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Alterations of microRNAs contribute to colon carcinogenesis

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Abstract

MicroRNAs are being evaluated as biomarkers and therapeutic targets for colon cancer. MicroRNAs have a functional role in the initiation and progression of colon cancer. Altered microRNA expression is found in tumors and their expression patterns may serve as useful cancer biomarkers. Polymorphisms in microRNAs or microRNA binding sites may modify ones risk of developing cancer. As we continue to improve our understanding of the role for microRNAs in the initiation and progression of cancer, one goal is to gain insights that will allow for the development of microRNAs as biomarkers and therapeutic targets for cancer. This review provides a current understanding of the connection between microRNAs and colon cancer. We will cover evidence that global microRNA expression patterns are altered in colon tumors, that specific microRNAs have a functional role colon carcinogenesis, that polymorphisms in microRNAs may be associated with risk of colon cancer, and the potential for using circulating microRNAs as a non-invasive biomarker for the detection of cancer.

Introduction

Throughout the world, ~1.2 million people are diagnosed with colon cancer and ~600,000 thousand die of this disease annually¹. This is the third most prevalent cancer and the third leading cause of cancer related mortality. As we improve our understanding of the biology of colon cancer, we hope to gain insights into potential biomarkers and therapeutic targets that can aid in the prevention, diagnosis and treatment of this deadly disease.

MicroRNAs are small non-coding RNAs that can lead to the translational repression of target genes. Since the original discovery connecting microRNAs to chronic lymphocytic leukemia², researchers have demonstrated with overwhelming evidence that microRNAs have a role in cancer. MicroRNAs can act as either tumors suppressors or oncogenes and their oncogenic activity can be tissue type dependent. MicroRNAs may be useful as both biomarkers for the diagnosis and as therapeutic targets for the treatment of colon cancer. This review will provide an overview of the connection between microRNA expression and colon cancer. We will cover the global expression differences in colon tumors, the function of specific microRNAs in carcinogenesis, and the potential use of microRNAs as diagnostic and prognostic biomarkers for colon cancer.

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MicroRNA expression patterns are altered in colon tumors

After the discovery that microRNAs were conserved in humans³, there was increased interest in the role that microRNAs have in the pathology of human diseases, including cancer. Several groups reported that global microRNA expression patterns were altered in colon cancer using a variety of methods⁴⁻⁸. The underlying theme of these reports indicated that microRNA expression patterns were distinctly different in tumors compared to nontumorous tissues. These results were consistent with the hypothesis that altered microRNA expression played an etiological role in the initiation and progression of cancer. Authors also noted that the global microRNA expression patterns could distinguish between tissue types and tumor types superior to mRNA expression patterns^{4, 5}. This led to a consensus that microRNA expression patterns have potential as biomarkers of cancer.

While it was originally reported that microRNA expression levels are globally reduced in cancers⁴, most studies examining colon tumors have found the opposite. The majority of published studies which have evaluated the global microRNA expression pattern of colon tumors indicate that more microRNAs show increased expression in colon cancer compared to those that have reduced expression^{5, 8-10}. These observations are consistent with findings that microRNA locations are over-represented in regions of genomic copy number gain and under-represented in regions of copy number loss in colon cancer^{11, 12}. While the meaning of this is unclear, there may be more oncogenic microRNAs than tumor suppressor microRNAs in colon cancer.

Based on expression studies, several microRNAs have been identified and validated as potential oncogenes or potential tumor suppressor genes in colon cancer. Some of these microRNAs are emerging as significant contributors to colon carcinogenesis. Examples of these include *miR-143* and *miR-145*, two of the first microRNAs identified as having reduced expression in colon tumors¹³. These findings have been validated in multiple studies and *miR-143* and *miR-145* may have tumor suppressor activity in colon cancer^{7, 14, 15}. *miR-21* is an oncogenic microRNA that has been found to be increased in colon tumors^{8, 16} and every other solid tumor type that has been examined⁵. This microRNA appears to be an important factor in the initiation, progression and metastasis of colon and other cancers. The first microRNA identified in humans was *let-7*³ and this microRNA has important tumor suppressor activity in multiple cancer types. Among the targets of *let-7* is the RAS oncogene^{17, 18}, consistent with its suggested tumor suppressor role. Other important microRNAs that appear to contribute to colon cancer are the *miR-17-92* cluster of microRNAs, *miR-135a/b* and *miR-200c*.

Phenotypic subgroups of colon cancer include microsatellite instability, DNA mismatch repair status and TP53 status. Different subgroups have varied survival prognoses and therapeutic responses to chemotherapy. Recently, there has been an emphasis on examining microRNA expression profiles of different subgroups of colon cancer. For example, Lanza and colleagues compared the microRNA expression patterns of tumors with microsatellite stability (MSS) versus those with microsatellite instability (MSI)¹⁹. The expression of 8 microRNAs could classify MSS from MSI tumors, including the expression of the *miR-17-92* cluster. Valeri and colleagues also demonstrated that *miR-155* could target the mismatch repair genes MLH1, MSH2 and MSH6 and contribute to MSI phenotype in colon cancer²⁰. Sarver and colleagues investigated the microRNA expression patterns of DNA mismatch repair proficient and DNA mismatch repair deficient tumors and identified 6 microRNAs that were differentially expressed between these subtypes of colon cancer²¹. Although most work with p53 regulated microRNAs has been performed *in vitro* with little support from human tissues, these studies have identified microRNAs regulated by p53 in colon cancer cell lines²²⁻²⁴. It will be of future interest to determine if these *in vitro*

differences are relevant to human disease in general. Taken together, all of these reports indicate that microRNA expression patterns may have the ability to distinguish different subgroups of tumors which supports the potential of microRNAs as diagnostic biomarkers. These findings also support that differences in microRNA expression may be partially responsible for some of the biological differences observed in these different subgroups.

Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, are associated with increased risk of developing colon cancer^{25, 26}. It will be interesting to study the microRNA expression patterns of tumors that arise in the context of inflammatory bowel disease. Studies have already demonstrated that microRNA expression patterns are altered in ulcerative colitis²⁷ and other chronic inflammatory conditions²⁸. Wu and colleagues found that 11 microRNAs were altered in active ulcerative colitis, including elevated levels of the oncogenic microRNA miR-21²⁷. These results suggest that alterations of microRNA expression may, in part, be responsible for the increased rates of colon cancer in inflammatory bowel disease. Studying the differences between sporadic tumors and inflammatory bowel disease-related tumors may also reveal differences in underlying biology of these tumors that have yet to be elucidated.

Altered microRNA expression contributes to colon carcinogenesis

There is a wealth of literature supporting a mechanistic role for altered microRNA expression patterns in the initiation and progression of colon cancer. It is not feasible to adequately review all of those findings in the context of this review, but instead we will present some of the important findings on specific microRNAs and their role colon cancer (Table 1A).

The first microRNAs to be implicated in colon cancer were miR-143 and miR-145. In 2003, Michael and colleagues cloned these microRNAs and found that both miR-143 and miR-145 had reduced expression in colon tumors¹³ indicating that these microRNAs were potential tumor suppressors. These microRNAs were found to affect cell growth and proliferation in colon cancer cell lines, consistent with a role tumor progression²⁹. Subsequent studies evaluated the specific mechanism of action of these microRNAs and found that miR-145 can target genes with important roles in carcinogenesis including insulin receptor substrate-1 (IRS-1)³⁰, c-Myc³¹, Yamaguchi sarcoma viral oncogene homolog 1 (YES1)³² and signal transducer and activator of transcription 1 (STAT1)³². In the first example, Shi and colleagues demonstrated that miR-145 could target IRS-1 and that over-expression of miR-145 would mimic the cell arrest phenotype of IRS-1 knockdown. This argued that the tumor suppressive effect of miR-145 was at least in part due to the targeting of IRS-1 by miR-145. Animal model experiments have also supported a role for miR-145 as a tumor suppressor. Recent findings have indicated a role for miR-145 in stem cell maintenance as well. Increased miR-145 expression can impair pluripotency in stem cells by targeting octamer-4 (OCT4), sex determining region Y-box 2 (SOX2) and Krüppel-like factor-4 (KLF4)³³. This is consistent with a tumor suppressor role for this miR-145. MiR-143 has also been studied in detail and among its targets are KRAS³⁴, DNA methyl transferase 3A (DNMT3A)³⁵ and extracellular signal-regulated kinase-5 (ERK5)²⁹. As an example, Chen and colleagues demonstrated that miR-143 could bind and suppress KRAS *in vitro* and that KRAS protein levels were inversely correlated with miR-143 expression levels in human tissues³⁴. These patterns are consistent with what one would expect of a microRNA target. The loss of miR-143 leads to increased KRAS and the subsequent phosphorylation/activation of MAPK. Furthermore, the reduction of KRAS by over expressing miR-143 resulted in reduced cell proliferation and blocked MAPK phosphorylation and activation³⁴. Additional studies have shown that intravenous treatment of DLD-1 xenograft mice with a

modified miR-143 reduced tumor growth³⁶ demonstrating that the loss of miR-143 expression is a critical step in colon cancer progression.

MiR-21 demonstrates another elegant example of a miRNA with an important role in colon carcinogenesis and tumor progression. MiR-21 is a highly studied, oncogenic microRNA that can target several tumor suppressor genes in various model systems³⁷ (Figure 1). Overexpression of miR-21 has been found in at least 18 malignancies indicating that altered expression of miR-21 may be a common mechanism in carcinogenesis^{5, 38}. Expression of miR-21 can be influenced by gene amplification¹², transcriptional silencing³⁹, genotoxic stress (i.e. H₂O₂ or ionizing radiation)⁴⁰, and various other transcriptional mechanisms including activation by EGFR pathway⁴¹, STAT3 dependent regulation through interleukin 6 (IL-6)⁴² or interferon (IFN)⁴³ and transcriptional repression by FOXO3A⁴⁴. Increased expression of miR-21 has been found to promote cell proliferation and decrease apoptosis⁴⁵. MiR-21 plays a role in cell migration, cell invasion and tumor metastasis. Its expression can directly inhibit the translation of many tumor suppressor genes including programmed cell death 4 (PDCD4)^{46, 47}, phosphatase and tensin homolog (PTEN)⁴⁸, reversion-inducing-cysteine-rich protein with kazal motifs (RECK)⁴⁹, TIMP3⁴⁹, maspin⁵⁰, nuclear factor 1 B-type (NFIB)⁵¹, tropomyosin 1 (TPM1)⁵², sprouty 2 (SPRY2)⁵³ and Ras homolog gene family, member B (RHOB)⁵⁴. The regulation of PDCD4 by miR-21 is especially relevant in colon cancer. PDCD4 is a tumor suppressor gene that suppresses cell proliferation, promotes apoptosis and inhibits tumor initiation, cell invasion and intravasation⁵⁵. Increased levels of miR-21 reduce the expression of PDCD4 and promote transformation, cell invasion and intravasation^{46, 47}. Based on *in situ* hybridization and immunohistochemistry, the expression of miR-21 and PDCD4 protein levels were inversely correlated in human colon tumors⁵⁶. These findings demonstrate that the *in vitro* interactions between miR-21 and PDCD4 are likely relevant to human disease. The interactions between miR-21 and PDCD4 have been replicated in many additional tumor types indicating that findings are likely relevant to multiple cancer types⁵⁵.

Additional microRNAs have been mechanistically implicated in colon cancer initiation and progression. The miR-17-92 cluster of microRNAs are overexpressed in colon cancer and target E2F1 to regulate cell proliferation indicating that this may have a role in neoplastic transformation of the colon epithelium⁵⁷. MiR-135a and -135b expression are increased in both colon adenomas and adenocarcinomas⁵⁸. These microRNAs target the adenomatous polyposis coli (APC) gene⁵⁸. APC is a component of the Wnt signaling pathway that is frequently mutated or repressed in colon adenomas, a precursor lesion to adenocarcinoma. Therefore up-regulation of miR-135a/b may be an early event in cellular transformation of colon cancer. Cyclooxygenase 2 (COX-2) can be negatively regulated by miR-101⁵⁹. Loss of miR-101 results in the overexpression of COX-2 and may contribute to the initiation and progression of colon tumors. MiR-451 overexpression can directly target the oncogene macrophage migration factor (MIF)⁶⁰. When miR-451 is over-expressed in colon cancer cell lines, MIF protein levels are decreased and cell proliferation is reduced, consistent with a tumor suppressive role for miR-451. Finally, the tumor suppressor retinoblastoma (RB) gene can be repressed by miR-675 in colon cancer and may serve as yet another mechanism for microRNAs to regulate the development of colon cancer⁶¹.

It is becoming clear that altered expression of microRNAs is causally associated with the initiation and progression of colon cancer. As these mechanisms are elucidated, we hope that it will become possible to develop therapeutic strategies that target specific microRNAs for the treatment of colon cancer.

MicroRNA expression as prognostic biomarkers for colon cancer

Prognostic biomarkers that can stratify patients into different risk groups may help physicians and patients with therapeutic decisions. MicroRNA expression patterns have potential in this arena. We have recently published that elevated levels of miR-21 were associated with prognosis and therapeutic outcome in colon cancer patients⁸. In this study we used microRNA microarrays on 84 pairs of tumors and noncancerous tissue from an American cohort to identify microRNAs that were associated with cancer-specific mortality. We then used qRT-PCR on pairs of tissue from an additional cohort of 113 Chinese patients to validate these findings. To our knowledge, this study represents the largest cohorts of colon cancer patients that have been profiled for microRNA expression. We identified 26 microRNAs that were significantly elevated in tumors and 11 that were significantly decreased in tumors. Of those microRNAs, high levels of miR-21, miR-20a, miR-181b, miR-203 and miR-106a were each associated with cancer specific mortality in the American cohort. These were measured in the Hong Kong cohort and miR-21 expression was significantly associated with prognosis, independent of other clinical characteristics. Therefore, miR-21 expression may be a useful prognostic biomarker for colon cancer. This was also true when examining only TNM stage II patients^{8, 62} indicating that miR-21 may be useful in identifying high risk, early stage cancer patients. Increased levels of miR-21 were observed in more advanced stage tumors, consistent with a role for miR-21 in the progression of cancer. High miR-21 expression was associated with worse therapeutic outcome for patients receiving 5 fluorouracil (5FU) indicating it may be useful in predicting response to 5FU. Because miR-21 was associated with prognosis in two independent cohorts, we had confidence these were likely representative of colon cancer. Other groups have also reported similar findings. In a cohort of 29 colon cancer patients, Slaby and colleagues noted miR-21 had increased expression in more advanced tumors¹⁶ which is consistent with our findings. Kulda and colleagues have recently reported that increased expression of miR-21 is associated with worse prognosis in a cohort of 46 colon cancer patients⁶³ further validating our findings. MiR-21 expression has now been associated with prognosis in at least lung^{64, 65}, breast⁶⁶, pancreatic⁶⁷, tongue⁶⁸ cancer and astrocytomas⁶⁹ indicating the miR-21 expression may be a general prognostic biomarker for many malignancies.

Additional studies have also identified microRNA expression patterns that have potential as prognostic biomarkers, although these studies have not been validated to our knowledge (Table 1A). Xi and colleagues found that miR-200c expression was associated with prognosis in 24 colon cancer patients⁷⁰. This microRNA has also been shown to target ZEB1 and ZEB2 in various cancer cell lines and thought to play a role in the epithelial to mesenchymal transition (reviewed by⁷¹) indicating it may play a functional role in the progression of cancer. In a study of 49 TNM stage II colon cancer patients, Schepler and colleagues found that reduced expression of miR-320 and miR-498 were each associated with worse progression free survival¹⁴.

While single prognostic biomarkers can be clinically useful, a combination of multiple validated biomarkers may provide more information and give better risk stratification of patients. Therefore, combination of microRNA biomarkers with other validated biomarkers may improve associations with clinical outcomes (Figure 2). As a proof of principle, we have recently reported that the combination *miR-21* expression data with an inflammatory gene biomarker and demonstrated that the combination of both biomarkers significantly improved association with cancer-specific mortality in colon cancer patients⁶². This was also true for TNM stage II colon cancer patients indicating that these may also be useful in identifying patients that are more likely to have undetectable micro-metastases.

These works demonstrate the potential for microRNA expression patterns to be used as prognostic biomarkers to help guide therapeutic decisions and help reduce the health burden caused by colon cancer. Prospective studies are warranted to investigate the associations of microRNA expression patterns with prognosis. Future research should also include focused efforts on microRNA expression patterns that can predict response to different therapeutics as they may aid in deciding which therapies are most appropriate for a given patient.

Potential of microRNAs for early detection of colon cancer

Early detection of colon cancer provides the best opportunity for successful treatment. While current chemotherapeutic strategies have significant clinical value for colon cancer, successful surgery is still the only curative form of treatment. Therefore there is a need to improve early detection methods for this disease. While colonoscopies are reliable and accurate as screening tools, they are invasive and costly which leads to reduced screening rates. Less invasive and less expensive strategies, such as fecal occult blood tests (FOBT), also have reduced sensitivity and specificity. Researchers have begun to determine if detection of microRNAs may be a non-invasive strategy to improve the early detection methods for colon cancer (Table 1B).

In the first study examining circulating microRNAs and colon cancer, Ng and colleagues performed PCR based microRNA profiling on plasma samples from a series of 25 colon cancer patients and 20 healthy controls⁷². They found that miR-17-3p and miR-92 were elevated in the plasma from colon cancer patients and then validated these findings in a cohort of 90 CRC patients and 50 healthy controls. The expression of miR-92 in plasma could distinguish colon cancer patients from healthy control patients with 70% specificity and 89% sensitivity. This provided the first evidence that expression levels of circulating microRNAs may be a useful, noninvasive strategy to improve screening for colon cancer. Another observation that these authors made was that the expression levels of both miR-17-3p and miR-92 were reduced following surgical removal of the tumor⁷². This argues that the elevated levels of these microRNAs in cancer patients are produced by the tumors themselves. These observations suggest that monitoring circulating levels of microRNAs may be used in combination with other biomarkers to measure disease recurrence or progression following surgery or chemotherapy.

Additional investigations are being performed to determine if microRNA expression levels in stool may be a useful diagnostic biomarker for colon cancer. Link and colleagues have reported that microRNAs can be efficiently extracted from stool and that miR-21 and miR-106a are elevated in patients with colorectal neoplasia⁷³. These results show promise for microRNA expression levels in stool to be used alone or in combination with FOBT as a screening biomarker for colon cancer. Additional studies will have to reproduce these findings and determine specificity and sensitivity of such assays.

Polymorphisms in microRNAs or microRNA binding sites and risk of colon cancer

It is clear that single nucleotide polymorphisms (SNPs) in certain protein coding genes can modify one's risk of developing cancer. It is now important to investigate if SNPs in microRNAs or microRNA binding sites are associated with increased risk of cancer⁷⁴. SNPs in microRNAs may affect microRNA processing, microRNA binding, or expression levels in general. A polymorphism in a microRNA binding site in the 3' untranslated region (UTR) of a gene may lead to increased protein production for that gene. Additionally, a SNP in the 3'UTR may also introduce new microRNA binding sites and cause repression of the

production of that protein. Any one of these scenarios can create a deregulation of networks of genes that can contribute to carcinogenesis.

Few studies have examined the association of SNPs in microRNAs and microRNA binding sites with colon cancer incidence (Table 1C). In one such study, Landi and colleagues examined the 3'UTRs of 104 candidate genes with known roles in colon carcinogenesis and identified 47 SNPs in potential microRNA binding sites⁷⁵. On a study of 697 cases and 624 controls, 2 of these SNPs (located in CD86 and INSR) were associated with the risk of colon cancer. While these findings clearly need to be validated in additional populations and the biological mechanism underlying these associations must be investigated, it shows potential for SNPs in microRNA binding sites to modify ones risk of colon cancer.

In a similar line of research, Lee and colleagues investigated if SNPs in microRNAs were associated with prognosis in colon cancer patients⁷⁶. They evaluated 40 polymorphisms in microRNA-related genes from 436 colon cancer patients. They found that a SNP in miR-492 was modestly associated with progression-free survival, but was not an independent prognostic marker. Future work should determine if there are other SNPs in microRNA-related genes that are associated with prognosis and if any could provide clinically useful data.

Conclusion

It is clear that microRNAs have role in colon carcinogenesis. Expression patterns are altered in tumors and these expressions are associated with cancer subtypes and clinical outcomes. MicroRNAs have functional roles in both the initiation and progression of cancer. Polymorphisms in microRNAs and microRNA binding sites may also modify ones risk of developing cancer. We are at the early stages studying the complex relationship between microRNAs and colon cancer and future research in this area will continue to improve our understanding of this disease. Hopefully, the end result will provide novel biomarkers and therapeutics to aid in all aspects of cancer screening, diagnosis and treatment.

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Figure 1. miR-21 plays an important role in carcinogenesis. Depicted are some of the ways in which miR-21 expression is regulated and how it contributes to cancer. The expression of miR-21 can be altered by a variety of mechanisms including gene duplication, transcriptional activation or repression, and genotoxic stress. In a variety of cancer models, increased expression of miR-21 has been shown to target tumor suppressor genes, thus contributing to tumor initiation and progression.

Figure 2.

The combination of multiple, validated biomarkers may improve predictions of clinical outcomes. This is a modified version from ²⁸. In this example, microRNA expression biomarkers and inflammatory gene biomarkers are combined to improve associations with prognosis. Theoretical Kaplan-Meier plots are shown with time on the X-axis and survival probability on the Y-axis. Each biomarker misclassifies a different subset of patients and combining these biomarkers provides a more accurate prediction. This proof of principle of this strategy has recently been shown in colon cancer patients to improve predictions of cancer-specific mortality by combining inflammatory gene and microRNA biomarkers

Table 1
MicroRNAs with roles in colon cancer

A: MicroRNAs whose expression are altered in colon tumors or associated with prognosis in colon cancer patients			
microRNA	Increased/decreased in colon tumors	Expression associated with prognosis?	Experimentally validated microRNA Targets
miR-143	decreased		KRAS ³⁴ , DNMT3A ³⁵ , ERK5 ²⁹
miR-145	decreased		IRS-1 ³⁰ , c-Myc ³¹ , YES1 ³² , STAT1 ³² , OCT4 ³³ , SOX2 ³³ , KLF4 ³³
miR-21	increased	Yes ^{8,63}	PDCD4 ^{46, 47} , PTEN ⁴⁸ , RECK ⁴⁹ , NFIB ⁵¹ , TPM1 ⁵² , SPRY2 ⁵³ , RHOB ⁵⁴ , TIMP3 ⁴⁹ , masin ⁵⁰
let-7	decreased		KRAS ^{17,18}
miR-17-92 cluster	increased	Yes ^{†8}	E2F1 ⁵⁷
miR-135a/b	increased		APC ⁵⁸
miR-451	increased		MIF ⁶⁰
miR-675	increased		RB ⁶¹
miR-101	decreased		COX2 ⁵⁹
miR-200c		Yes ^{†70}	ZEB1 ⁷¹ , ZEB2 ⁷¹
miR-320		Yes ^{†14}	
miR-498		Yes ^{†14}	

B: MicroRNAs whose expression are altered in plasma of colon cancer patients		
microRNA	Increased/decreased blood plasma	Associated with Cancer Risk
miR-17	Increased	Yes ^{†62}
miR-92	Increased	Yes ^{†72}

C: Polymorphisms in microRNAs or microRNA binding associated with cancer risk or prognosis in colon cancer patients		
microRNA or target gene	Increased risk of cancer	Associated with prognosis
CD86 (targeted by miR-337, miR582, miR-200a*, miR-184, miR-212)	Yes ^{†75}	
INSR (targeted by miR-612) miR-492	Yes ^{†75}	Yes ^{†76}

[†] indicates associations that have been reported in a single study that has yet to be validated in additional cohorts/studies