

Role of MicroRNA Molecules in Colon Cancer Etiology

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Abstract

The biogenesis of micro (mi) RNA suggests that nearly 3% of human genes are encoded for micro (mi) RNAs, and computer predictions indicate that more than 30% of protein coding genes in humans are coded by miRNAs through an imperfect binding to the 3' untranslated region (UTR) of target messenger (m) RNA affecting gene silencing and leading to either transcription repression, or induction of messenger (m) RNA degradation. The expression of several miRNAs in noninvasive body fluids/excrements has been linked to development of colorectal cancer (CRC) and its progression. The majority of miRNA genes are oriented antisense to neighboring genes, and miRNA genes are transcribed by polymerases II and III. The rate of evolution of miRNAs has been very slow, which has permitted morphological innovation by making gene expression specific, a process that permitted the genesis of complex organisms. Bacteria lack true miRNAs. Bioinformatics approaches to predict putative miRNA target genes has been facilitated by finding that miRNA target recognition is partly based on simple sequence complementarity, and exact base pairing between miRNAs and their targets is required only in the first six to eight bases from the 5' end of the miRNA.

Keywords: Gene expression; Histopathology; Metastasis; Risk factors; Staging

Introduction

CRC is the 2nd and 3rd most common malignancy worldwide in men and women, respectively, in developing and developed countries, including USA and Europe, with an estimated one million new cases and half million deaths yearly [1,2]. In USA, CRC represents 10% of incident cancers and cancer deaths. About 6% of the population will develop CRC in their lifetime. In 2009, 102,900 new cases and 51,370 deaths were estimated [3,4] (Table 1). Globally, there are about 1 million new cases and about 500,000 deaths/year, and these numbers are destined to dramatically increase because of worldwide adoption of

a Western-type diet, i.e., diet rich in calories, carbohydrates and fats, and poor in essential nutrients, vitamins and minerals [5,6]. Although CRC incidence has declined from about 60 per 100,000 in 1975 to ~ 50 per 100,000 in 2004, and the decrease in USA mortality has recently accelerated, it has changed little for African American men and women [7-9] (Table 2). Moreover, data show that early-onset carcinomas (<40 years) have increased during the past four decades and pathologic features became more biologically aggressive [10,11]. Five-year survival rates for CRC are strikingly different by stage, ranging from 90% for localized-disease to 10% for distant-disease (www.cdc.gov), clearly arguing for an early detection for this cancer. In spite of the billions of dollars spent in the USA over the last three decades, there is currently no validated reliable sensitive or specific biomarker for CRC screening [12].

Test Specification	FBOT● Guaiac Immunologic	Methylated gene■ & chromosomal loci	Promoter◆ methylation	Mutated DNA markers	Colonoscopy examination▲	Proteomic Approach▶	mRNA miRNA Approach▼
Noninvasive	Yes Yes	Yes	Yes	Yes	No	Yes	Yes Yes
Sensitivity	10.8%†16.3%†	87.5%■	31%†	18.2%	87%	75%	>80% >90%¶
Specificity	95% 94.5%	82%	95%	94.4	100%	>95%	>95% >95%¶
Automation	No No	No	No	No	No	Yes	Yes Yes
Cost	\$15‡ \$25‡	\$400‡	\$150‡	\$695‡	\$900‡	\$650‡	\$250‡ \$100‡

Table 1: Comparison of tests employed for premalignant† and malignant human colon cancer screening. †For polyps ≥ 1 cm in diameter, villous or tubovillous, or the presence of high grade dysplasia, ●,◆ from refs 13,, 22, 44; ■Only for advanced cancer, but not adenoma, based on vimentin gene, DY loci 5p21 and OC91199, from refs 39, 40; ◆Based on one gene, from ref. 37; ■From refs 36, 38, 41, 42; ▲From refs. 23, 24, 222, 223; ▶From refs 42, 224; ¶ Based on our data, ref. 35; ▼Based on our miRNA data, ref. 25, ¶preliminary miRNA results are based on a limited

number of genes and a small sample size; †Estimates are based on contacts with other test developers, and our experience. with assay requirements and developments.

Estimated New Cases**				Estimated Deaths***		
Cancer	Both Sexes	Males	Females	Both Sexes	Males	Females
Colon	102900	49,470	53,430	51,370	26, 580	24,790
Rectum	40,870	23,580	17,290	†	†	†

Table 2: Estimated New Cancer Cases & Deaths by Sex due to CRC in the USA in 2009* Source: American Cancer Society (www.cancer.org).

The traditional understanding of the evolution of CRC was based on a model emphasizing progression from an adenoma to a carcinoma stage through genetic alterations involving tumor suppressor genes [13]. An alternative pathway has been the serrated one, which involves hyperplastic polyps or related lesions; however, our understanding of this newer model is currently incomplete [14].

Differences between colon and rectal cancer

Epidemiologic evidence suggests that colon cancers (CCs) and rectal cancers (RCs) differ in their morbidities and etiologies. RC is more common in Asia (China) where it accounts for over 50% of CRC, compared with <30% in Western countries [15]. In contrast, CC was shown to account for over 60% of CRC cases in the USA and Europe, is related mostly to environmental factors (e.g., consumption of fatty foods and less exercise) and to a Caucasian ethnic origin [16-19], all are factors which collectively suggest differences in carcinogenesis between CC and RC. Moreover, several structural and molecular studies have also indicated differences in etiology, clinical manifestation, pathological features and genetic abnormalities between CC and RC [20-22].

Studies using molecular biology assays have found that tumor suppressor genes, point mutations and genetic instability due to epigenetic components differ according to the subsite colorectum. CC has been reported to more likely have CpG island methylator phenotype and k-ras mutations, whereas rectal and distal colon tumors are more likely to have p53 and APC mutations [23-26]. Gene hybridization techniques have shown amplification of 20q in CC, compared with amplification of 12p in RC [27]. Another study indicated significant differences between rectal and colon cancer in the amplification of genes for cell cycle such as cyclin-A2, -B1, -D1 and -E [28]. An omic study using Illumina HT-12 V4.0 Expression Bead chip oligonucleotide microarrays (San Diego, CA) found RC to be more complex than CC as 676 genes related to 11 signal pathways were differentially expressed in CC development, compared to 1,789 genes related to 30 signal pathways altered in RC, with 824 common differentially expressed genes up- or down-regulated in both CC and RC [15] all are facts that have collectively lead to the conclusion that colon and rectal cancers represent two distinct types of tumors. In this review, we have focused on colon cancer because it is more prevalent in the USA.

Public health implications

Mortality and morbidity from CRC represent a major health problem involving a malignant disease that is theoretically preventable through screening. Early detection would be greatly enhanced if accurate, practical and cost effective diagnostic biomarkers for this malignancy were available. Current screening methods lack sensitivity (e.g., fecal occult blood test, FOBT) [17-19], costly and have low compliance, or could result in mortality (e.g., colonoscopy) [20,21]. Our data and others [22 -34] have shown that quantitative changes in the expression of few miRNA genes in tissue, stool or plasma that are associated with colon cancer would permit development of more sensitive and specific CRC molecular markers than those currently available on the market for a cancer that is deadly if not diagnosed before metastasis. Using stable molecules such as miRNAs that is not easily degradable when extracted from stool or blood and manipulated thereafter, a miRNA -approach is preferable to a transcriptomic mRNA-, mutation DNA-, epigenetic- or a proteomic-based test [35-44]. If performance criteria are met in a validated undertaking, the non-invasive miRNA-based test in a noninvasive medium such as stool, or a semi-invasive medium human blood plasma based on high throughput automated technologies and quantitative expression measurements, commonly used in the diagnostic clinical laboratory, would be advanced to the clinical setting and will make a significant impact on the preventive oncology of CRC. Table 3 presents a comparison of tests that are currently in use, or those under development for CRC screening, compared to a proposed test using miRNAs after further development and validation of this test.

Race/Ethnicity	Men				Women			
	Local	Regional	Distal	Unstaged	Local	Regional	Distal	Unstaged
Non-Hispanic White	42	35	19	4	40	36	19	5
Non-Hispanic African American	36	34	25	5	36	34	24	6
Asian American/Pacific Islander	40	37	19	4	38	41	18	3
American Indian/Alaska Native	36	37	24	3	40	39	17	3
Hispanic	37	36	22	5	37	36	21	5

Table 3: Colorectal Cancer Stage Distribution (%) by Race/Ethnicity, 1999-2006 Source: Reference (5) Alekruse S, Kosary C, Krapcho M et al. eds (2010) SEER Cancer Statistics Review 1975-2007, <http://seer.cancer.gov/csr/1975-2007/>, based on November 2009 SEER data submission, posted on the SSR web site, April 2010, Bethesda, MD, NCI.

MiRNAs discovery, nomenclature, biogenesis, function and evolution

MiRNAs are small RNA molecules that regulate gene expression post-transcriptionally. The first miRNA was discovered in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum while working on genes involved in developmental timing in the millimeter-long roundworm *Caenorhabditis elegans* [45,46]. One of the genes, termed *lin-4*, did not encode a protein, but instead a novel 22-nucleotide small RNA. Seven years later (year 2000), Reinhart and his colleagues discovered a second 22-nucleotide small RNA of the same type, *let-7*, which repressed *lin-41*, *lin-14*, *lin-28*, *lin-42* and *def-12* expression during developmental stage transition in *C. elegans* timing [47]. Homologs of *let-7* gene were identified in other animals, including humans [48]. The conservation of *let-7* across species suggested an important biological role for this small RNA and pointed out to the existence of a wider phenomenon. At that time, the mechanism of RNA interference (RNAi) linking miRNA and RNAi pathways and showing that both mechanisms share common components was discovered. In the following year, more than 100 additional small regulatory RNAs resembling *lin-4*, and *let-7*, now named miRNAs, were identified in worms, the fruit fly and humans [49-51]. Subsequently many more miRNAs were also identified in DNA viruses [52], algae [53], and almost all multicellular organisms such as flowering plants, worms, flies, fish, frogs and mammals [54,55]. Computer predictions of miRNA targets suggest that ~ 30% of human protein-coding genes are regulated by miRNAs [56,57]. These discoveries culminated in 2007 to the award of the Nobel Prize in Physiology and Medicine to Andrew Fire and Craig Mello. Today, more than 1000 human miRNAs have been experimentally identified. Computer predictions of miRNA targets suggest that about 3% of human genes encode for miRNAs, and in excess of 30% of human protein coding genes are regulated by miRNAs [58], through imperfectly binding to the 3' untranslated region (UTR) of target mRNAs resulting in prevention of protein accumulation by either transcription repression, or by inducing mRNA degradation, making miRNAs as one of the most abundant classes of regulatory genes in humans.

MiRNAs are sequentially processed from longer precursor molecules that are encoded by the miRNA genes [59]. A miRNA gene is written as prefix (*mir*) to distinguish it from the corresponding mature miRNA (called miR) followed by a dash and a number (e.g., *mir-1* versus miR-1) to indicate the order of naming. MiRNAs with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case. For example, miR-123a would be closely related to miR-123b. Pre-miRNAs that lead to 100% identical mature miRNAs but that are located at different places in the genomes are indicated with an additional dash-number suffix. For example, the pre-miRNA has-mir-194-1 and has-mir-194-2 lead to an identical mature miRNA (has-miR-194) but are located at different regions of the genome. Species of origin is designated with a three-letter prefix, e.g., has-miR-123 is a human (*Homo sapiens*) miRNA and oar-miR-123 is a sheep (*Ovis aries*) miRNA. Other common prefixes include "V" for a miRNA encoded by a viral genome, and "d" for the fruit fly *Drosophila*. When two mature miRNAs originate from opposite arms of the same pre-miRNA, they are denoted with a -3p (sense) or -5p (antisense) suffix. When relative expression levels are known, an asterisk following the name indicates a miRNA expressed at low levels relative to the miRNA in the opposite arm of the hairpin. For example, miR-123 and miR-123* would share pre-miRNA, but more miR-123 would be found in the cell [60].

The majority of characterized miRNA genes are intergenic (or oriented antisense to neighboring genes) and are therefore suspect to be transcribed as independent units [61]. On the other hand, a miRNA gene could be transcribed with its host gene, providing a mean for coupled regulation of miRNA and protein-coding gene [62]. As much as 40% of miRNA genes may lie in the introns of protein and non-protein coding genes, or even in exons of long nonprotein-coding transcripts, which are usually found –although not exclusively– in the same orientation, and are thus often regulated together with their host genes [63]. Other miRNA genes showing a common promoter include the 42-48% of all miRNAs originating from polycistronic units containing multiple discrete loops from which mature miRNAs are processed [64], although this does not imply that the mature miRNAs of a family will be homologous in structure and function. These promoters have similarities in their motifs to promoters of other genes transcribed by RNA polymerase II such as protein coding genes [65]. Six percent of human miRNAs show RNA editing (IsomiRs), the site-specific modification of RNA sequences, to yield products different from those encoded by this DNA, which increases the diversity and scope of miRNA action beyond that implicated from the genome alone.

MiRNA genes are usually transcribed by RNA polymerase II (Pol II) [64]. The polymerase often binds to a promoter found near the DNA sequence encoding what will become the hairpin loop of the pre-miRNA. The resulting transcript is capped with a specially modified nucleotide at the 5' end, polyadenylated with multiple adenosines a poly (A) tail, [66] and spliced. Animal miRNAs are initially transcribed as part of one arm of an ~80 nucleotide RNA stem-loop that in turn forms part of a several hundred nucleotides long miRNA precursor termed a primary miRNA (pri-miRNA). When a stem-loop precursor is found in the 3' UTR, a transcript may serve as a pri-miRNA and an mRNA [67]. RNA polymerase III (Pol III) transcribes some miRNAs, especially those with upstream Alu sequences transfer RNAs (tRNAs), and mammalian wide interspersed repeat (MWIR) promoter units. A single pri-miRNA may contain from one to six miRNA precursors. These hairpin loop structures are composed of about 70 nucleotides each. Each hairpin is flanked by sequences necessary for efficient processing.

The double-stranded RNA structure of the hairpins in a pri-miRNA is recognized by a nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8), named so for its association with DiGeorge Syndrome. DGCR8 associates with the enzyme Drosha, which cuts RNA to form the "Microprocessor" complex [68]. In this complex, DGCR8 orients the catalytic RNase III domain of Drosha to liberate hairpins from pri-miRNAs by cleaving RNA about eleven nucleotides from the hairpin base (two helical RNA turns into the stem). The product resulting has a two-nucleotide overhang at its 3' end : it has 3' hydroxyl and 5' phosphate groups. It is often termed precursor-miRNA (pre-miRNA). Pre-miRNAs that are spliced directly out of introns, bypassing the Microprocessor complex, are known as "mitrons". Although originally thought to exist only in *Drosophila* and *C. elegans*, mirtrons are now found in mammals [69]. Perhaps as many as 16% of pri-miRNAs may be altered through nuclear RNA editing [70]. RNA editing can halt nuclear processing (for example, of pri-miR-142), leading to degradation by the ribonuclease Tudor-SN and alter downstream processes including cytoplasmic miRNA processing and target specificity (e.g., by changing the seed region of miR-376 in the central nervous system) [71]. Pre-miRNA hairpins are exported from the nucleus in a process involving the nucleocytoplasmic shuttle Exportin 5 a member of the

karyopherin family, which recognizes a two-nucleotide overhang left by the RNase III enzyme Drosha at the 3' end of the pre-miRNA. Exportin-5-mediated transport to the cytoplasm is energy-dependent, using GTP bound to the Ran protein [72]. In cytoplasm, the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer, yielding an imperfect miRNA:miRNA* duplex about 22 nucleotides long. Overall hairpin length and loop size influence the efficiency of Dicer processing, and the imperfect nature of the miRNA:miRNA* pairing also affects cleavage. Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact.

Two ribonuclease enzymes, Drosha and Dicer, process the primary transcript (pri-miRNA) to generate the mature miRNAs. The primary transcripts contain one or more double-stranded stem-loop RNA structures of ~ 70 nucleotide sequence bases that can fold back on itself to form a double helix with a region of imperfect base pairing that forms an open loop at the end (Figure 1). The enzyme Drosha (Pasha in plants) excises the stem-loop structure to form the precursor miRNA (pre-miRNA) [71]. After export into the cytoplasm, the pre-miRNA is cleaved by the ribonuclease Dicer to form a short RNA duplex [72]. Following untwisting one RNA strand becomes the mature single-stranded miRNA, while the complementary strand (called miRNA*) is rapidly degraded (Figure 2). MiRNAs recognize their targets based on sequence complementarity in which a mature miRNA is partially complementary to one or more mRNAs [73]. In humans, the complementary sites are normally within the 3'-untranslated region of target mRNA. The mature miRNA forms a complex with proteins known as RNA-induced silencing complex (RISC). The miRNA incorporated into the silencing complex binds to target mRNA by base pairing, a process that causes inhibition of protein translation and/or degradation of mRNA [74] (Figure 3), leading to inhibition of protein translation of target genes, whereas mRNA levels are infrequently degraded or cleaved [59] by imperfectly binding to the 3' UTR of target mRNAs resulting in prevention of protein accumulation by either transcription repression, or by inducing mRNA degradation. Each miRNA generally targets hundreds of conserved mRNAs and several hundreds of nonconserved targets that operate in a complex regulatory network, and it is predicted that miRNAs together regulate thousands of human genes, as well as regulating protein expression.

RISC is also known as a miRNA ribonucleoprotein complex (miRNP); RISC with incorporated miRNA is sometimes referred to as "miRISC. Dicer processing of the pre-miRNA is thought to be coupled with unwinding of the duplex. Generally, only one strand is incorporated into the miRISC, selected on the basis of its thermodynamic instability and weaker base-pairing relative to the other strand [74]. The position of the stem-loop may also influence strand choice [75]. The other strand, called the passenger strand due to its lower levels in the steady state, is denoted with an asterisk (*) and is normally degraded. In some cases, both strands of the duplex are viable and become functional miRNA that target different mRNA populations' strand [72,76].

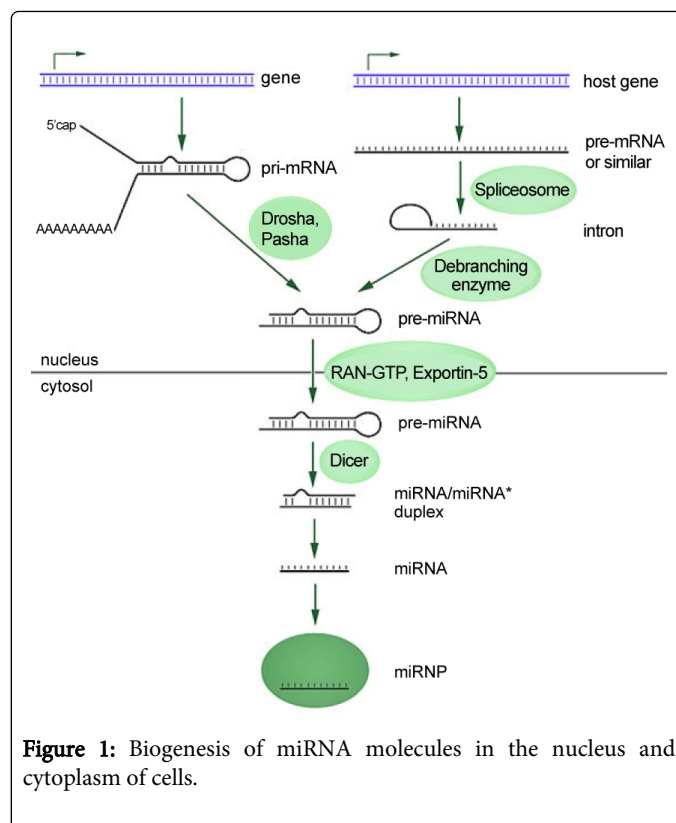


Figure 1: Biogenesis of miRNA molecules in the nucleus and cytoplasm of cells.

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Members of the Argonaute (Ago) protein family are central to RISC function. Argonautes are needed for miRNA-induced silencing and contain two conserved RNA binding domains: a PAZ domain that can bind the single stranded 3' end of the mature miRNA and a PIWI domain that structurally resembles ribonuclease-H and functions to interact with the 5' end of the guide strand. They bind the mature miRNA and orient it for interaction with a target mRNA. Some argonautes, for example human Ago2, cleave target transcripts directly; argonautes may also recruit additional proteins to achieve translational repression [77]. The human genome encodes eight Argonaute proteins divided by sequence similarities into two families: AGO (with four members present in all mammalian cells and called EIF2C/hAgo in humans), and PIWI (found in the germ line and hematopoietic stem cells) strand [77,78].

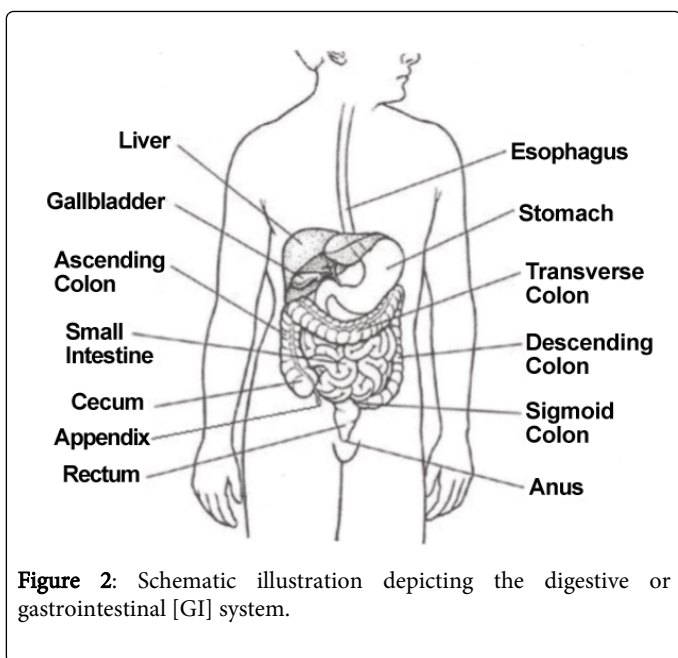


Figure 2: Schematic illustration depicting the digestive or gastrointestinal [GI] system.

Additional RISC components include TRBP (human immunodeficiency virus) (HIV) trans activating response RNA (TAR) binding protein, protein activator of the interferon induced protein kinase (PACT), the SMN complex, fragile X mental retardation protein (FMRP), Tudor staphylococcal nuclease-domain-containing protein (Tudor-SN), the putative DNA helicase MOV10, and the RNA recognition motif containing protein TNRC6B [78]. Gene silencing may occur either via mRNA degradation or preventing mRNA from being translated. If there is complete complementation between the miRNA and target mRNA sequence, Ago2 can cleave the mRNA and lead to direct mRNA degradation. Yet, if there isn't complete complementation, the silencing is achieved by preventing translation [76,79].

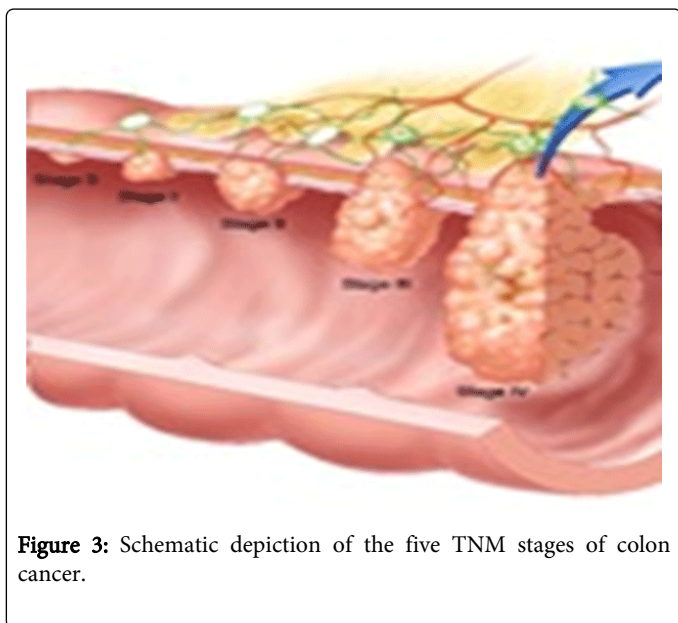


Figure 3: Schematic depiction of the five TNM stages of colon cancer.

Turnover of mature miRNA is needed for rapid changes in miRNA expression profiles. During miRNA maturation in the cytoplasm,

uptake by the Argonaute protein is thought to stabilize the guide strand, while the opposite (* or "passenger") strand is preferentially destroyed. In what has been called a "use it or lose it" strategy, Argonaute may preferentially retain miRNAs with many targets over miRNAs with few or no targets, leading to degradation of the non-targeting molecules [80]. Decay of mature miRNAs in *C. elegans* is mediated by the 5'-to-3' exoribonuclease XRN2 [81]. In plants, SDN (small RNA degrading nuclease) family members degrade miRNAs in the opposite (3'-to-5') direction. Similar enzymes are encoded in animal genomes, but their roles have not yet been elucidated [82].

Several miRNA modifications affect miRNA stability. Uridylation of some animal miRNAs has been reported and both plant and animal miRNAs may be altered by addition of adenine (A) residues to the 3' end of the miRNA. An extra A added to the end of mammalian miR-122, a liver-enriched miRNA important in Hepatitis C, stabilizes the molecule, and plant miRNAs ending with an adenine residue have slower decay rates [80].

The function of miRNAs in the cells appears to be gene regulation. For that purpose, a miRNA is complementary to a part of one or more mRNAs. Animal miRNAs are usually complementary to a site in the 3'-UTR, whereas plant miRNAs are usually complementary to coding regions of mRNAs. Perfect or near perfect base pairing with the target RNA promotes cleavage of the RNA, as often occurs in plant miRNAs, whereas animals miRNAs more often have only partly the right sequence of nucleotides to bond with the target mRNA [83]. The match-ups are imperfect. For partially complementary microRNAs to recognize their targets, nucleotides 2-7 of the miRNA (its 'seed region') still have to be perfectly complementary. Animal miRNAs inhibit protein translation of the target mRNA [84]. MiRNAs that are partially complementary to a target can also speed up deadenylation causing mRNAs to be degraded sooner [85]. While degradation of miRNA-targeted mRNA is well documented, whether or not translational repression is accomplished through mRNA degradation, translational inhibition, or a combination of the two is hotly debated. Recent work on miR-430 has shown that translational repression is caused by the disruption of translation initiation, independent of mRNA deadenylation [86]. MiRNAs occasionally also cause histone modification and DNA methylation of promoter sites, affecting the expression of target genes [87].

Nine mechanisms of miRNA action are discerned using a unified mathematical model: a) cap-40S initiation inhibition, b) 60S ribosomal unit joining inhibition, c) elongation inhibition, d) ribosome drop-off (premature termination), e) cotranslational nascent protein degradation, f) sequestration in P-bodies, g) mRNA decay (destabilization), h) mRNA cleavage, and i) transcriptional inhibition through miRNA-mediated chromatin reorganization followed by gene silencing (Figure 4). It is often impossible to discern these mechanisms using the experimental data about stationary reaction rates nevertheless; they are differentiated by dynamics and have different kinetic signatures [88].

Unlike plant miRNAs, the animal miRNAs target a diverse set of genes [89]. However, genes involved in functions common to all cells, such as gene expression, have relatively fewer miRNA target sites and seem to be under selection to avoid targeting by microRNAs [90]. Double strands (ds) RNA can also activate gene expression, a mechanism that has been termed "small RNA-induced gene activation" or "RNAa dsRNAs". Targeting gene promoters can induce potent transcriptional activation of associated genes. This was demonstrated in human cells using synthetic dsRNAs termed small activating RNAs

(SaRNAs) [91], as well as for endogenous microRNA [92]. Interactions between miRNAs and complementary sequences on genes and pseudogenes that share sequence homology are thought to be a back channel of communication regulating expression levels between paralogous genes. Given the name "competing endogenous RNAs (ceRNAs)", these miRNAs bind to miRNA response elements on genes and pseudogenes, providing another explanation for the persistence of non-coding DNA [93].

MiRNAs are considered significant phylogenetic markers because of their very low rate of evolution [94]. Their origin may have permitted the development of morphological innovation, and by making gene expression more specific and fine-tunable, this could have permitted the genesis of complex organs [95] and ultimately complex life [96]. Indeed, rapid bursts of morphological innovation have generally been associated with a high rate of miRNA accumulation [94,95]. MiRNAs originate predominantly by the random formation of hairpins in non-coding sections of DNA (i.e. introns or intergene regions), and also by duplication and modification of existing miRNAs [97].

The rate of evolution (i.e. nucleotide substitution) in recently originated miRNAs is comparable to that elsewhere in the non-coding DNA, implying evolution by neutral drift; however, older miRNAs have a much lower rate of change (often less than one substitution per hundred million years) [95], suggesting that once a miRNA gains a function it undergoes extreme purifying selection [97]. At this point, a miRNA is rarely lost from an animal's genome, although miRNAs which are more recently derived (and thus presumably non-functional) are frequently lost [5,95]. This makes them a valuable phylogenetic marker and a possible solution to such outstanding phylogenetic problems as the relationships of arthropods [98]. MiRNAs feature in the genomes of most eukaryotic organisms, from the brown algae [99] to the animals. Latest miRBase release (v20, June 2013), <http://mirbase.org> contains 24 521 microRNA loci from 206 species, processed to produce 30 424 mature microRNA products [100]. Whilst short RNA sequences (50 – hundreds of base pairs) of a broadly comparable function occur in bacteria, bacteria lack true miRNAs [101].

Role of MiRNAs in Cancer and Biomarker Development

Three observations in the early history of miRNAs suggest a potential role in human cancer: a) the earliest miRNAs discovered in *C. elegans* and the *Drosophila* were found to control cell proliferation and apoptosis [102,103], implying that their deregulation could contribute to proliferative diseases such as cancer, b) many miRNA genes were located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common breakpoint regions in the genome, suggesting their involvement in carcinogenesis [104], and c) malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal tissues [105,106]. An unanswered question is whether the altered miRNA expression observed in cancer is a cause or a consequence of malignant transformation?

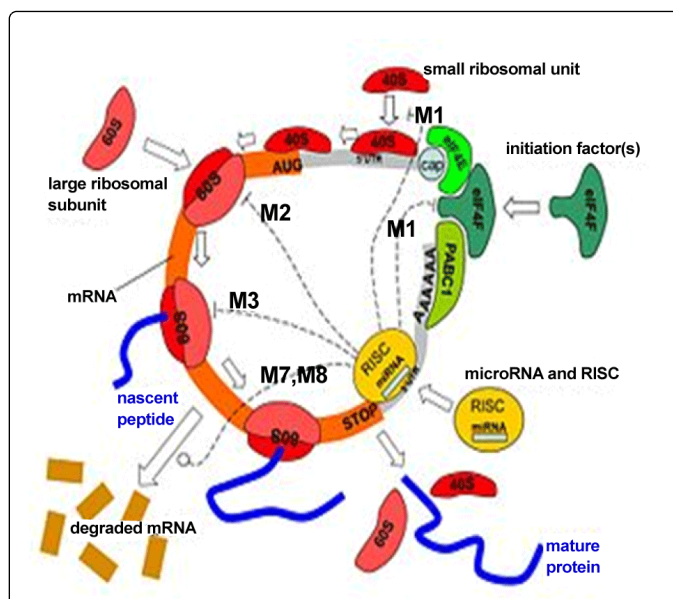


Figure 4: Nine mechanisms of translation repression are shown: M1) on the initiation process, preventing assembling of the initiation complex or recruiting the 40S ribosomal subunit; M2) on the ribosome assembly; M3) on the translation process; M7 and M8) on the degradation of mRNA. There exist other mechanisms of miRNA action on protein translation (transcriptional, transport to P-bodies, ribosome drop-off, co-translational protein degradation and others) that are not visualized here. The 40S and 60S are light and heavy components of the ribosome, 80S is the assembled ribosome bound to mRNA, eIF4F is a translation initiation factor, PABC1 is the Poly-A binding protein, and "cap" is the mRNA cap structure needed for mRNA circularization (which can be the normal m7G-cap or artificial modified A-cap). The initiation of mRNA can proceed in a cap-independent manner, through recruiting 45 40S to IRES (Internal Ribosome Entry Site) located in 5'UTR region. The actual work of RNA silencing is performed by the RISC in which the main catalytic subunit is one of the AGO, and miRNA serves as a template for recognizing specific mRNA sequences.

Deletion on chromosome 13, which is the most frequent chromosomal abnormality in chronic lymphocytic leukemia (CLL), had been suspected for long to contribute to leukemogenesis, although studies had failed to identify a causal gene. Calin and his colleagues observed that the expression two miRNA genes, *mir-15* and *mir-16*, located within this 30-kb deletion in blood samples from patients with chronic lymphocytic leukemia (CLL) were shown to be absent or down regulated in 68% of cases compared to normal tissue or lymphocytes [107], suggesting that the two miRNAs were causally involved in the pathogenesis of CLL. Three reports provided the first mechanistic insight into how miRNAs might contribute to carcinogenesis in 2005. Two independent studies described the relationship between a miRNA cluster, *mir-17-91*, and the Myc oncogenic pathway [108,109], and a third report showed an interaction between *let-7* miRNA and the RAS proto-oncogene [110].

In mammals, miRNAs play key roles in diverse biological processes, including embryogenesis and stem cell maintenance [111,112], hematopoietic cell differentiation [113], development [47,114] and

apoptosis [115], and specific miRNAs are critical in oncogenesis [116], effective in classifying solid [117,118] and liquid tumors [107,113], and serve as oncogenes or suppressor genes [108,119]. MiRNAs have great promise serving as biomarkers for cancer diagnosis, prognosis and/or response to therapy [22–24,106]. MiRNA expression has been found to be deregulated in a wide range of human diseases, including cancer [116]. Profiles of miRNA expression differ between normal tissues and tumor types, and evidence suggests that miRNA expression profiles can cluster similar tumor types together more accurately than expression profiles of protein-coding mRNA genes [22,24,30]. However, it is uncertain whether altered miRNA expression is a cause or a consequence of pathological processes. A study on the role of miRNA and gene expression identified miRNAs that classify molecular cancer subtypes [120]. Although the natural mechanisms for the dysregulation of miRNAs is still largely unknown, one theory tested in colon cancers proposes that DNA hypermethylation leads to down-regulation of certain miRNAs [121].

A global decrease in miRNA levels has been observed in human cancers, indicating these molecules may have an intrinsic function in tumor suppression. Lu et al. [30] in an analysis of 217 human and mouse miRNAs across 334 human cancers, cancer cell lines, and normal tissues [30] were the first to show that the expression levels of many miRNAs were significantly reduced in cancers compared to the corresponding normal tissues. Cancers had significantly reduced global miRNA expression. Poorly differentiated tumors had lower miRNA levels compared with more-differentiated tumors, which lead to the hypothesis that miRNAs functions to drive terminal differentiation and prevent cell division. Global changes in miRNA expression may reflect the degree of cell differentiation [30]. A study that investigated the expression of 241 human miRNAs in NCI-60 panel of human cancer cell lines and in normal tissues confirmed that most miRNAs were expressed at lower levels in human tumor-derived cell lines compared with the corresponding normal tissue [112].

Until recently, it was uncertain whether the altered miRNA expression observed in cancer was a cause or consequence of malignant transformation. Kumar et al. [119] proved for the first time that widespread reduction in miRNA expression promotes carcinogenesis. Global reduction in the production of mature miRNAs through a knockdown of the enzymes Droscha and Dicer in mouse and human cancer cell lines showed a decreased steady-state miRNA level, which was correlated with an enhanced cellular growth *in vitro*. These cells generated faster growing and more invasive tumors compared to controls upon injection into nude mice. To assess the effect of global miRNA loss *in vivo*, the authors deleted the miRNA-processing enzyme Dicer in a mouse model of lung cancer, and showed that these mice had an increased tumor burden, correlated with an increase in tumor number and size, as well as the presence of tumors tumors which were less well differentiated when compared to controls. The study also demonstrated that loss of miRNAs leads to upregulation of proto-oncogenes RAS and c-Myc, although it remains unknown whether loss of all miRNAs is necessary or whether reduction of a subgroup of key tumor is required for tumorigenesis [118].

Transcriptional networks are often deregulated in cancer and may lead to altered transcription of miRNA genes. A study showed that miR-34 is regulated by the p53 transcription factor [115]. The p53 protein, known as the guardian of the genome, regulates the cellular response to stress and cancer, initiating DNA damage. He and colleagues [120] found that miR-34 is directly activated by transcription factor p53 after DNA damage. Expression of miR-34

induces cell cycle arrest and thereby acts together with other effectors of the p53 tumor suppressor network to inhibit cell proliferation. An independent group showed an upregulation of miR-34 by p53 upon DNA damage, which promoted apoptosis [115]. These data indicate that altered expression of miRNAs is not simply a secondary event that reflects the less differentiated state of cancer cells, but at least, in some cases, miRNA expression is specifically driven by oncogenes and tumor suppressor genes.

Transcriptional networks may drive miRNA expression in cancers. Studies by Ma et al. [121] suggested a model by which a pleiotropic transcription factor, Twist, induces expression of miR-10b, which suppresses its direct target and in turn activates a pro-metastatic gene, leading to tumor cell invasion and metastasis in mouse and human breast cancer cells. Moreover, the expression level of miR-10b in primary human breast carcinomas correlated with clinical progression, suggesting that specific miRNAs have a role beyond the tumor-initiation and could directly participate in tumor progression and metastasis.

The transcription of *mir-124a* gene was shown to be inactivated by hypermethylation of its promoter in various human tumors. Epigenetic silencing is a well-known mechanism to inactivate protein-coding genes in cancer cells and could similarly inactivate miRNAs. MiR-127 was found to be highly induced in cultured human cancer cells after treatment with demethylating drugs, suggesting its subjugation to epigenetic silencing through promoter hypermethylation [122].

Two independent groups showed that disrupting the interaction of a single miRNA and its target can produce an abnormal phenotype in mammalian cells. Mayr et al. [123] and Lee and Dutta [124] demonstrated that miRNA function could be regulated through loss of miRNA binding sites in the target gene through chromosomal translocations in a high mobility group A2 (Hmga2) oncogene, leading to loss of the *let-7* miRNA binding sites in its mRNA. Disrupted repression of Hmga2 by *let-7* was shown to promote oncogenic transformation and growth in mammalian cells. Moreover, evidence suggests that miRNAs are regulated indirectly through control of their processing enzymes. Thomson et al. [125] showed that a down regulation of miRNAs in human cancer was not associated with reduced levels of the primary miRNA transcripts, suggesting that regulation of miRNAs could occur during subsequent processing steps, e.g., through altered function of the enzyme Droscha.

Cancers of epithelial and hematopoietic origin show distinct miRNA profiles. A subgroup of gastrointestinal tumors, which arise from endoderm, was distinguished by their miRNA expression patterns. Moreover, tumors within a single cell lineage such as acute lymphoblastic leukemia were further differentiated according to their underlying genetic abnormality into BCR/ABL-positive tumors, T-cell tumors, and those with MLL gene rearrangement. When a miRNA expression profile of 217 miRNAs established to an independent series of 17 poorly differentiated tumors of unknown origin was applied, a correct diagnosis was established in 12 out of 17 of the tumors. On the other hand, transcriptomic gene expression profiling based on ~ 16,000 mRNAs did not accurately classify the tumors [30]. This finding has an important clinical implication because if miRNAs prove useful for clinical diagnosis, their advantage lies in the stability of these molecules compared to easily fragmented mRNAs. Besides, miRNAs are long-lived *in vivo* [126] and very stable *in vitro* [127], which might allow analysis of paraffin-embedded samples for routine diagnostic applications.

Conclusions

Many researchers had long thought that miRNAs played a role in cancer development and progression. In the late 1990s and early 2000, the focus was on stool colonocytes. But in 2008, some investigators noticed that miRNAs could also be detected in the blood, possibly paving the way for their use as cancer biomarkers in a semi-invasive medium [128]. The expression of a number of miRNAs has been linked to different cancers and their progression. But contrary to the stool, the origins of these miRNAs and how they arrive in the blood are not yet fully known [129]. Still, miRNAs as biomarkers of cancer are being validated and are winding their way toward the clinic.

MiRNAs are interesting biomarkers that are functionally important, play a significant role in gene regulation, are amplifiable, and are stable molecules. Moreover, miRNA expression profiles allow for distinguishing malignant and non-malignant tissue, as well as distinguishing different tumor entities, and measuring miRNA molecules provides a lot of information content [130]. There is also a small number of miRNAs, about 1000 validated miRNAs, which gives a global picture of a given tumor, compared to, for example, hundreds of thousands of messenger RNAs. Furthermore, because they are nucleic acids, there are a number of extant tools that can be used to analyze miRNAs, like for example microarrays and PCR techniques. Most circulating miRNAs are associated with Argonaute2, which is part of the RISC silencing complex. But whether these circulating miRNAs come from normal tissue or tumor tissue and how they are released into body fluids — through cell death or some other process — are mostly unanswered questions. It is possible that cell death could cause miRNAs to be released into the blood or other body fluids, or the cells might selectively release miRNAs in vesicles or exosomes. In healthy tissue, there is evidence that cells release miRNAs, both in vesicles and in protein complexes. Those miRNAs can then act as intercellular signaling molecules. If they are taken up by a recipient cell, they could possibly modulate gene expression of that recipient cell. Tumor tissue cells may act similarly as they could release miRNAs that promotes a microenvironment that helps the tumor to survive, giving tumors a selective advantage. Work is needed to determine how or what the balance is between passive release by various ways and release that is programmed within the cell. Host cells, like immune cells, might also be sources of miRNAs in cancer patient. Many circulating miRNAs linked to solid tumors are also expressed in blood cells [24]. It often does not matter where miRNAs come from, as long as they are well-validated markers.

A novel miRNA-profiling based screening assay for the detection of early-stage CRC has been developed and is currently in clinical trials. Early results showed that blood plasma samples collected from patients with early, resectable (Stage II) colorectal cancer could be distinguished from those of sex-and age-matched healthy volunteers. Sufficient selectivity and specificity could be achieved using small (less than 1 mL) samples of blood. The test has potential to be a cost-effective, non-invasive way to identify at-risk patients who should undergo colonoscopy [131].

A number of sophisticated bioinformatics approaches are being developed to predict putative miRNA target genes based on the fact that miRNA target recognition is at least partly based on simple sequence complementarity [132–135]. This has been facilitated by the finding that exact base pairing between miRNAs and their targets commonly appears to be required only in the first six to eight bases from the 5' end of the miRNA. The short nature of the seed region allows a single miRNA to act on up to a hundred different target sites,

and all human miRNAs together may regulate up to one third of protein coding genes [80,135], suggesting suggests a highly complex network of miRNA-target interactions. Another approach to discovering miRNA target genes is to knock out or overexpress a particular miRNA and use conventional microarrays to identify genes that show changes in expression. This approach is based on the observation that some miRNAs can also downregulate mRNA levels in addition to down regulating protein levels of their target genes [136]. An experimental validation of miRNA target sites has been to express a miRNA in vivo while simultaneously expressing and monitoring the target mRNA linked to a reporter gene, i.e., Luciferase [134,137-144].

Recommendations

Most studies carried out for diagnosing the role miRNAs in cancer employed small numbers of subjects. Although there are fewer miRNA than mRNA genes, we and others [140,141] are in the opinion that diagnostic studies employing large numbers of subjects (in the hundreds of individuals) are needed to evaluate potential application of miRNA in early detection and diagnosis of colon cancer [142], whereas for analysis of transcriptomic mRNA expressions, the number of subjects needed will be at least in the thousand because there are much more transcripts than miRNAs. When working with body fluids such as plasma, a challenge is to establish standardized protocols for extracting and quantifying circulating miRNAs. However, thus far it has been difficult to do so as the technology keeps changing and improving; however, it is expected that in 5 to 10 years, scientists carrying out this work will have worked out the best way to quantitate miRNAs in blood and also in other body fluids/excrements.

An additional approach towards data integration aimed at the identification of conserved changes to reduce background noise in expression profiling studies, and subsequently in miRNA studies, is represented by cross-species comparison of tumors from human patients and animal models of cancer [143]. This approach is expected to pinpoint genes of conserved functional relevance in the process of tumor formation and progression in mammalian intestinal tumorigenesis. Although mice mainly develop small intestinal tumors [144-147], comparison with human may be useful in further reducing the noise generated by organ-specific genes. Other animals such as pigs, which resemble humans more than rodents, can be used, but these studies cannot employ large numbers of pigs because of cost.

Key Issues

CoIon and rectal cancers are two different diseases as shown by differences in epidemiology, ethnic origin, embryologic differences, and different responses to molecular tests.

It is important to develop cost-effective markers for the early diagnosis of this disease in order to enhance screening and decrease disease mortality.

Risk factors for colon cancer include factors that cannot be changed such as age, history of IBDs, diabetes, and life-style factors such as diet, physical inactivity, smoking and alcohol consumption.

The genetic heterogeneity in colon cancer is increased by the influence of mismatch DNA repair.

MiRNA genes are usually transcribed by RNA polymerase II, and some with Polymerase III, especially those with upstream A₀ sequences, transfer RNAs and mammalian wide interspersed promoter units.

Because of their low rate of evolution, miRNAs are significant phylogenetic markers, making gene expression specific, which allowed for the genesis of complex organs, and subsequently complex life.

Bioinformatics approaches are developed to predict putative miRNA target genes, as miRNA target recognition is partly based on simple sequence complementarity and the finding that exact base pairing between miRNAs and their targets is required only in the first six to eight bases from the 5' end of the miRNA.

There are 24 521 microRNA loci from 206 species, processed to produce 30 424 mature microRNA products in *miRBase*, release (v20), in June 2013.

MiRNA molecules are expressed in colon cancer tissue, as well as from stool and blood taken from human subjects, although the expression in the later non-invasive and semi-invasive media is less than in tissue.

MiRNA molecules can serve as sensitive and specific biomarkers for the early noninvasive diagnosis of colon cancer, more economically and conveniently than any other test on the market. However, a large, well designed epidemiologic study that employs large numbers of subject will be needed to validate results.

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