Chapter 5
Epigenetic Regulation of MicroRNA in Colon Cancer

Qiong Zhang, Mukesh Verma, and Sharad Khare

Abstract Colorectal cancer (CRC) is the leading cause of death from cancer all over the world. New and improved approaches are needed to detect CRC early and develop effective therapeutic approaches. Recently, the role of non-coding small microRNAs (miRs) in CRC initiation and progression has been proposed. In this article, we have summarized which miRs are commonly regulated by epigenetic mechanisms in CRC and their contribution towards gene regulation leading to carcinogenesis. The potential implications of using miR as a marker of risk, therapeutic target and survival are discussed. Future studies should emphasize translational research including the validation of key findings from different laboratories and investigators and the implications of these findings for clinical practice.

Keywords Biomarker • Colon cancer • Epigenetics • Methylation • Micro RNA

5.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 [1]. Early detection of CRC is needed because the

Q. Zhang • S. Khare, Ph.D. (✉)
Harry S. Truman Memorial Veterans' Hospital, Columbia, MO 65201, USA

Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Missouri, CE440 Clinical and Educational Support Building, One Hospital Drive, Columbia, MO 65212, USA
e-mail: khares@health.missouri.edu

M. Verma
Epidemiology and Genomics Research Program, Division of Cancer Control and Population Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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majority of patients who develop CRC have no specific risk factors for the disease and the best indicator of prognosis is based on the stage of the disease. Epigenetic biomarkers, including microRNAs (miRs), may provide tools to detect CRC early as well as opportunities for therapeutic intervention and evaluating survival [2]. Noncoding RNAs, miRs regulate gene expression post-transcriptionally. These miRs are 19– 25-nt noncoding RNAs that are cleaved from 70- to 100-nt hairpin-shaped precursors [3]. Pri-miRs are transcribed in the nucleus and after processing by Drosha and its cofactor DGCR8 transported into the cytoplasm. They undergo processing by the RNAse III enzyme Dicer resulting in the mature miRs, which are incorporated into the RNA-induced silencing complex (RISC). Each step of the process is well regulated, and dysfunction at any level can result in inapt miR functions. To date, more than 1,000 human miRs have been identified and each miR control hundreds of genes [4]. MiRs function as endogenous suppressor of gene expression by binding of RISC to the 3′-untranslated region (3′-UTR) of target mRNAs and inducing either mRNA degradation or translational repression. The mRNA degradation is induced if miR binds completely or almost completely, however, if the binding is incomplete, miR represses translation of mRNA. It has been predicted that miRs regulate the translation rate of more than 60 % of protein coding genes. MiRs have important functions in basic biological processes such as cellular differentiation, proliferation, apoptosis, migration and invasion that constitute the hallmarks of cancer. MiR expression profiles are distinctive between normal tissue and derived tumors and between diverse tumor types. Intriguingly, down-regulation of subsets of miRs is a common finding in many of these studies, indicating that some of these miRs may act as putative tumor suppressor genes. Gene silencing is the most thoroughly studied role of miRs, however, they can upregulate gene transcription during cell cycle arrest [5] and, therefore, overexpression of miRs in human cancers suggested potential oncogenic functions of miRs. In addition, some miRs bind directly to 5′-UTR of target genes and activate rather inhibit gene expression or induce gene expression by binding to the promoter regions [6].

5.2 Aberrant DNA Methylation

The molecular mechanism underlying regulation of miR expression in cancer is not completely understood. Several mechanisms, for example, specific transcriptional regulation, epigenetic mechanisms including methylation and histone deacetylation, mutations affecting proteins involved in the processing and maturation of miRs, or changes in miR stability have been proposed. Cancer cells undergo global DNA hypomethylation [7], which contributes to genome instability. This leads to transcription of silenced transposable sequences, furthering chromosomal rearrangements and genome disruption, a characteristic feature of tumor promotion. Contrary to global hypomethylation, CpG islands of the promoter regions of tumor suppressor genes experience DNA hypermethylation leading to gene silencing and tumor promotion [8]. DNA methylation refers to the covalent addition of a methyl group to the five-position of cytosine usually in a CpG dinucleotide in differentiated cells. Epigenetic mechanisms accounting for the observed down-regulation of tumor suppressive miRs

in human cancer are CpG island hypermethylation or histone modifications in a similar manner that is now well accepted for classic tumor suppressor genes. MiRs that are transcribed from CpG islands undergo DNA hypermethylation-coupled repression due to binding of the transcriptional repressor methyl CpG binding proteins and histone modifications such as loss of acetylation of histones H3 and H4 [9]. About 16 % of the annotated human miRs are located within 1,000 bp of a CpG island and thus epigenetic regulation of miRs might be more common than reported so far.

5.3 Epigenetically Silenced miRs in Colon Cancer

The list of miRs that undergo CpG island promoter hypermethylation in CRC is growing rapidly [10, 11]. A recent epigenomic survey of 450,000 CpG sites revealed that 30–40 miRs underwent hypermethylation in CRC cells [11]. Epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transitions occur in tumorigenesis. EMT is a complex process that includes disbanding of cell-cell junctions, and loss of apicobasal polarity, which gives rise to migratory and invasive mesenchymal cells. During this process loss of adhesion proteins such as E-cadherin and γ-catenin and gain of mesenchymal proteins such as Vimentin and N-cadherin is invariably observed. The role of E-cadherin repressors such as SNAIL, ZEB1, ZEB2 and E47 is very well established. Once metastasized, mesenchymal cells require MET conversion for proliferation of dispersed cancer cells at secondary locations. It has been suggested that methylation dependent regulation of miR-200 family plays a crucial role in EMT and MET [12]. The putative DNA methylation regulated inactivation of various miR-200 members has been described in cancer [13]. The miR-200 family is consists of five members that can be divided into two clusters: miR-200a/b/429 on chromosome 1 and miR-200c/141 on chromosome 12. The miR-200 family is recognized as a key regulator of the epithelial phenotype by targeting ZEB1 and ZEB2, two important transcriptional repressors of the E-cadherin (cell adherence) and *CRB3* and *LGL2* (polarity) genes. The miR-200a/b/429 and miR-200c/141 transcripts endure an active epigenetic regulation linked to EMT or MET phenotypes in tumor development. The 5′-CpG islands of both miR-200 loci are normally unmethylated and linked to epithelial features, such as low expression of ZEB1/ZEB2 and high levels of E-cadherin, CRB3 and LGL2. However, 5′-CpG island hypermethylation-associated silencing is observed in transformed cells with mesenchymal characteristics. The stable transfection of miR-200a/b/429 and miR-200c/141 in the hypermethylated cells reinstated the epithelial markers with inhibition of cell migration, tumor growth and metastasis in nude mice. Furthermore, the miR-200 epigenetic silencing is a dynamic process, which can be shifted to hypermethylated or unmethylated 5′-CpG island status corresponding to the EMT and MET phenotypes, respectively. Laser micro-dissection revealed that normal colonic crypts (epithelia) and stroma (mesenchyma) are unmethylated and methylated at these loci, respectively. However, the colorectal tumors undergo selective miR-200 hypermethylation in epithelia during EMT. These findings signify that the epigenetic manipulability of the miR-200 family contributes to the adapting phenotypes of CRC [13]. Other studies also

demonstrated that DNA methylation dependent silencing of miRs is involved in the metastatic phenotype [14]. Treatment of cancer cells with DNA methylation inhibitor exhibited cancer-specific hypermethylation of the CpG islands in the promoter regions of miR-148a, miR-9 family and miR-34b/c [15]. Mir-34 family, tumor suppressor miR family, is regulated by transcriptional factor p53 and DNA methylation [16]. Metastatic genes such as c-Myc, cyclin-D kinase-6 (CDK6) and E2F3 are target genes of miR-34b/c whereas TGIF2 is target of miR-148a.

A recent study first identified 23 miRs that are down-regulated in human primary CRC compared with matched normal colorectal epithelium [17]. Of the 23 miRs down-regulated in CRC, 5 of them miR-9, miR-124, miR-129, miR-137 and miR-149 are located with-in 1,000 bp of a CpG island. Treatment with DNA methyltransferase and HDAC inhibitors restored the expression of 3 of the 5 miRs in 3 colon cancer cell lines. Subsequently it was shown that miR-9-1, miR-129-2 and miR-137 genes were silenced in CRC and this was mediated at least in part by epigenetic mechanisms including DNA hypermethylation and histone acetylation [18]. After treatment with HDAC inhibitor, CRC cell lines were subjected to chromatin immunoprecipitation with an antibody against acetylated H3 (a mark of transcriptional active chromatin) followed by Q-PCR. Results demonstrated an increase in AcH3 in the case of miR-9-3 and miR-127 but no significant changes in miR-9-1, miR-129-2 and miR-137. These results indicated that expression of mature miR-9 could be regulated by hypermethylation of miR-9-1 locus and H3 de-acetylation of the miR-9-3 locus [18]. The association between miR-9-1 methylation and more advanced stages and nodal involvement suggested a role of miR-9-1 as a potential tumor marker of poor prognosis in CRC. However, the precise functions and targets of these miRs in CRC were not investigated [18]. Another study also exhibited that miR-137 is hypermethylated in the early stages of CRC and targets CDK6, lysine-specific histone demethylase-1 (LSD-1) and E2F6, a transcription factor, which is involved in cell cycle [19].

Intronic regions of coding genes can also be transcribed into miRs and this may also explain that tumor phenotype is due to the hypermethylation of a gene that in turn down regulates miR it harbors. The example of this scenario is miR-342 that is transcribed from EVL gene. The EVL gene affects cell polarity, cytoskeleton remodeling and cell motility. The EVL promoter is hypermethylated in the early stages of CRC and thus the gene and miR-342 both are downregulated [20]. The EVL/miR-342 locus is methylated in 86 % of colorectal adenocarcinomas and in 67 % of adenomas, indicating that it is an early event in colorectal carcinogenesis.

The genetic disruption by homologous recombination of DNA Methyltransferase1 (DNMT1) and DNMT3b (DKO, double knock out) of wild type colon cancer cell line HCT-116 showed a drastic reduction of DNMT activity, 5′-methylcytosine DNA content, and, most significant, a release of gene silencing associated with CpG island hypermethylation [9]. Further, the comparison of miR expression profile of HCT-116 wild type cells with DKO cells established that epigenetic silencing of the tumor-suppressor miR-124a by aberrant DNA hypermethylation leads to CDK6 overexpression a bona fide oncogenic factor [9]. Transfection of the tumor cell line with miR-124a reduces CDK6 protein levels and retinoblastoma (Rb) phosphorylation, one of the targets of CDK6 [9]. Rb, an antiproliferative tumor-suppressor gene when phosphorylated by CDK6, abrogates its antiproliferative activity.

A recent study identified silencing of DNA methylation-regulated miR-941, miR-1237 and miR-1247 in CRC cells [21]. Ectopic expression of miR-1247 significantly reduced cancer cell proliferation and migration in HCT-116 and DLD-1 cells suggesting that miR-1247 may work as tumor suppressor. Several predicted targets of miR-1247 were proposed, for example, Citron (CIT), a serine threonine kinase that regulates G2/M transition in cell cycle, FosB that dimerizes with Jun protein to activate transcription and transmembrane glycoprotein ADAM15, which has a role to play in cancer metastasis. These computationally predicted targets need validation in future studies. Overexpression of miR-941 significantly inhibited cell migration in both HCT-116 and DLD-1 cells. Predicted target for miR-941 was metallopeptidase24 (MMP24), which facilitates tissue remodeling and cell migration [21].

Treatment of colon cancer cell lines with 5-aza-2′-deoxycytidine, a methylation inhibitor resulted in greater than two-fold increased abundance of 10 miRs [22]. Amongst them transfection of miR-373 in colon cancer cells resulted in decreased cell proliferation. In patient biopsies, miR-373 was down regulated in colon cancers (29/40, 72.5 %) relative to control samples, whereas a member of Ras oncogene family RAB22A, predicted target gene of miR-373 was upregulated (24/40, 60 %). Aberrant methylation of miR-373 in colon cancers (35/40, 87.5 %) relative to controls (8/40, 20 %) was also noted [22]. These studies established that one mechanism accounting for the transcriptional down regulation of miRs in human cancer is CpG island hypermethylation.

5.4 Methods

The mirVana RNA isolation (Ambion, Texas) or Exiqon miRNA isolation kits are frequently used for miR isolation and enrichment. TaqMan real time QPCR for miR quantification and microarrays are commonly used for miR profiling. The gold standard method to evaluate the methyl status of cytosine residues is bisulfite sequencing. For this DNA is treated with sodium bisulfite (CpGenome DNA modification kit) and then sequenced using primers specific to bisulfite sequence. These methods, routinely used in miR research, are discussed in detail earlier [23]. Mature miR mimics are transiently transfected or miR genes are stably transfected to increase miR expression levels in cancer cells. To confirm target genes of miR, luciferase vectors (empty luciferase vector or luciferase vector containing wild-type or mutant-type target gene 3′-UTR) are transfected for luciferase reporter assays.

5.5 Translational Applications and Future Directions in MiR Research

The DNA methylation profile of miRs can delineate tumor type, clinical prognosis and treatment response. miRs have great potential at the diagnostic and therapeutic levels. miRs circulate in blood stream in a stable form and thus can function as early

biomarkers in a clinical setting. More than 100 studies have been reported so far to assess the potential role of serum and plasma miRs as biomarkers for different types of cancer [24]. To improve CRC diagnosis, use of miR DNA methylation in biological fluids such as colorectal mucosal wash fluids [25] and feces [26] has been described. On the basis of clinical trials, DNA methyltransferase inhibitor azacytidine (Vidaza™) was approved for the treatment of myelodysplastic syndrome. Azacytidine is being evaluated in clinical trials for other malignant diseases. Similarly, Vorinostat (Zolinza™) and Romidepsin (Isotodax™) both novel HDAC inhibitors have been approved for the treatment of refractory cutaneous T cell lymphoma [24]. More studies are required to find out whether these epigenetic drugs would modulate miR expression in colon cancer.

Information about miRs profiling and/or single nucleotide polymorphism (SNP) have been used in colon cancer epidemiology studies [27–32]. For example, high miR-21 expression in colon adenocarcinoma was associated with a low survival rate and resistance to therapy in 196 patients [32]. Another study demonstrated role of miR-106a in prediction of survival of 110 colorectal cancer patients [27]. The prognostic value of miR-215 was noted in stage II and III of colon cancer when patient's samples were analyzed by quantitative PCR [28]. MiR-143 was noted as a novel prognosticator and a promising drug target in 77 colorectal patients with KRAS mutations and undergoing EGFR targeted therapy with the monoclonal antibodies cetuximab or panitumumab [30].

A SNP rs2737 in the IC53 gene (gene associated with the grade and depth of adenocarcinoma) affected expression of miR-379, and this polymorphism was found to be associated with late onset of colorectal cancer in 222 patients [33]. Thus, miRs may have role in cancer protection. However, case–control study (Central-European Caucasian population) evaluated SNPs in miR-196a, miR-27a, and miR-146a and their association with CRC, but no significant association was observed [34]. Polymorphism rs4919510 in miR-608 was associated with CRC outcome but not with risk [35]. MiRs usually regulate gene expression by binding to the 3′-UTR of mRNAs. Due to the stringent recognition requirement needed by the miR and the binding region on its target gene, it is quite conceivable that SNPs could have functional implications on the post-transcriptional regulation of target genes. A SNP could either weaken a known miR target or create a sequence match to the miR that was not previously associated with the given mRNA. Changes in the expression pattern of a gene could therefore influence a person's risk of disease.

Altered miR expression is a hallmark of a number of tumor types including CRC. Hundreds of miRs have been identified to date; however, computer models suggest there may be thousands more. As bench-work continues to verify *in silico* predictions, miR profiling will remain a prominent tool for identification of differentially expressed miRs in normal cellular courses and human disorders including CRC. Now, the results should be validated in a large number of samples collected from a variety of geographically dispersed populations. As with other cancers, screening of populations at high risk of developing CRC, using miR profiling might be the first step towards preventing these cancers. Emphasis should be placed on the identification of polymorphisms in the double-stranded region of CRC specific miRs. Since the cost of high throughput sequencing has decreased significantly, this objective is now

Table 5.1 Selected miRs in colon cancer and their characteristics

Micro RNAs	Characteristics
miR-1	Under-expressed [36]; inhibits cell proliferation and viability [37]
miRs-122, -214, -372, -15b, let-7e, -17	Differentially expressed and affect p53 pathway [38]
miR-195	Down-regulated in CRC and correlates with lymph node metastasis and poor prognosis [39]
miRs-15b, -181b, -191, -200c	Over-expressed [40]
miR-499	Under-expressed [41]
miR-9-1	Expression is inversely associated with its promoter methylation; associated with lymph node metastasis [18]
miR-21	Acts as an oncomiR; inflammation-mediator in CRC [42]; interacts with PTEN/PI-3 K/Akt signaling pathway [43]; over-expressed in high-risk stage II CRC patients [44]
miRs-34a, -34b/c	Inactivation due to promoter methylation [45]; in Wnt-signaling [46]; regulate Axl receptor expression [47]
miR-92	Higher levels in adenomas and carcinomas than other miR-17-92 cluster members (miR-17, miR-18a, miR-19a, miR-19b, miR-92a) [48]
miRs-31, -223	Over-expressed in CRC of patients with hereditary non-polyposis colorectal cancer syndrome (Lynch Syndrome) [49]
miRs-192, -215, -26b, -143, -145, -191, -196a, -16, let-7a	Under-expressed in CRC [49]
miRs-31, -183, -17-5p, -18a, -20a, -92	Over-expressed in CRC [50]
miR-135b	Correlated with the degree of malignancy [51]
miRs-105, -549, -1269, -1827, -3144-3p, -3177, -3180-3p, -4326	Upregutaed in CRC [52]
miR-126	Under-expressed in CRC [53]
miR-129	Regulates cell proliferation; interacts with Cdk6 [54]
miRs-17-92 cluster, miRs-21, -135	Could be detected in exfoliated colonocytes isolated from feces for CRC screening; upregulated in CRC [55]; interaction of miR-135 with APC expression and Wnt pathway [56]
miRs-182, -17, -106a, -93, -200c, -92a, let-7a, -20a	Upregulated in CRC [57]
miRs-215, -375, -378, -422a	Decreased in CRC [58]
miRs-127-3p, -92a, -486-3p, -378	Down-regulated in KRAS mutation positive samples [59]
miR-200c	Involved in epithelial to mesenchymal transition [12]

achievable and few reports are already available [34, 35]. For future research, we should take into account miRs and their regulatory networks in order to understand the complex processes underlying malignant transformation. Additional research investments are needed in this area of science (Table 5.1).

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