changes are potentially modifiable. Epigenetic alterations are heritable changes in gene expression which do not alter the DNA sequence. Epigenetic mechanisms include DNA methylation, microRNA (miRNA) expression, histone modification and chromatin remodelling. Epigenetic alterations can increase, decrease or silence gene expression[19]. There has recently been a surge in the scientific literature of epigenetic biomarkers which are potentially associated with colorectal cancer[19-21]. In particular, there has been a focus on DNA methylation and miRNA expression patterns, the latter of which will be the subject of this review.

The majority of CRCs arise from localised pre-cursor lesions called adenomas. Thus, individuals with colorectal adenomas are at increased risk of CRC[17]. Removal of this adenoma can prevent it from developing into cancer. An epigenetic biomarker which is differentially expressed in adenoma patients compared to healthy controls would identify those people who have an increased risk for developing CRC. As the presence of adenomas is known to increase risk for CRC, epigenetic biomarkers which detected adenomas have been included in this review.

BIOMARKERS

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[22].

Environmental factors have been linked to aberrant epigenetic modifications which contribute to cancer formation. In colorectal cancer, there is the potential to link lifestyle and diet risk factors to aberrant epigenetic alterations in the very early stages of colorectal cancer development. As both epigenetic changes and lifestyle choices are modifiable, there is the possibility that positive lifestyle interventions can alter epigenetic mechanisms to change gene expression and reduce the risk for colorectal cancer. This modified level or expression of the epigenetic markers could be measured as a biomarker to reflect the risk status of the individual.

Many biomarkers in the literature are either tissue, stool or blood-based[22]. Tissue based biomarkers require a sample of tissue in which to measure the expression of the epigenetic biomarker. This is usually taken through a biopsy during colonoscopy and is thus an invasive technique which requires colonoscopy and excision of tissue. An ideal biomarker is non- invasive so this review only included biomarkers which were measured from a stool or blood sample. The details of the studies are provided in Table 1[23-36], and discussed subsequently.

MICRORNA IN CRC RISK

While some studies had considered peripheral blood mononuclear cells which are important for studying DNA or gene expression changes, the small size of miRNAs means that they are more readily detected in plasma samples, or in faecal samples. Thus, the following discussion mostly considers such samples.

MiR-21 has been investigated in several different studies. Link *et al*[23] showed that miR-21 was increased in stool samples from adenoma subjects compared to normal colonoscopy subjects. However, in another stool-based study, no difference was found in miR-21 levels between patients with polyps and controls[26]. Two studies have demonstrated that miR-21 is overexpressed in the serum of patients with advanced adenomas (AA), as compared to controls[24,25]. Two findings from one of the serum-based studies indicated that miR-21 in the serum of CRC patients is secreted from CRC tissue, which is significant as it increases the diagnostic specificity of blood-based levels of miR-21[25]. Initially, Toiyama *et al*[25] observed a statistically significant correlation between tissue miR-21 level and matching serum samples from a small number of patients with CRC. Surgical removal of CRC led to a statistically significant reduction of miR-21 in the serum of these same patients. However, they identified two possible limitations of miR-21 as a colorectal neoplasia biomarker. Firstly, it is presently difficult to be certain that changed expression levels of circulating miR-21 are specifically caused by colorectal neoplasia, because circulating miR-21 has been reported to be associated with other cancers, such as breast cancer, glioblastoma and pancreatic cancers[25]. Secondly, all their study samples were from subjects of Japanese origin[25]. Hence, the lack of ethnic diversity may hamper this study's applicability to other populations. Luo *et al*[27] did not find any differences in plasma miR-21 levels of AA patients, compared to controls. However, these authors acknowledged that their observed lack of differences in miRNA expression levels may have been because of inadequate power to distinguish modest differences due to small sample sizes.

MiR-92a has also been examined in a number of studies. It was shown to be overexpressed in the serum[24], plasma[31] and stool[26] of AA patients,

Table 1 Summary of experiments relevant to microRNA detection of colorectal cancer

miRNA	Sample size		Findings	Specimen type	Ref.
	Cases (*n*)	Controls (*n*)			
miR-21 miR-106a miR-17 miR-143 miR-622 miR-654-3p	9 non-advanced adenomas and AA 10 CRC	10 controls (normal colonoscopy)	miR-21, miR-106a: Colorectal neoplasia (adenoma, CRC) patients had higher stool expression of these two miRNA compared to normal colonoscopy subjects ($P < 0.05$). Adenoma patients had higher stool miR-21 and miR-106a expression compared to CRC patients miR-17, miR-143, miR-622, miR-654-3p: No differences between groups	Stool	Link *et al*[23]
miR-21 miR-92a miR-31 miR-18a miR-106a	50 AA 200 CRC	80 controls (do not have a current or previous malignancy or inflammatory condition)	miR-21, miR-92a: miR-21 and miR-92a levels in CRC patients and AA patients were significantly higher compared to controls (all $P < 0.05$). miR-21 yielded an AUC of 0.709 in differentiating AA from controls. miR-92a yielded an AUC of 0.701 in differentiating AA from controls. Both miRNA together yielded an AUC of 0.722 in differentiating AA from controls miR-18a, miR-31, and miR-106a: No significant differences between groups	Serum	Liu *et al*[24]
miR-21 miR-31	43 AA 60 postoperative patients 186 CRC	53 controls (negative colonoscopic examination, no prior diagnosis of any other malignancy)	miR-21: Serum levels were increased in adenomatous polyp patients compared with controls ($P < 0.001$). Serum miR-21 levels yielded an AUC of 0.803 (95%CI: 0.669-0.869) in differentiating AA from controls. The sensitivity, specificity, positive predictive value and negative predictive values were 76.8 % and 81.1%, 76.7%, and 81.1%, respectively, at a cut-off value of 0.0013	Serum	Toiyama *et al*[25]
miR-92a miR-21	44 patients with minor polyp (defined as hyperplastic polyp or adenoma less than 1 cm in diameter) 13 AA 88 CRC	101 controls (asymptomatic individuals)	miR-92a: Stool miR-92a was significantly increased in polyp patients compared with controls ($P < 0.0001$). Sensitivity of 56.1% for polyp, specificity of 73.3%. Higher sensitivity for AA than minor polyps ($P < 0.05$). The removal of AA led to a decrease in stool miR-92a level ($P < 0.05$). miR-21: No difference between polyps and controls	Stool	Wu *et al*[26]
miR-29a, miR -106b, miR -133a, miR -342-3p, miR -532-3p miR-18a, miR -20a, miR -21, miR -92a, miR -143, miR -145, miR -181b	Marker validation phase 50 AA	Marker validation phase 50 controls (free of colorectal neoplasms)	No statistically significant differences between AA patients and controls for any of the investigated miRNA	Plasma	Luo *et al*[27]
miR-10a, miR-29a, miR-31, miR-92a, miR-100, miR-125b, miR-184, miR-187, miR-196a, miR-200b, miR-203, miR-17-3p	73 non-advanced adenoma 43 AA 8 CRC	48 controls (polyp-free)	No statistically significant associations with non-advanced adenoma or AA for any of the investigated miRNA	Plasma	Adams *et al*[28]
miR-34a miR-150 miR-923	Discovery set 8 polyp 16 adenoma 8 CRC (stage I/Ⅱ) 8 CRC (stage Ⅲ/Ⅳ) Validation set 20 polyp 20 adenoma 23 CRC (stage I/Ⅱ) 14 CRC (stage Ⅲ/Ⅳ)	Discovery set 8 controls Validation set 20 controls	miR-34a: Validation cohort: Significantly higher in adenoma group compared to controls (FC 2.09, $P = 0.028$). Significantly higher in adenoma group compared to the polyp group (FC 2.71, $P = 0.002$). miR-923: Validation cohort: No significantly different levels	Plasma	Aherne *et al*[29]

miR-18a miR-15b miR-19a miR-19b miR-29a miR-335	Set 1 20 AA 21 CRC Set 2 40 AA 42 CRC	Set 1 20 controls Set 2 53 controls	miR-18a: Set 1 and Set 2: Significantly overexpressed in AA patients compared to controls in both sets. Set 1: Good discriminative capacity in AA patients (AUROC, 0.84; 95%CI: 0.72-0.96; sensitivity [S], 80%; specificity [Sp], 80%). Set 2: Lower discriminative capacity in AA patients (AUROC, 0.64; 95%CI: 0.52- 0.75; S, 72%; Sp, 57%)	Plasma	Giráldez *et al*[30]
miR-29a, miR-92a,	Large-scale validation 37 AA 100 CRC	Large-scale validation 59 controls (negative results of health examination including blood test, chest X-ray, abdominal ultrasound examination, fecal occult-blood testing, rectal touch, CT scan and colonoscopy. None of these controls had previously been diagnosed with any types of malignancy previously)	miR-29a and miR-92a: Significantly higher in AA compared to controls (P < 0.0001 for miR-29a, P < 0.0001 for miR-92a). Both miRNAs together yielded an AUC of 0.773 (95%CI: 0.669-0.877), sensitivity 73.0% and specificity 79.7%, in discriminating AA. miR-29a: Yielded an AUC of 0.769 (95%CI: 0.669-0.869) for differentiating AA from controls. The sensitivity was 62.2% and specificity 84.7%, at a cut-off value of 1.210 for miR-29a. The odds ratio for cases with miR-29a > 1.210 being associated with AA was 12.20 (95%CI: 4.350-34.237). miR-92a: Yielded an AUC of 0.749 (95%CI: 0.642-0.856) for differentiating AA from controls. Sensitivity 64.9% and specificity 81.4%, at a cut-off value of 1.682 for miR-92a. The odds ratio for cases with miR-92a > 1.682 being associated with AA was 4.56 (95%CI: 1.893-10.988)	Plasma	Huang *et al*[31]
A panel of 8 miRNAs miR-532-3p + miR-331 + miR-195 + miR-17 + miR-142-3p + miR-15b + miR-532 + miR-652	Initial Screening 9 adenoma 20 CRC (stage Ⅲ/Ⅳ) Validation 16 adenoma 15 CRC (stage Ⅰ/Ⅱ) 15 CRC (stage Ⅲ) 15 CRC (stage Ⅳ)	Initial Screening 12 controls (without CR neoplasia) Validation 26 controls (without CR neoplasia)	Initial Screening 15 out of 380 screened miRNAs most dys-regulated in plasma of adenoma patients compared to controls (P < 0.05, FDR: 5%). Validation A panel of 8 plasma miRNAs yielded an AUC of 0.868 (95%CI: 0.76-0.98), sensitivity 88% and specificity 64% in differentiating adenoma from controls	Plasma	Kanaan *et al*[32]
miR-601 miR-760	Large scale validation 43 AA 90 CRC	Large scale validation 58 controls	miR-601: AUC of 0.638, sensitivity of 72.1% and specificity of 51.7% in differentiating AA from controls miR-760: AUC of 0.682, sensitivity of 69.8% and specificity of 62.1% in differentiating AA from controls miR-601 + miR-760: Significantly decreased in colorectal neoplasia (AA and CRC) compared to controls. Both miRNAs together yielded AUC of 0.683, sensitivity 72.1% and specificity 62.1% in differentiating AA from controls	Plasma	Wang *et al*[33]
miR-135b miR-31	110 adenomas < 1 cm in size 59 AA 42 IBD 104 CRC	109 controls (normal colonoscopy)	miR-135b: Significantly increased in adenoma subjects (median, 28.4; IQR, 0.2-79.7; P < 0.0001) compared to controls (median, 0; IQR, 0-30.8). No significant difference in IBD subjects compared to controls. AUC of 0.71 for detection of adenoma. Sensitivity of 73% for AA, 61% for adenoma < 1 cm in diameter, 65% for any adenoma and specificity of 68%, at a cut-off of 14 copies/ng of stool RNA. Sensitivity of 44% for adenoma < 1 cm, 46% for AA, and specificity of 80%, at a cut-off of 38 copies/ng of stool RNA. Removal of AA or CRC resulted in a significant reduction of stool miR-135b. miR-31: No significant differences between groups	Stool	Wu *et al*[34]
miR-18a miR-221	151 adenoma 48 AA 198 CRC	198 controls (normal colonoscopy)	miR-18a, miR-221: No significant up-regulation in adenoma or AA	Stool	Yau *et al*[35]
A panel of 4 miRNAs miR-19a-3p + miR-223-3p + miR-92a-3p + miR-422a	Validation of the diagnostic performance of the miRNA panel: 73 adenoma 117 CRC	Validation of the diagnostic performance of the miRNA panel: 102 controls (healthy individuals seeking a routine health check- up)	Validation of the miRNA panel The miRNA panel yielded an AUC of 0.765 (95%CI: 0.669-0.845) in differentiating adenoma from controls	Serum	Zheng *et al*[36]

CRC: Colorectal cancer; AA: Advanced adenomas; IBD: Inflammatory bowel disease.

compared to controls. However, Luo *et al*[27] did not show a difference of miR-92a levels in the plasma of AA patients compared to neoplasm-free controls. Furthermore, Adams *et al*[28] did not find plasma miR-92a levels to be associated with non-advanced adenomas or AA. The cause of these discrepancies is unclear, but may have been due to differing samples sizes and patient characteristics including ethnicity. A notable finding that supported the involvement of miR-92a in CRC came from the stool based study of miR-92a from Wu *et al*[26], as they found that following the removal of AA or cancer, there was a significant reduction in stool miR-92a.

Giráldez *et al*[30] found that miR-18a was over-expressed in the plasma of AA, compared to controls. However, these findings were not concordant with another study which found no differences in plasma miR-18a levels in AA samples, compared to neoplasm-free controls[27]. Another study did not find differences in miR-18a levels between serum samples from AA individuals and controls[24]. Furthermore, no upregulation of miR-18a was seen in stool samples from adenoma subjects[35]. Thus, the present balance of evidence does not support miR-18a being a useful biomarker.

Similarly, miR-31 also does not seem like a useful biomarker at present. A number of studies have not been able to find a difference in miR-31 expression levels between adenoma patients and controls in serum[24,25], plasma[28] or stool[34] samples. A large case-control study revealed the potential utility of miR-135b for detecting adenoma[34]. The group's findings suggested that overexpression of this miRNA is specific for colorectal neoplasia, as removal of AA and CRC led to a significant reduction in the expression of stool miR-135b. Furthermore, this study included controls with inflammatory bowel disease and a lower level of stool miR-135b was found in these controls, giving further evidence that miR-135b upregulation is specific for colorectal neoplasia. The initial findings for miR-135b appear promising and some strengths of this study include larger cohort numbers and inclusion of IBD controls. However, some limitations of this study were identified by the authors; the findings of this study may not be representative of the community screening setting due to recruitment from limited locations and the inclusion of some symptomatic patients[34]. Also, the impact of attaining a stool sample before compared to after colonoscopy on miRNA levels was not analysed and in this study, stool was sampled one week before colonoscopy in all AA patients.

Researchers have investigated the discriminative capability of both single miRNAs and panels of miRNAs in differentiating adenoma patients from controls. It may be more useful in clinical practice to use panels of miRNA rather than a single miRNA to discriminate individuals with colorectal adenomas from those without, as there is doubt that a single miRNA has high enough specificity for it to be used alone as a biomarker of colorectal neoplasia[32]. The reports of specific miRNA being differentially expressed in multiple cancers lend support to this idea[27]. Panels of plasma and serum miRNA have been tested for their ability to discriminate adenomas from controls. A panel of 8 plasma miRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, miR-652) yielded an AUC of 0.868 (95%CI: 0.76-0.98), sensitivity 88% and specificity 64% in differentiating adenoma from controls[32]. However, the authors pointed out that to investigate specificity more thoroughly, individuals with inflammatory diseases and other tumours should have been included among their controls. A serum miRNA panel composed of 4 miRNA (miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a) yielded an AUC of 0.765 (95%CI: 0.669-0.845) in differentiating adenoma from controls[36].

MiR-760 had an AUC value of 0.682, sensitivity of 69.8% and specificity of 62.1% in differentiating AA from controls[33]. MiR-601 had an AUC of 0.638, sensitivity of 72.1% and specificity of 51.7% in this same study. When both miRNA were combined, an AUC of 0.683, sensitivity of 72.1% and specificity of 62.1% was achieved in differentiating AA from controls. The 2 miRNA together demonstrated an AUC, sensitivity and specificity similar to miR-760 alone and therefore, the addition of miR-601 to miR-760 was not considered to be useful[33]. This highlights the importance in analysing the individual contribution of panel components to the discriminatory power of the panel overall to identify those markers which should or should not be included.

As noted in sections on specific miRNAs above, there were inconsistences in findings between studies for the same miRNA. Firstly, this may have been caused because of the different type of sample used (stool, plasma or serum). Methodologic differences in collection procedures, specimen preparation/processing, miRNA extraction, miRNA detection and measurement, data acquisition, data normalisation, quantification methods may have also contributed to different results for studies. For example, the collection of blood samples before or after colonoscopy may have affected results. MiRNA levels normalised to different internal controls could also contribute to disparities in results. The differences in results may also be due to differences in subject characteristics, including age, gender and ethnicity.

One group has reported that most of the reported circulating miRNA biomarkers for cancer are highly expressed in blood cells and therefore, haemolysis and variations in blood cell counts can significantly alter miRNA levels in plasma[37]. Thus, altered circulating miRNA levels discovered in cancer studies reported to have been associated with cancer may in

fact reflect blood cell effects, instead of cancer tissue specific origin[37]. This may suggest that miRNAs that are not expressed in blood cells should be investigated if attempting to identify circulating miRNAs biomarkers that are very specific for cancer[37].

PROSPECTS FOR MODULATING THE MICRORNAS IMPLICATED IN THESE STUDIES

While early detection of CRC is desirable for enabling early surgical or pharmacologic intervention, there is also considerable interest in the question as to whether diet or other lifestyle changes could affect the relevant microRNAs. A summary of some relevant dietary factors is provided in Table 2, and discussed below.

Tarallo *et al*[38] studied a panel of seven human microRNAs in plasma and stool samples from 24 healthy individuals with differing dietary habits. Eight of these were vegans, eight vegetarians and eight on an omnivorous diet, and the groups had similar age and sex distributions. They found that miR-92a was differentially expressed in both plasma and stool samples, and was very significantly affected by diet. This MicroRNA was also associated with low body mass index. Although miR-16, miR-21, mir-34a and miR-222 showed associations with diet and lifestyle factors, the data were not consistent between stool and plasma.

Davidson *et al*[39] used a hypothesis-drive approach to study dietary modulation of microRNA expression. Specifically, they considered the effects of a long chain omega-3 polyunsaturated fatty acid (lc n-3 PUFA)-enhanced diet on the development of carcinogen- induced CRC in a Sprague-Dawley rat model. The animals were fed diets containing corn oil or fish oil, and injected with the colon-specific carcinogen, azoxymethane, or saline as negative control. They quantified the effects of the diets on expression of 368 different microRNAs in the colonic mucosa. It appeared that let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were the most strongly significantly affected by diet X carcinogen interactions.

The fish oil-fed animals showed the smallest number of differentially expressed miRNAs between carcinogen and control treatments, probably because the fish oil was protecting against carcinogen-induced inflammation. Using a mouse transplantable tumour model, Tsoukas *et al*[40] also related protection against tumour growth and progression associated with microRNA dysregulation, to levels of lc n-3 PUFAs. These nutrients have also been shown to have a beneficial effect on the modulation of MiR-21 expression in breast cancer cells[41]. Dietary lipid intake will also modulate serum lipoproteins, and there is evidence that miRNAs are transported to target cells *via* binding to lipoproteins such as LDL and HDL cholesterol[42].

MiR-155 is carried mainly by HDL. Although not one of the main miRNAs specifically identified in CRC, it is well characterized as an inflammatory regulator[43]. Kim *et al*[43] studied the levels of miR-155 among HDL isolated from smokers and non-smokers, following eight weeks daily consumption of high dose Vitamin C. They reported several potentially beneficial effects in MiR-155 expression and consequent inflammation through an improvement of lipoprotein parameters.

Singh *et al*[44] studied Vitamin D regulation of miRNA across different cancer cell models, representing non-malignant and malignant cells. They exposed the cells to 30 min of treatment with vitamin D3 (1,25-dihydroxyvitamin D3) or 1,25(OH)2D3. Across all cells, 111 miRNAs were significantly modulated by the vitamin treatment, but only 5 miRNAs were modulated in more than one cell model, and of these only 3 miRNAs were modulated in the same direction. An integrative network-based analysis used a publicly available data set to study the role of 1,25(OH)2D3 in cancer cells on levels of microRNAs[45]. Pathway analysis revealed 15 significantly altered pathways, including eight somewhat general, mostly cell cycle-related pathways, and seven cancer-specific pathways. The authors identified a new vitamin D-microRNA network, including six differentially expressed microRNAs (MiR-29a, MiR-371-5p, MiR-1915, MiR-663, MiR-134 and MiR-542-5p). All six were up-regulated in 1,25(OH)2D3-treated cancer cells in the studies by Kutmon *et al*[45].

MiR-155 is a key regulator of Toll-like receptor (TLR) signaling that plays a pivotal role in immune response and through this, may also play a role in CRC. Li *et al*[46] used a rodent model to show that 1,25(OH)2D3 modulates innate immune response by targeting the miR-155-SOCS1 axis. Jorde *et al*[47] supplemented human volunteers with high doses of 1,25(OH)2D3 for 12 mo. They found significant changes with miR-532-3p and miR-221 from baseline to 12 mo that was significantly different between the vitamin D and placebo group. In colon human cancer cell models, MiR-22 and several other miRNA species have been identified as 1,25(OH)2D3 targets[48].

Vitamin E has also been shown to play a role in miRNA regulation, at least in rat liver[49]. These authors fed rats for 6 mo with vitamin E-sufficient or deficient diets, after which they estimated the hepatic concentrations of miRNAs that had been previously associated with this vitamin (miRNA-122a in lipid metabolism and miRNA-125b in cancer and inflammation). Concentrations of both these miRNAs were decreased in conditions of vitamin E deficiency.

Minerals may also affect miRNA expression and activity. In particular, Selenium (Se) showed effects on the expression of a number of genes, especially

Table 2 Summary of dietary regulation of microRNAs, potentially relevant to colorectal cancer

MicroRNA	Study population	Diet or nutrient	Analysis method	Findings	Specimen type	Ref.
miR-16 miR-21 miR-34a miR-92a miR-106a miR-146 miR-222	Italian-based 8 vegans 8 vegetarians 8 omnivores	Meat, processed meat, fish, cheese	Food frequency and lifestyle questionnaire	miR-92a was significantly decreased by meat and dairy products, and associated with low body mass index. Weaker associations found between miR-21 levels and vegetable intake	Plasma and stool	Tarallo *et al*[38]
Let-7d miR-15b miR-107 miR-191 miR-324-5	Sprague-Dawley rats, treated with saline or the carcinogen, azoxymethane	Corn oil *vs* fish oil in the diet	Effects of diets on the expression of 368 miRNAs in the colonic mucosa	The five identified miRNAs were the most strongly affected by diet X carcinogen actions. The fish fed animals showed the smallest number of differentially expressed miRNAs - interpreted as due to a reduction in inflammation	Colonic mucosa	Davidson *et al*[39]
miR-1903 miR-467c miR-368 miR-927c	Female athymic nude mice, injected with HT-29 colon cancer cells	Corn oil *vs* ground walnuts in the diet	Effects of the diets on the expression of four microRNAs in the colon tumours	The first three of these microRNAs were down-regulated and the latter up-regulated in expression. These data were related to significant increases in α-linolenic, eicosapentaenoic, docosahexaenoic and total omega-3 acids, and a decrease in arachidonic acid in the walnut fed mice	Colorectal tumour tissue	Tsoukas *et al*[40]
miR-155	Young subjects (22 + 2 yr), smokers and non-smokers	High dose vitamin C daily for 8 wk	Expression level of miR-155 in HDL3	miR-155 reduced in HDL fraction by 49% in non-smokers and 75% in smokers after 8 wk supplementation. This effect was related to a reduction in reactive oxygen species	Serum lipoprotein levels	Kim *et al*[43]
miR-98 miR-92a miR-30e miR-140-5p miR-138	7 different prostate cell models including malignant and non-malignant	30 min treatment with 1a,25(OH)2D3	MiRNA microarray analyses	111 miRNAs showed changed expression levels, but only 5 were seen affected in more than one cell line and only 3 were changed in the same direction	Total mRNA and miRNA from each cell line.	Singh *et al*[44]
miR-22 miR-29ab miR-134 miR-1207-5p miR-371-5p miR-17 miR-20a	LNCaP human prostate cancer cells	48 h treatment with 100 nmol/L 1,25(OH)2D3 compared with non-treated control, cells	Agilent human microRNA v3 microarrays to measure microRNA expression	Four hundred and twenty genes were up-regulated and 413 genes down-regulated in the 1,25(OH)2D3-treated cells. The most strongly affected are those identified in column 1 (the last two of these miRNAs is downregulated)	Integrative network-based analysis using a publicly available data set	Kutmon *et al*[45]
miR-155	RAW264.7 macrophage cells stimulated with lipopolysaccharide (LPS)	24 h in the presence of EtOH or 20 nmol/L 1,25(OH)2D3	miRNA profiling by microarrays	Several miRNAs were induced by LPS and suppressed by 1,25(OH)2D3, of which miR-155 was on the top of the list, suppressing about 50% of the LPS induction	Total mRNA and miRNA from each cell line	Li *et al*[46]
miR-22	SW480-ADH and HCT116 colon cancer cells	10^{-7} mol/L 1,25(OH)2D3 for 24, 48 or 96 h	miRNA profiling by microarrays	Although there were 12 microRNAs that showed differential expression with and without vitamin D, miR-22 showed the most consistent differences	Total miRNA from each cell line	Alvarez-Díaz *et al*[48]
Let-7f Let-7a miR-151-5p miR-22 miR-221 miR-28-5p miR-552-3p miR-766 miR-99b	Males, generally in good health, with no diabetes or other concomitant diseases	High dose vitamin D3 (20000-40000 IU per week)	Quantitative real-time PCR	In 10 pilot subjects, 136 miRNAs were changed in expression in one or more plasma samples drawn at baseline and after 12 mo of vitamin D supplementation. The twelve miRNAs that showed the greatest change in expression in the pilots were further measured in RNA from baseline and 12 mo plasma samples in 40 subjects given vitamin D and 37 subjects given placebo	Plasma	Jorde *et al*[47]
miR-122a miR-125b	Fischer 344 rats	0, 12 or 24 mg/kg	Quantitative real-time PCR	Vitamin E sufficiency resulted in increased concentrations of miRNA-122a and miRNA-125b	Liver tissue	Gaedicke *et al*[49]

miR-625 miR-492 miR-373 miR-22, miR-532-5p miR-106b miR-30b miR-185 miR-203 miR1308 miR-28-5p miR-10b	CaCO2 human colon cancer cells	Selenium-deficient or sufficient medium	Microarray validated with quantitative real-time PCR	Selenium deficiency resulted in altered expression of 12 genes	Total miRNA from combined cells of each treatment	Maciel-Dominguez *et al*[50]
miR-21	U251 human glioblastoma cells	10, 50 or 100 umol/L Resveratrol for 72 h	Quantitative real-time PCR	Resveratrol inhibited miR-21 expression which in turn suppressed NF-κB activity. However, over-expression of miR-21 could reverse the effect of resveratrol on NF-κB activity and apoptosis	Cell extracts	Li *et al*[54]
miR-21	Estrogen-dependent MCF-7 and estrogen receptor-negative p53 mutant MDA-MB-468 human breast cancer cells	0, 30 or 60 umol/L	Quantitative real-time PCR	Cells were studied either in tissue culture or as a xenograft in BALB/C female athymic mice miR-21 was up-regulated in DIM-treated MCF-7 cells, but not in the ER negative, p53 mutant MDA-MB-468 cells	Cell extracts	Jin[55]
miR-30b miR-1224-3p miR-197 miR-523-3	HepG2 human hepatocarcinoma cells	3-3′-Diindoyl-methane for 24-96 h 50 mg/L of epigallocatechin gallate (EGCG), 100 mg/L of grape seed extract (GSPE) or 100 mg/L of cocoa proanthocyanidin extract (CPE)	Microarray analysis validated by quantitative real-time PCR	MiR-30b was downregulated by all three treatments, while treatment with GSPE or CPE upregulated miR-1224-3p, miR-197 and miR-532-3p	Cell extracts	Arola-Arnal *et al*[57]
miR-210 (plus 13 other miRNAs upregulated and 7 down-regulated)	Tobacco carcinogen-induced lung cancer in A/J mice	Purified mouse chow containing 0.4% EGCG	Microarray analysis validated by quantitative real-time PCR	MiR-210 had been previously found upregulated by EGCG in *in vitro* experiments, but this ranked behind the 13 most strongly upregulated miRNAs (miR-2137, miR-449a, miR-144, miR-486, miR-3107, miR-193, miR-5130, miR-2861, miR-511-3p, miR-763, miR-3473, miR-211, miR-210) or seven most down regulated in this *in vivo* study	Tumour tissue, all tumours from a single mouse combined to a single sample	Zhou *et al*[58]

glutathione peroxidase 2 and selenophosphate synthetase 2, through altering the profile of miRNAs in an intestinal cell line[50]. Following exposure of CaCO2 cells to Se-deficient medium for 72 h, there were alterations in the levels of twelve miRNA: miR-10b, miR-22, miR-28-5p, miR-30b, miR-106b, miR-185, miR-203, miR-373, miR-492, miR-532-5p, miR-625 and miR-1308. In particular, silencing of miR-185 increased GPX2 and SEPSH2 expression.

A range of polyphenols have been found to interact with and be affected by miRNAs. Of particular interest to CRC may be the regulatory effects of curcumin on miR-21[51]. MiR-21 has been found to mediate a range of effects of curcumin on cancer cells, including cell proliferation, cellular senescence or apoptosis, metastasis and anti-cancer drug resistance. MiR-21 was found to suppress the anticancer activities of curcumin by targeting the PTEN gene in human non-small cell lung cancer A549 cells[52]. In turn, curcumin has been shown to decrease the levels of miR-21 through increasing miR-21 exosome exclusion from cancer cells, and also through inhibiting the transcription of the miR-21 gene by binding to its promoter. At a 20-40 mmol dose, curcumin treatment led to a significant reduction of microRNA-21 expression, as compared to that in untreated cells.

Resveratrol (trans-3,4′,5-trihydroxystilbene) has been shown to induce the expression of miR-663, a tumor-suppressor and anti-inflammatory microRNA, while downregulating miR-155 and miR-21[53]. These authors suggest that the use of resveratrol in therapeutics may be optimised by considering the effects of the selected dose on the expression of miR-155 or miR-21. The authors also suggested that the activity of resveratrol might be enhanced by finding ways to manipulate the levels of its key target microRNAs, such as miR-663. More generally, resveratrol is known to play an important role in inhibiting proliferation and inducing apoptosis of cancer cells[54]. These authors found a dose-dependent decrease in cancer cell viability following resveratrol

treatment. As well as effects on cellular signalling pathways, resveratrol inhibited miR-21 expression, which in turn could suppress nuclear factor-kappaB activity. Conversely, over-expression of miR-21 was found to inhibit the beneficial antitumour effects of resveratrol.

3,3'-Diindolylmethane (DIM) is a cancer-preventive phytochemical that is found in Brassica vegetables. At least in a human breast cancer cell line, DIM was shown to inhibit cell growth through a miR-21-mediated mechanism[55]. These effects were related to differential modulation of cellular signalling pathways that led to arrested cell-cycle progression of the human cancer cells.

Proanthocyanidins are highly abundant and found in a range of food plants including cinnamon, cocoa beans, grape seed, grape skin and various berries. They have been found to have positive health effects on a variety of metabolic disorders associated with inflammation, largely through their effects on genomic stability[56]. Arola-Arnal and Blade studied the effects of proanthocyanidin-rich natural extracts in modulating miRNA expression[57]. They used microarray analysis and Q- PCR, to study miRNA expression in colonic HepG2 cells treated with a grape seed proanthocyanidin extract (GSPE), cocoa proanthocyanidin extract (CPE) or the green tea polyphenol epigallocatechin gallate (EGCG). They found that miR-30b was downregulated by all three treatments, while treatment with GSPE or CPE upregulated miR-1224-3p, miR-197 and miR-532-3p.

In other models, Zhou *et al*[58] found that EGCG upregulated the expression of miRNAs such as miR-210, thereby reducing cell proliferation. They used functional genomic approaches to study the role of miRNA in EGCG inhibition of carcinogen-induced mouse tumors. They identified changes in the expression levels of 21 microRNAs for which they found 26 potential targeted genes relating to cancer inhibition.

CONCLUSION

The available studies summarised herewith begin in 2010, at a time when miRNA methodologies were becoming increasingly more sensitive. While not all hypothesised miRNAs have proved as useful as initially hoped, it would seem that either individual miRNAs or a panel of these would have very significant prognostic value and enable an intensive lifestyle intervention to prevent what would otherwise be the natural course of disease. The identification of potential biomarkers that reliably detect or diagnose early stages of CRC or evidence of CRC progression is urgently needed.

An increasing body of evidence suggests that epigenetic changes contribute to carcinogenesis, and miRNAs are prominent among these. There is no question but that deregulation of miRNAs plays an important role in human carcinogenesis. Overall, miRNAs appear to be a promising class of biomarkers for CRC. However, further research is needed to validate previous findings and increase our current understanding of the identified miRNAs. The applicability of some studies in other populations is uncertain as many subjects were recruited from a single ethnic group. Thus, future studies could confirm the utility of miRNA for other ethnic groups by recruiting subjects from multiple centres and various populations.

Many of the studies had small sample sizes and this may have contributed to the contradictory findings of some of the reports. Hence, stronger studies in the future would do well to increase their sample sizes to determine whether certain miRNA levels are consistently modified. As well as determining that specific miRNA levels are modified in patients with AA, it would also be useful to determine how specific these changes are for colorectal neoplasia. Future studies may also consider whether the asymptomatic or symptomatic status of subjects causes differences in miRNA expression levels. Analyses of miRNA expression should be separated for asymptomatic and symptomatic individuals to examine any differences which may exist. For most of the miRNA studies so far, controls consist of individuals who were determined to be free from colorectal neoplasia following a colonoscopy. However, it is known that miRNA are dysregulated in different cancers and other diseases. Hence, to investigate the specificity of miRNA for colorectal neoplasia, groups should include control individuals with different diseases including conditions affecting various organs to confirm the specificity of individual miRNA transcripts. On another note on controls, it is important to obtain comprehensive information on the health of potential controls to confirm those in the "healthy" control groups are indeed healthy to the best available knowledge. Another way to confirm the specificity of miRNA for colorectal neoplasia is to analyse the relationship between miRNA expression in neoplastic tissue and miRNA expression from blood and stool samples. If there is concordant expression between the two, it makes it more likely that the miRNA was secreted from the colorectal neoplastic lesion. This would be an important finding as this would increase the specificity of this miRNA for colorectal neoplasia[20]. Another issue for circulating miRNA research is the lack of consensus on precise and robust internal controls[27,31]. Luo *et al*[27] chose miR-16 as an internal control in their plasma-based miRNA tests. There is also room for improvement in the assay technologies used to measure miRNAs. qRT-PCR cannot precisely quantify many miRNAs

in plasma which are present in too low levels so some miRNAs cannot be included in studies or were included but their performance may not be accurately reflected in results[27,28]. Other methods to detect and quantify low-level miRNAs with higher sensitivity would be desirable in future investigations. MiRNA with initial promising results should be further studied in larger study populations to verify reproducibility. To validate miRNA further, the performance of selected miRNA should be compared to the performance of current accepted screening tools, such as fecal occult blood tests. All promising miRNA should undergo testing in large prospective trials if they are to be accepted as a screening tool in routine clinical practice.

A biomarker will be useful for screening or the early detection of cancer only if it can be detected in a non-invasive or minimally invasive fashion without tissue biopsy. Increasing evidence has indicated that miRNAs in serum, stools or other body fluids may become important biomarkers for the detection of early CRC. It is hoped that such miRNA markers will be translated into clinical use in the near future, enabling early diagnosis of CRC development and an accurate assessment of disease progression. Such advances would allow patients to receive early treatment and ultimately improve survival.

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