The Diagnostic and Prognostic Role of microRNA in Colorectal Cancer – a Comprehensive review

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Abstract

The discovery of microRNA, a group of regulatory short RNA fragments, has added a new dimension to the diagnosis and management of neoplastic diseases. Differential expression of microRNA in a unique pattern in a wide range of tumor types enables researches to develop a microRNA-based assay for source identification of metastatic disease of unknown origin. This is just one example of many microRNA-based cancer diagnostic and prognostic assays in various phases of clinical research.

Since colorectal cancer (CRC) is a phenotypic expression of multiple molecular pathways including chromosomal instability (CIN), micro-satellite instability (MIS) and CpG islands promoter hypermethylation (CIMP), there is no one-unique pattern of microRNA expression expected in this disease and indeed, there are multiple reports published, describing different patterns of microRNA expression in CRC.

The scope of this manuscript is to provide a comprehensive review of the scientific literature describing the dysregulation of and the potential role for microRNA in the management of CRC. A Pubmed search was conducted using the following MeSH terms, "microRNA" and "colorectal cancer". Of the 493 publications screened, there were 57 papers describing dysregulation of microRNA in CRC.

Key words: microRNA, colorectal cancer

Introduction

Colorectal cancer (CRC) is the second most common cancer in females and the third in males with 1.2 million annual new cases worldwide.¹ The incidence and mortality caused by CRC have been slowly decreasing in the United States. Over 143,000 new cases of CRC are diagnosed annually and approximately 52,000 Americans die of the disease every year. These deaths account for approximately 9% of all
cancer mortality.1

The lifetime incidence of CRC in the average-risk population living in North America and Western Europe is 5%. The vast majority of cases (90%) occur after the age of 50.1 Due to this age-associated risk, current screening guidelines recommend routine testing after the age of 50. Patients with risk factors require age adjustment. The strongest risk factor is presence of hereditary CRC syndromes such as familial adenomatous polyposis (FAP) or its variants, MYH-associated polyposis, Lynch syndrome (hereditary non-polyposis colon cancer - HNPCC), BRCA2, juvenile polyposis, or any personal or family history of sporadic CRC or adenomatous polyps. Other risk factors that currently influence screening recommendations include presence of a long standing inflammatory bowel disease and history of abdominal radiation. Screening colonoscopy is recommended by the age of 45 in African Americans however, current screening recommendations do not stratify individuals by gender.2,4

Despite the increased awareness with improved screening recommendations and techniques, CRC remains the second leading cause of cancer-related death of both men and women. Early detection was shown to have an important role in reducing CRC-related mortality rate. When detected at stage I or II, five year survival rates are 90% and 75%, respectively. Nevertheless, diagnosis is often delayed until patients become symptomatic with more advanced lesions and substantially increased associated mortality. Accordingly, the 5 year survival rates for stage III and IV are 65% and 5%, respectively.5

Pathogenesis of colorectal cancer

In most CRC patients, the progression of normal colonic mucosa to invasive cancer requires several molecular changes. The estimated time interval of the malignant transformation from normal mucosa through adenomatous polyp into an invasive carcinoma is 5-10 years in most cases.6 This long time-interval provides the ground for early detection and even prevention of CRC as was shown by the National Polyp Study. In this study, 1,418 patients were followed after all identifiable polyps were removed by colonoscopy. Within this follow-up period, only five subsequent malignancies were discovered and there were no deaths attributed to CRC. When compared to three other control groups, the study group had a decreased incidence of CRCs. The authors concluded that CRC arises mostly from adenomas, and colonoscopy-based screening may reduce both the incidence and the stage at diagnosis of CRC compared to non-screened populations.7

Screening for colorectal cancer

Fecal Occult Blood Testing (FOBT)

Guaiac-based fecal occult blood testing using rehydrated or nonrehydrated stool specimens in people aged 50 to 80 decreases mortality from colorectal cancer. In 1993 and 1996, 3 clinical trials provided evidence that FOBT screening reduces CRC-related mortality. A reduction of 33% in mortality was demonstrated among subjects who had annual rehydrated FOBT testing in a US clinical trial9, and approximately 15% mortality-rate reduction was demonstrated in two European studies using a bi-annual nonrehydrated FOBT testing protocol.1,9,10 The fact that occult gastrointestinal bleeding is more common in benign conditions and the fact that bleeding is not universal in adenomas and early cancers, results in relatively low sensitivity and specificity of FOBT for CRC detection. In recent years, there are multiple reports of improved sensitivity and specificity of immuno-based FOBT assays.11 A large clinical trial is currently conducted in Spain with the main aim of comparing Ig-FOBT to colonoscopy-based screening.12

Endoscopic procedures

Flexible sigmoidoscopy

The role of flexible sigmoidoscopy for CRC screening is controversial. The 60 cm sigmoidoscope can reach the splenic flexure and potentially evaluate one-half of the colon. The benefit of flexible sigmoidoscopy is that it is cheaper and safer compared to colonoscopy and a full bowel preparation is not necessary. In a case-controlled study, CRC related mortality reduction was shown with screening by flexible sigmoidoscopy. However, this benefit was limited to the portion of colon examined.13 It is important to determine what percentage of malignancies is proximal to the splenic flexure and would, therefore, be missed by sigmoidoscopy alone. Imperiale et al. showed that, a strategy based on flexible sigmoidoscopy followed by full colonoscopy in cases where adenomatous polyp was found in the range of the sigmoidoscope, would detect additional invasive cancers and increase the detection rate to 70-80% of all cases of invasive colon cancer.14

Colonoscopy

Since colonoscopy has been shown to prevent CRC by the removal of large polyps prior to their conversion to invasive cancer, in recent years, colonoscopy has become the preferred choice for CRC screening. Additionally, colonoscopy can identify and biopsy most cancers during the initial procedure.
However, procedural competence varies among examiners and the cecum is reached in 80–98% of procedures, with the depth of penetration depending mainly on the experience of the endoscopist and the adequacy of bowel preparation. To date, there have been no studies examining the effectiveness of colonoscopy as a screening modality for CRC with reduction in CRC-related mortality as a study endpoint. Additionally, there are no controlled trials that address the question of how frequent colonoscopy should be performed. The National Polyp Study demonstrated that the removal of polyps reduces the incidence of CRC. Additionally, CRC mortality is lowered through the detection of early cancers and precancerous lesions. The limitations of colonoscopy remain its complication rate as well as compliance. The incidence of complications during colonoscopy is: 1 in 1,000 patients suffers perforation; 3 in 1,000 suffer major hemorrhage; and 1–3 in 10,000 die as a result of the procedure. The data on compliance with screening colonoscopy is even more distressing. When physicians, nurses, and spouses of the nurses were invited by letter to undergo a free screening colonoscopy, less than 15% complied. Colonoscopy has an important role in CRC screening, since it was an integral part of the FOBT screening trials that demonstrated a significant reduction in mortality in screened patients.

Imaging modalities for CRC screening

Barium Enema

Double contrast barium enema (DCBE), was the primary technique for the detection of polyps before colonoscopy became available. Despite the fact that it is the safest of the structural screening tests for CRC and the risk of serious complications is 1 in 10,000, it is hardly used anymore. The sensitivity of DCBE is variable and ranges between 70–90% for polyps >1 cm but only 50–80% for polyps <1 cm in diameter. The lack of sensitivity has been attributed to inadequate visualization of parts of the bowel as well as to errors in interpretation. DCBE has been considered an inaccurate examination for the rectum and sigmoid colon. No prospective randomized trials of DCBE screening have been completed, and most published studies of DCBE relate to asymptomatic rather than asymptomatic patients. A randomized trial of DCBE plus flexible sigmoidoscopy versus colonoscopy in 383 symptomatic patients with gastrointestinal bleeding, suspected to be from the colon, found that colonoscopy detected more cases of polyps <9 mm than DCBE plus sigmoidoscopy. There was no difference between strategies in the number of patients detected with cancer or polyps >9 mm. In summary, this method is currently used only in very selective cases and was replaced by colonoscopy or CT colonography.

Computed Tomography (CT) colonography (Virtual Colonoscopy)

In recent years, the utilization of virtual colonoscopy or CT colonography (CT-Co), is rapidly rising. Here the colon is prepared in the same manner as it is prepared for colonoscopy, distended with air inflated through the anus, and studied with the help of a computerized tomography (CT). In recent studies, CT-Co's sensitivity for the detection of large polyps ranged from 50 to 91%. The theoretical advantage to CT-Co is that it is rapid and completely visualizes the colon with a negligible risk. No sedation is required and the patient’s acceptance is better than fibro-optic colonoscopy or barium enema. CT-Co can image the right colon and cecum in cases where fibro-optic colonoscopy was not completed, either due to technical difficulties or in a more common case scenario, because of a nearly obstructing lesion. Finally, it may also provide additional information by imaging the abdominal structures outside the colon. Its most significant limitation is that the colon should be thoroughly clean and that polypectomy is not possible with virtual colonoscopy. The size of polyp is also important, since the sensitivity of CT-Co to detect lesions smaller than 10mm is limited. There is also a concern regarding a flat adenoma, which cannot be detected by this modality.

Fecal DNA and RNA screening

Approximately one-sixth to one-third of normal adult colonic epithelial cells (colonocytes) are shed daily from the lower two-thirds of colon crypts, this corresponds to the daily exfoliation of approximately 10^{10} cells, each having a lifespan of 3–4 days.

Screening the feces for specific cancer-related DNA or RNA is appealing. However, technical complexity as well as the lack of specific CRC molecular markers prevented the development of a robust, commercially available, fecal DNA screening kit.

Since CRC is a common phenotype of several molecular diseases. Understanding the molecular genetics of colorectal cancer is essential for the analysis of fecal DNA or RNA. The vast majority of colorectal cancers result from chromosomal instability (CIN), with mutations progressively accumulating in the adenomatous polyposis coli (APC) gene, the p53 tumor-suppressor gene, and the K-ras oncogene. The second most common pathway (6%-25% of CRC) leads to a loss of genes involved in DNA-mismatch
repair, manifested by microsatellite instability (MSI).24

Other molecular pathways leading to CRC are the CpG islands promoter hypermethylation (CIMP) including several different molecular pathways characterized by CIMP.25-30 Colorectal cancer may also be detectable through the use of DNA markers associated with disordered apoptosis.31 Several studies using fecal-based DNA testing have reported a sensitivity of 62-91% for cancer and 27-82% for advanced adenomas, with a specificity of 93-96% in persons with normal findings on colonoscopy.32-36 However, those studies were conducted in patients with advanced, symptomatic lesions. In a large-scale study comparing fecal DNA to FOBT, 5486 patients were screened.36 The fecal DNA panel detected 16 of 31 invasive cancers, whereas FOBT identified 4 of 31 (51.6% vs. 12.9%, P≤0.003). The DNA panel detected 29 of 71 invasive cancers, and adenomas with high-grade dysplasia, whereas FOBT identified 10 of 71 (40.8% vs. 14.1%, P<0.001). Specificity in subjects with negative findings on colonoscopy was 94.4% for the fecal DNA panel and 95.2% for the FOBT.36

Another molecular screening strategy is to screen for fecal RNA and amplify cancer specific molecular markers using RT-PCR. Ahmed et al. described a method that permits extraction of intact non-degraded total RNA from human colonocytes in stool by utilizing commercially available kits.37 They showed that RNA can be adequately reverse transcribed, making high-quality copy cDNA. This was followed by PCR using colon cancer-specific gene primers (guanylyl cyclase C and PYPAF5 genes).37

Serum (blood) test for the detection of colon cancer

Currently, there is no commercially available FDA-approved blood test for CRC diagnosis. Literature search identified 93 studies evaluating an overall of 70 different blood markers for CRC, including CEA and carbohydrate antigens, as well as newly introduced ones, including mainly proteins identified through mass spectrometry analysis. The majority of studies evaluated protein markers, but in recent years, there is increasing interest in genetic and epigenetic (mRNA, DNA, microRNA) markers.38-43

Overall, a broad range of sensitivity and specificity was reported for the various markers. A direct comparison of results from different studies is complicated due to the diverse populations used and use of different cutoff points for the same marker. Furthermore, the majority of markers were evaluated in a single study. In addition, many studies had small sample size. Only in very few studies overall sample size was above 300 subjects. Another concern refers to comparability of results across studies given potential differences in serum collection, processing and storage methods, and uncertainties in the stability of several biomarkers. Other concerns are the lack of patients with adenomatous polyps in most of these studies, and the fact that control patients in most studies did not undergo mandatory colonoscopy ruling out the presence of adenoma or carcinoma.

The current stage of evidence calls for prospectively planned and systematic evaluations of both the most promising blood tests and the most promising stool tests in a well-defined, large-scale screening population, in which special attention is drawn to standardized sample collection, processing, and storage.

microRNA

microRNAs (miRs) are a class of endogenous small, noncoding, RNA fragments (18-24 nucleotides in length) involved in multiple intracellular processes by regulating gene expression. Since the first miR discovery in the Caenorhabditis Elegans worm in 1993, numerous different miRs were identified in plants, animals, and humans.44 At present, 2042 mature human miRs are listed in the updated database (http://www.mirbase.org). Approximately half of all human miR genes are contained within the introns of protein-coding genes, whereas others reside apart from known genes or in the exons of untranslated genes.45,46

miRs biosynthesis begins with long double-stranded primary transcripts converted to a 70 nucleotide precursor. As many as 1600 human miR precursors are documented. miR precursors are cleaved in the cytoplasm in the short 22 nucleotide double stranded miR. The double strand is then unwound to produce single stranded active miRs. Mature miRs incorporate into the RNA-induced silencing complex (RISC) and are able to bind to the 3-untranslated region (UTR) of the target gene. This miR binding may cause either block of translation or mRNA degradation depending on the level of complementarity with the target DNA sequence.47 It is thus that the effect of miRs on their target genes is based on the degree of homology between the sequences of the miR and the target gene. The homology that dictates the specificity of miRs is dependent on 6-7 nucleotides that bind to the 3-UTR of their target mRNAs. As a result a single gene can be targeted by many different miRs and a single miR can potentially affect hundreds of genes.48

The homology diversity places the miRs in a perfect position to regulate multiple different intracellular processes. It is predicted that up to 30% of
protein-coding genes are controlled by miRs. It was also shown that miRs are involved in cellular development, differentiation, proliferation, and apoptosis. As a result, miR expression level may affect tumor pathogenesis and behavior. Low expression of a specific miR may lead to upregulation of oncogenes and in contrast, high expression of other miRs may lead to downregulation of tumor-suppressor genes. In a similar manner, dysregulation of other miRs may have the opposite effect.

In the last decade, a rapidly increasing number of studies have identified miR-dysregulation in multiple malignancies. Since the first description of miR-15a and -16-1 in B-cell chronic lymphocytic leukemia other malignancies followed. To date, numerous miR dysregulations have been described in glioblastoma, breast cancer, acute myeloid leukemia, melanoma, pancreatic cancer, lung cancer, ovarian cancer, hepato-cellular carcinoma, thyroid cancer, bladder and kidney cancer, gastric cancer, colorectal cancer, and others.

While the exact mechanism and target gene for every miR are yet to be elucidated, many oncologic aspects are currently being studied. The first step is to document the presence of miR-dysregulation in tumor versus control non-malignant tissues. Commercial kits are now available for RNA extraction and miR profile analysis. The mere documentation of miR-dysregulation in a specific malignancy does not shed light on the suspected target gene (or genes) nor on the specific mechanism of contribution to malignancy development. Lastly, targeting specific miRs may prove to have therapeutic value.

Due to their small size, miR levels are remarkably stable in tissue samples, serum, plasma, and even stool. Turchinovich et al. showed that extracellular miR remains stable for at least one month. This stability may contribute to the value of specific miRs as biomarkers for malignancy. The focus of this review is the dysregulation of miR in CRC. The role of miRs as potential biomarkers for CRC will be investigated through studies that show miR dysregulation in the plasma and stool of patients with CRC. Furthermore, the prognostic role of miRs in CRC will be critically evaluated.

**miR-dysregulation in tumor samples**

The first study of miR expression in colorectal tumor tissue compared to normal colonic tissue was reported in 2003 by Michael et al. In this study, miR-200c was first isolated in normal colonic tissue and miR-143 and -145 were found to be downregulated in tumor tissue compared to normal colon tissue. Since then, many studies have compared the dysregulation of different miRs in CRC samples in comparison to normal colonic mucosa. We have evaluated 46 different studies. The studies differ dramatically in the number of miRs evaluated (Range 1-723 miRs), number of samples used (4-197 samples), and number of dysregulated-miRs found (1-71). The fold dysregulation for each specific miR also differed between the studies. Table 1 summarizes the studies that were evaluated and the miR-dysregulation that was found.

Overall, 170 different miRs were found to be upregulated in CRC. Upregulation of miR-21 was demonstrated in 15 studies followed by miR-31, miR-135b, and miR-183 (upregulated in 11, 9, and 8 studies, respectively). Other 110 different miRs were found to be upregulated in only one study each (Table 1). A total of 127 different miRs were found to be downregulated in CRC. Downregulation of miR-145 was demonstrated in 15 studies, followed by miR-143, downregulated in 9 studies, followed by miR-1, miR-195, and miR-378 downregulated in six studies each.

**miR-dysregulation in serum or plasma samples**

The first report of miRs detected in the serum of colorectal patients was by Chen et al in 2008. Although the miRs detected are not specified, there were 69 miRs detected in sera of CRC patients but not in sera of normal controls. Interestingly, the authors note that a large number of miRs were commonly detected in both sera obtained from colorectal and lung cancer patients. The only specific miRs mentioned are miR-23a, miR-134, miR-146a, miR-221, and miR-222. Since then, 13 reports were published of 33 miRs dysregulated in the plasma of CRC patients. In several studies, miR upregulation was found to be both in plasma and tumor tissue of CRC patients.

Out of 95 miRs analyzed, Ng et al identified five miRs (miR-17-3p, -92, -95, -135b, -222) that were upregulated in both plasma and tissue CRC samples. Furthermore, miR-17-3p and miR-92 plasma levels significantly decreased following surgery for CRC, indicating these specific miRs as potential markers for response to therapy. In 2010, Huang et al. identified that out of 12 miRs studied, only miR-29a and -92a were upregulated in plasma obtained from CRC patients. It was further demonstrated that these two miRs were also upregulated in patients with advanced adenomas, indicating a possible role in CRC diagnosis. Out of the three miRs reported as upregulated in CRC most often (miR-21, miR-221, and miR-222) only miR-221 levels were upregulated to a degree that could serve as biomarker. In the same study, conducted by Pu et al, the high levels of
miR-221 also correlated with prognosis as well as with p53 expression. Wang et al confirmed the upregulation of miR-21 in CRC serum samples and further demonstrated its upregulation in breast, esophageal, gastric, and lung cancer.113 Cheng et al identified that plasma upregulation of miR-141 in the plasma of CRC patients, was associated with metastatic disease, and correlated with CEA levels and poor prognosis.114 In a study that analyzed 380 miRs, Kanaan et al identified only miR-21 as a possible serum marker accurately differentiating between CRC patients and healthy individuals, with 90% sensitivity and high specificity.76 Most recently, Wang et al identified a panel of 22 miRs (miR-10a, miR-19a, miR-22*, miR-24, miR-92a, miR-125a-5p, miR-141, miR-150, miR-188-3p, miR-192, miR-210, miR-221, miR-224*, miR-376a, miR-425*, miR-495, miR-572, miR-601, miR-720, miR-760, miR-let-7a, and -let-7e) that were dysregulated in CRC plasma samples with a fold changes greater than five.115 After validation of this panel in a cohort of 191 CRC patients, it was noted that miR-601 and miR-760 were significantly downregulated in CRC plasma samples and could serve as markers accurately differentiating between plasma samples of CRC patients and of healthy controls, as well as between the plasma of patients with advanced adenomas and the plasma of normal controls (AOC of 0.792 and 0.683, respectively).115 All the data of miR-dysregulation in plasma or serum of CRC patients are summarized in Table 2.

miR-dysregulation in stool samples

The first study reporting miR-dysregulation in stool samples of patients with CRC was conducted by Ahmed et al in 2009.116 Fourteen miRs were dysregulated, of which, seven miRs were upregulated (miR-20a, miR-21, miR-92, miR-96, miR-106a, miR-203, and miR-326), and seven downregulated (miR-16, miR-125b, miR-126, miR-143, miR-145, miR-320, and miR-484-5p). Link et al also identified differentiated upregulation of miR-21 and miR-106b in stool samples obtained from CRC patients as compared to stool samples obtained from healthy controls.117 Koga et al using a large cohort of 206 CRC patients and 134 healthy controls, identified upregulation of miR-17-92 cluster, miR-21, and miR-135 in stool samples.118 The overall sensitivity and specificity of a stool assay based on upregualtion of these three miRs were 74% and 79%, respectively.

Hypermethylation may occur not only in promoters of protein-coding genes but also in miRs. Hypermethylation of miR-34a/b and miR-148a was found by Kalimutho et al in stool samples of 98% and 75% of the patients with CRC, respectively.119 Another report by the same group showed miR-144 to be upregulated in stool samples of CRC patients. Therefore it may be used for CRC detection with a sensitivity and specificity of 74% and 87%, respectively.120 Wu et al. further validated the upregulation of miR-21 and miR-92a in both tissue and stool samples and following surgical removal of the tumor, stool levels of miR-21 and miR-92a were significantly reduced.72 Nevertheless, only miR-92a was significantly upregulated in stool of patients with adenomatous polyps as compared to healthy controls. Li et al. identified downregulation of miR-143 and -145 in stool samples of CRC patients as compared to healthy control subjects.121 However, there was no statistically significant difference in the expression levels of both miR-21 and miR-106a between stool samples of CRC patients and stool samples of the healthy human subjects.121 Data on miR dysregulation in feces samples are summarized in Table 2.

In summary, RNA can be extracted from stool samples, and dysregulated miRs in CRC may be amplified and provide a platform for a stool assay for early detection or follow up of CRC patients. The value of such as assay in detection of adenomatous polyps is yet to be defined.

miR-dysregulation and prognosis

Since the costs of modern therapy are rapidly increasing to a point that health authorities will not be able to fund therapy for cancer patients, a substantial research effort is carried out in order to identify patients that will benefit most from a given therapy. Despite this research effort, very few prognostic or predictive biomarkers exist in clinical practice. miRs may serve as prognostic or predictive markers in CRC patients.

In 2006 Xi et al reported that CRC patients in stage I & II (n=4 each) and 16 patients in stage III & IV (n=16 each) with higher expression of miR-200c in their tumor samples had a shorter median survival compared to patients with lower expression (26 vs. 38 months, respectively).93 Schetter et al demonstrated that miR-21 was associated with advanced AJCC-TNM stage, poor survival independent of other clinical covariates, and poor therapeutic outcome.67 Diaz et al showed that downregulation of miR-106a predicted shortened disease-free survival and overall survival regardless of tumor stage.122 Downregulation of miR-17-5p correlated with disease-free survival in CRC patients diagnosed in early stages of disease, however this did not reach statistical significance.122 In a different study, Schepeler et al demonstrated that the expression levels of eight miRs (upregulation of miR-1, -let-7b, -let-7d, and miR-126, as well as down-

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regulation of miR-320, miR-420, miR-498, and miR-526c) correlated with recurrence-free survival in AJCC-stage II CRC patients by a multivariate analysis. Kaplan-Meier survival curves showed that patients diagnosed with AJCC-stage II CRC bearing tumors with high expression of miR-320 or miR-498 showed a significant difference in progression-free survival compared to patients bearing tumors with low expression. Yantiss et al demonstrated that CRC patients younger than 40 years of age had more aggressive tumors and upregulation of miR-20a, miR-21, miR-145, miR-181b, and miR-203. Wang et al identified positive correlation between miR-31 and advanced AJCC-TNM stage. Schetter et al identified that miR-21 expression was associated with inflammatory cytokines expression (IL-6, IL-8, IL-10, IL-12a, and NOS2a) as well as cancer-specific mortality. Pu et al showed that circulating plasma levels of miR-221 are associated with poor overall survival in CRC patients. Chiang et al identified that low expression of miR-203 was correlated with tumor size and pT stage. Nielsen et al using a multivariate analysis, showed that upregulation of stromal miR-21 significantly correlates with shorter disease-free survival in AJCC-stage II colon (but not rectal) cancer patients. Feng et al confirmed that upregulation of miR-21 was associated with advanced tumor stage. Wang et al showed that downregulation of miR-195 was an independent predictor of overall survival and it was upregulated more often in CRC patients with lymph node metastasis and advanced tumor stage, compared to patients with early, node-negative disease. Upregulation of miR-125b was shown by Nishida et al to be an independent predictor of advanced tumor size, tumor invasion, and poor prognosis of CRC patients using a multivariate analysis. In multiple studies, miR-21 was shown to be associated with advanced disease and worse outcome. Furthermore, Shibuya et al demonstrated that upregulation of miR-21 was associated with venous invasion, liver metastasis, and advanced tumor stage. Another predictor of tumor spread was reported by the same group, showing upregulation of miR-155 was correlated with lymph node metastases. The downregulation of miR-124 correlated with tumor grade and poor overall and disease-free survival. Another downregulated miR associated with poor outcome is miR-144, shown by Iwaya et al to correlate with venous invasion, liver metastasis, liver recurrence, and poor survival. Upregulation of miR-10b was shown by Nishida et al to be associated with high incidence of lymphatic invasion and poor prognosis, as well as resistance to fluorouracil-based therapy. Once again, miR-21 expression levels in tumor tissue as well as miR-155 were independent prognostic factors for both overall survival and disease-free survival in CRC patients, regardless of clinical stage. Cheng et al, confirmed once again, that CRC patients with disease recurrence had higher levels of miR-141 in their tumor-tissues. Zhu et al identified miR-9 to be upregulated in CRC patients with distant metastasis in comparison to CRC patients diagnosed in earlier disease stages (without distant metastasis). Akçakaya et al reported that upregulation of miR-185 and downregulation of miR-133b were correlated with distant metastasis and poor survival in CRC patients. Downregulation of miR-345 was associated with lymph node metastasis and worse histological type in CRC patients in a study conducted by Tang et al. A panel of miRs containing upregulation of miR-31, miR-10b, and miR-139-5p and downregulation of miR-143 was typical for mucinous phenotype. Furthermore, progressively increasing levels of miR-10b expression in tumor tissues were observed in correlation with AJCC T-stage, T1 to T4 lesions and with AJCC stage I to IV disease. K araayvaz et al reported that miR-215 upregulation in tumor tissue is closely associated with poor CRC patients’ overall survival. Ma et al identified that miR-150 downregulation was associated with decreased overall survival and a worse response to adjuvant chemotherapy. Similarly, Nie et al demonstrated that downregulation of miR-365 is correlated with cancer progression and poor survival in CRC patients. Upregulation of a small panel of three miRNA reported by Vickers et al, including miR-21, miR-135a, and miR-335, correlated with a stage-associated differential upregulation in CRC patients, while upregulation of miR-206 in tumor tissue was associated with better outcome. They also reported that upregulation of miR-let7a was associated with metastatic disease. Both Liu et al and Horiiuchi et al showed once more that miR-21 upregulation significantly correlates with advanced clinical stage and poor cell differentiation. Wiessmann-Brenner et al analyzed 903 miRs and their association to recurrence in patients with either AJCC stage-I or II CRC. Only miR-29a was associated with increased recurrence rate. High expression of miR-29a was associated with a longer disease-free survival, on both univariate and multivariate analyses. Yamashita et al used a cohort of 144 CRC patients and identified that upregulation of miR-372 was associated with synchronous liver metastasis and was an independent prognostic factor. Pichler et al confirmed that downregulation of miR-143 was an independent prognostic factor with respect to cancer specific survival. Single nucleotide polymorphisms (SNP) occur-
ring in various regions of the human genome were shown to have significant value in both risk for developing cancer in a given population and as predictive markers. Ryan et al analyzed a single nucleotide polymorphism (SNP) in miR-608 in a large cohort of 245 CRC samples and 446 control patients. A specific genotype (GG) was associated with increased polymorphism (SNP) in miR-608 in a large cohort of developing cancer in a given population and as predictive factors.

Table 1. miR dysregulation in colorectal tissue samples

| Dysregulation | Number of studies | miRs and references |
|---------------|-------------------|---------------------|
| Upregulated   | 15                | miR-21[6,7]         |
|               | 11                | miR-31[4,6,10,11,12,15] |
|               | 9                 | miR-135[1,2,3,4,5,6,7,8] |
|               | 8                 | miR-183[9,10,11,12,13,14,15] |
|               | 7                 | miR-19[1,2,3,4,5,6,7] |
|               | 5                 | miR-18[1,2,3,4,5,6,7,8] |
|               | 4                 | miR-15[1,2,3,4,5,6,7,8] |
|               | 3                 | miR-106[1,2,3,4,5,6,7,8] |
|               | 2                 | Let-7[1,2,3,4,5,6,7,8] |
|               | 1                 | miR-19[1,2,3,4,5,6,7,8] |
| Downregulated | 15                | miR-145[10,11,12,13,14,15] |
|               | 9                 | miR-1[10,11,12,13,14,15] |
|               | 5                 | miR-19[1,2,3,4,5,6,7,8] |
|               | 4                 | miR-15[1,2,3,4,5,6,7,8] |
|               | 3                 | miR-12[1,2,3,4,5,6,7,8] |
|               | 2                 | Let-7[1,2,3,4,5,6,7,8] |
|               | 1                 | miR-19[1,2,3,4,5,6,7,8] |

The same SNP (rs4919510) in miR-608 was associated with increased risk for both recurrence and death. Also, miR-219-1:rs213210 showed consistent association with CRC-related death. Zhou et al confirmed that upregulation of miR-92a correlated with advanced disease-stage, lymph node and distant metastases, and poor overall survival. Nishimura et al identified that upregulation of miR-181a was an independent significant prognostic factor for CRC. These data are summarized in Table 3.
### Table 2. miR dysregulation in serum or fecal samples

| Source | Dysregulation | Number of studies | miRs and references |
|--------|---------------|-------------------|---------------------|
| Serum  | Upregulated   | 3                 | miR-221<sup>100,102,115</sup> |
|        |               | 2                 | miR-21<sup>7,113</sup>; miR-92a<sup>8,115</sup>; miR-222<sup>10,111</sup> |
|        |               | 1                 | miR-Let-7e<sup>115</sup>; miR-17-3p<sup>111</sup>; miR-19a<sup>115</sup>; miR-22<sup>115</sup>; miR-23a<sup>115</sup>; miR-29a<sup>86</sup>; miR-92<sup>115</sup>; miR-95<sup>111</sup>; miR-125-5p<sup>115</sup>; miR-134<sup>110</sup>; miR-135b<sup>111</sup>; miR-141<sup>114</sup>; miR-146<sup>110</sup>; miR-210<sup>115</sup>; miR-576<sup>a115</sup> |
|        | Downregulated | 1                 | miR-10a<sup>115</sup>; miR-141-3p<sup>115</sup>; miR-150<sup>115</sup>; miR-188-3p<sup>115</sup>; miR-192<sup>115</sup>; miR-224<sup>115</sup>; miR-245<sup>115</sup>; miR-495<sup>115</sup>; miR-572<sup>115</sup>; miR-601<sup>115</sup>; miR-720<sup>115</sup>; miR-760<sup>115</sup>; Let-7a<sup>115</sup> |
| Feces  | Upregulated   | 4                 | miR-21<sup>7,116-118</sup> |
|        |               | 1                 | miR-17<sup>118</sup>; miR-18a<sup>118</sup>; miR-19a<sup>118</sup>; miR-19b-1<sup>118</sup>; miR-20a<sup>116</sup>; miR-92<sup>116</sup>; miR-92a<sup>27</sup>; miR-96<sup>118</sup>; miR-106a<sup>116</sup>; miR-106b<sup>117</sup>; miR-135<sup>118</sup>; miR-144<sup>120</sup>; miR-203<sup>116</sup>; miR-326<sup>116</sup> |
|        | Downregulated | 2                 | miR-143<sup>116,121</sup>; miR-145<sup>116,121</sup> |
|        |               | 1                 | miR-16<sup>118</sup>; miR-125b<sup>119</sup>; miR-126<sup>118</sup>; miR-320<sup>116</sup>; miR-484-5p<sup>116</sup> |

### Table 3. Studies on the association between miR dysregulation and staging or prognosis

| Author  | Year | miR | N   | Endpoint                                                                 |
|---------|------|-----|-----|--------------------------------------------------------------------------|
| Schetter<sup>124</sup> | 2009 | miR-21 | 115 | High miR-21 expression was associated with higher rates of cancer-specific mortality (P <0.0001) |
| Horiuchi<sup>126</sup> | 2011 | miR-21 | 326 | PDCD4 mRNA levels were negatively regulated by miR-21 in each tumor stage of CRC. OS and DFS rates of low PDCD4 patients were significantly worse than those of patients with high expression. |
| Schetten<sup>67</sup> | 2008 | miR-21 | 197 | 5-year cancer-specific survival rate: 57.5% - Maryland cohort and 49.5% - Hong Kong cohort High miR-21 expression associated with advanced TNM staging, poor survival, and poor therapeutic outcome. |
| Feng<sup>126</sup> | 2011 | miR-21 | 54  | Highly expressed miR-21 was more common in stage IV cancer than in stage II and stage III cancer |
| Liu<sup>136</sup> | 2011 | miR-21 | 42  | High expression of miR-21 was significantly correlated with advanced clinical stage and poor cell differentiation. miR-21 may predict pathological tumor response to chemotherapy. |
| Shibuya<sup>128</sup> | 2010 | miR-21, miR-155 | 156 | High miR-21 expression was significantly associated with venous invasion, liver metastasis and tumor stage. High miR-155 expression was significantly correlated with lymph node metastases. The OS and DFS rates of patients with high miR-21, and high miR-155 expression were significantly worse than those of patients with low expression. |
### miRNA Expression Levels and Prognostic Significance in CRC

**Nielsen**<sup>25</sup> 2011  miR-21  130 - stage II colon cancer 67 - stage II rectal cancer  
**Vickers**<sup>34</sup> 2012  miR-335, miR-206, miR-135a, miR 21, miR let7a  
**Xia**<sup>93</sup> 2006  hsa-miR-200c  24  
**Diaz**<sup>122</sup> 2008  miR-17-5p, miR-106a  110  
**Schepeler**<sup>48</sup> 2008  miR-320, miR-498  37  
**Wang**<sup>90</sup> 2009  miR-31  98  
**Pu**<sup>112</sup> 2010  miR-221  103  
**Chiang**<sup>71</sup> 2011  miR-203  107  
**Wang**<sup>94</sup> 2012  miR-195  85  
**Nishida**<sup>127</sup> 2011  miR-125b  89  
**Cheng**<sup>114</sup> 2011  miR-141  102 - First cohort 156 - Second cohort  
**Zhu**<sup>130</sup> 2012  miR-9  25  
**Akçakaya**<sup>132</sup> 2011  miR-185, miR-133b  50  
**Tang**<sup>109</sup> 2011  miR-345  31  
**Chang**<sup>74</sup> 2011  miR-31, miR-139-5p, miR-143, miR-10b  102  
**Karaayvaz**<sup>133</sup> 2011  miR-215  34  
**Ma**<sup>101</sup> 2012  miR-150  239  
**Nie**<sup>105</sup> 2011  miR-365  97

**miR-21 and miR-155 expression levels in CRC tissue were independent prognostic factors for OS and DFS.**

Higher miR-21 expression correlated significantly with shorter DFS (p = 0.004) in the stage II colon cancer patient group, but not in the stage II rectal cancer group.

Increased expression of miR-21, mir-135a and miR-335 was associated with clinical progression of CRC.

miR-206 demonstrated an opposite trend.

miR-let7a, showed elevated expression in metastatic disease compared to normal mucosa or non-metastatic disease, and only in KRAS mutation positive tumors.

Prognostic signature of miR 21,135a, 335, 206 and let-7a for detecting the presence of metastases had a specificity of 87% and sensitivity of 76% for the presence of metastases.

Shorter median survival- (26 vs. 38 months) for patients with hsa-miR-200c over expression.

Lower levels of miR-17-5p and miR-106a were associated with pathological tumor features of poor prognosis.

Downregulation of miR-106a predicted shortened DFS (P = 0.03) and OS (P = 0.04), independent of tumor stage.

Stage II colon tumors with high expression of miR-320 or miR-498 showed a significant difference in progression-free survival compared with tumors with low expression.

Higher miR-31 expression was positively related to advanced TNM stage (p = 0.026) and deeper invasion of tumors (p = 0.024).

Patients with higher plasma miR-221 levels have a dramatically lower survival rate than that in the low expression group (P < 0.05).

Significant low expression of miR-203 associated with increased tumor size (p=0.015) and an advanced pT stage (p=0.005).

Reduced expression of miR-195 occurred more often in patients with lymph node metastasis and advanced tumor stage (all P < 0.01).

Patients with reduced miR-195 had a poor OS (P < 0.01).

Reduced expression of miR-195 was an independent predictor of OS.

The high miR-125b expression group showed a greater incidence of advanced tumor size and tumor invasion, as well as a significantly poorer prognosis compared to the low miR-125b expression group (P<0.05).

Multivariate analysis indicated that high miR-125b expression was an independent prognostic factor for survival.

Circulating plasma miR-141 was significantly associated with stage IV colon cancer.

High levels of plasma miR-141 predicted poor survival.

Significant up regulation of miR-9 expression was observed in patients with distant metastasis (P < 0.001).

High expression of miR-185 and low expression of miR-133b were correlated with poor survival (p=0.001 and 0.028, respectively) and metastasis (p=0.007 and 0.036, respectively).

Low expression of mir-345 was associated with lymph node metastasis and worse histological type.

Elevated expression of miR-31, miR10b (p=0.004) and miR-139-5p (p<0.001) and reduced expression of miR-143 (p=0.016) were associated with aggressive mucinous phenotype.

Progressively increasing levels of miR-10b expression were observed from T1 to T4 lesions and from stage I to IV disease.

High levels of miR-215 expression (P=.025) are closely associated with poor patient’s OS.

Patients with low miR-150 expression had shorter survival and a worse response to adjuvant chemotherapy than patients with high expression.

Decreased levels of miR-365 were related to colon cancer progression. Low miR-365 levels significantly correlated with reduced DFS.
### Summary

Large body of evidence has accumulated in recent years regarding the dysregulation of miRs in CRC. Multiple different miRs were shown to be upregulated or downregulated in tumor tissue (Table 1). The variability in results is caused by the fact that most studies were small sampled in size and used heterogenic groups of patients. Different sources of tissue used for the studies, paraffin-embedded tissue versus frozen tissue, and the timing from colon resection to fixation either in formaldehyde or in liquid nitrogen may all have a significant impact on the quality, quantity, and composition of RNA extracted. The most frequently upregulated miRs are miR-21 in fifteen studies, miR-31 in eleven studies, and miR-135b in nine studies while the most downregulated are: miR-145 in fifteen studies and miR-143 in nine (see Table 1 for more details).

In stool samples, the content of microRNA may change following bowel preparation. Therefore, stool samples used from patients undergoing colonoscopy may vary in their miR content compared to stool samples obtained from patients that did not undergo bowel preparation. Again, the time interval between stool sampling and freezing may have a major impact on RNA degradation. The most frequently upregulated miR in stool samples is miR-21 in four studies, while the most downregulated are miR-145 and miR-143 in two studies each (see Table 2 for more details).

The variability in the results obtained from blood sampling may be attributed to many factors: some studies report plasma dysregulation of miRs while

| Author          | Year | miR   | No. | Description                                                                 |
|-----------------|------|-------|-----|-----------------------------------------------------------------------------|
| Nishida         | 2012 | miR-10b | 88  | miR-10b over expression was associated with high incidence of lymphatic invasion (P = 0.0257) and poor prognosis (P = 0.0057). High miR-10b expression is an independent prognostic factor for survival. Over expression of miR-10b confers chemoresistance in colorectal cancer cells to 5-FU. |
| Wiessmann-Brenner | 2012 | miR-29a | 110 | High expression of miR-29a was associated with a longer DFS in stage II CRC patients. |
| Yamashita       | 2012 | miR-372 | 144 | High miR-372 expression was an independent prognostic factor (p = 0.006). High miR-372 expression was associated with synchronous liver metastasis (p = 0.035). |
| Pichler          | 2012 | miR-143 | 77  | Low levels of miR-143 were an independent prognostic factor with respect to cancer specific survival (p=0.024). miR-143 expression levels serve as an independent prognostic biomarker for CRC in KRAS wild-type patients. |
| Ryan            | 2012 | miR-608 | 245 | A specific genotype (GG) was associated with increased survival in African Americans and decreased survival in Caucasians. |
| Lin             | 2012 | miR-608, mir219-1 | 1097 | mir608:rs4919510 was associated with increased risk for both recurrence and death in patients with stage III disease. mir219-1:rs213210 showed consistent association with death. Patients carrying the variant genotypes at both sites exhibited a 5.6-fold increased risk of death. |
| Zhuo            | 2012 | miR-92a | 82  | High expression of miR-92a correlated with advanced clinical stage (p = 0.025), lymph node metastases (p = 0.015), distant metastases (p = 0.046), and poor OS (p = 0.001). Increased expression of miR-92a was an independent predictor of OS. |
| Wang            | 2012 | miR-124 | 96  | Decreased miR-124 expression correlated significantly with the grade of differentiation (P = 0.021). Downregulated miR-124 was significantly correlated with worse prognosis, both in terms of OS (P = 0.017) and DFS (P = 0.014). Downregulated miR-124 was demonstrated as an independent prognostic factor for OS (P = 0.002) and DFS (P = 0.002). |
| Iwaya           | 2012 | miR-144 | 137 | Low expression levels of miR-144 were associated with enhanced malignant potential such as venous invasion (P = 0.0013), liver metastasis (P = 0.08), liver recurrence (P = 0.0058) and poor prognosis (P = 0.0041). Low miR-144 expression was an independent prognostic factor for survival. |
| Nishimura       | 2012 | miR-181a | 162 | Patients with high expression of miR-181a had a significantly poorer prognosis than those with low expression (P=0.011). High miR-181a expression was an independent significant prognostic factor for CRC. However, no correlation was observed between miR-181a expression and clinicopathological parameters. |
others used serum, the timing between blood sampling and separation of the serum/plasma is critical, and so is the methods in which the samples were stored. On top of that, different microRNA-enriched RNA extraction kits were used with variability in performance as well as heterogeneous patient populations. The most frequently upregulated miRs are miR-221 in three studies, miR-21, miR-92a, and miR-222 in two studies each, and there were many miRs downregulated in one study each (see Table 2 for more details). There is new and interesting data showing that miRs are carried in the plasma by small micelles, therefore may resist the hostile environment of peripheral blood.

The current AJCC staging system is based on histopathologic parameters. Additional molecular prognostic markers are essential for better stratification of CRC patients. The accumulated data of dysregulated miRs associated with better or worse outcome may be incorporated into staging CRC patients and used for better treatment selection. There is also important data regarding the potential role of miRs in the regulation of the carcinogenesis process with possible implications for therapy; however, this is beyond scope of the current review. Overall, the vast amount of data generated by multiple studies should be verified in a uniform and controlled manner in order to establish miRs with a potential to become future predictive or prognostic markers.

Competing Interests

The authors have declared that no competing interest exists.

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