MicroRNA in Colorectal Cancer: New Perspectives for Diagnosis, Prognosis and Treatment

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INTRODUCTION

Colorectal cancer (CRC) represents the third most common tumor worldwide, and is still burdened by significant morbidity and mortality [1] despite several therapeutic improvements. In fact it has been estimated that in 2013 CRC will cause more than 50,000 deaths in the United States and more than 500,000 worldwide [2]. Colorectal cancer incidence rates, however, show high variability (over 10-fold) worldwide, with the highest values in Australia and New Zealand, Europe and North America, and the lowest in Africa and South-Central Asia [3]. Such differences depend on life styles, environmental influence and genetic background.

Risk factors for CRC are well known, including age, previous CRC or polyps, family history of CRC or adenomatous polyps, inflammatory bowel diseases, chemoprevention, and preventable risk factors (high-fat diet, diet poor in fruits and vegetables, physical inactivity, obesity, smoking, alcohol). The development of CRC has been established and follows the sequential progression from adenoma to carcinoma (i.e., autonomous cellular growth) to carcinoma (i.e., development of features of invasion and metastasis by intestinal epithelial cells). Thus,
there are opportunities to interfere with the natural course of CRC at different levels, including screening in the general population, chemoprevention in high risk groups, tailored chemotherapies in diagnosed or resected CRC, and palliative therapies in advanced stage cancer.

In the last two decades, several randomized controlled trials have shown a reduction in CRC incidence, as well as mortality rates (American Cancer Society. Cancer Facts & Figures, 2010. American Cancer Society. http://www.cancer.org/Research/CancerFactsFigures/index. Accessed April 26, 2011). A major role has been played by the introduction of structured screening programs which have led to improvements in early detection and therapeutic strategies [4-6].

Although several CRC screening programs (colonoscopy, occult fecal blood, X-Ray barium enema) may decrease CRC burden, the best screening strategy is not yet available. Moreover, despite the unquestionable benefit of CRC screening programs, adherence to such programs is still poor and unsatisfactory, approaching only 50% in patients at high risk [7]. Thus, novel screening tools are being developed as potential biomarkers of CRC. Recent investigations have shown that the expression of distinct small RNA molecules, named microRNAs (miRNAs) are strongly correlated to the genesis, progression and prognosis of CRC [8, 9].

The present paper was designed to discuss the role of miRNAs as novel molecular tools in the diagnosis and prognosis, as well as therapy of CRC.

**GENERAL FEATURES OF miRNAs**

MiRNAs are a class of endogenous, small (17-25 nucleotides), noncoding, single-stranded, evolutionarily conserved RNA molecules involved in the post-transcriptional regulation of gene expression in vertebrates, plants and protozoa [10]. MiRNAs function as negative regulators of target genes, binding imperfectly to the 3' untranslated region of target mRNA's, and lead to mRNA degradation or translational inhibition [11]. Let-7 was the first miRNA to be characterized in 1993 as regulator of Caenorhabditis elegans developmental timing [12, 13]. However, a major role for miRNAs was identified in 2000, when it was demonstrated that let-7, the second identified miRNA, was conserved across species [14].

MiRNAs belong to the wider epigenetic mechanism of RNA interference (RNAi), the biological phenomenon that regulates gene expression through interaction between the mature transcript and smaller molecules of dsRNA [15].

Since their discovery, it has been demonstrated that miRNAs are involved in several biological processes, including cellular development, proliferation, migration, differentiation, and apoptosis. Recently, it has been suggested that miRNAs can play an important role also in tumorigenesis, since they may function as oncogenic as well as oncosuppressor molecules [11, 16]. To date, a miRNA database is a searchable online repository for published miRNA sequences and associated annotations (www.mirBase.org/index.shtml). The last version of miRNA internet database counts 24,521 human miRNAs, which are able to regulate the expression of almost one-third of human genome.

**BIOGENESIS OF miRNAs**

MiRNA biogenesis is a complex multi-step process that involves several enzymes and different cellular compartments (Fig. 1). The process starts in the nucleus, where miRNAs are encoded by genomic DNA and transcribed by a RNA polymerase II into the long primary transcript pri-miRNAs of variable length (1 kb-3kb) [17]. This step occurs in the genomic regions located within the introns or exons of protein-coding genes (70%) or in intergenic areas (30%) [18]. Pre-miRNAs are thereafter processed by a nuclear ribonuclease enzyme called microprocessor complex which contains RNase III Drosha and its cofactor DGCRC8(DiGeorge Syndrome Critical Region 8), also known as Pasha. This step leads to a stem-loop precursor of approximately 70-100 nucleotides (pre-miRNA) [19-21]. Until this moment, miRNA biogenesis takes place in the cell nucleus.

Pre-miRNA is exported into the cytoplasm through nuclear pore complexes, by a shuttle protein, exportin 5, which depends on RanGTP protein, a member of the Ras superfamily [22]. The hydrolyzation of GTP in GDP allows pre-miRNA to pass into the cytoplasm, where pre-miRNA is cleaved by the ribonuclease enzyme Dicer RNase III into a mature double-stranded miRNA (miRNA/miRNA* complex) of 21-23 nucleotides in length [23]. When the two strands of the duplex are divided by Dicer processing, one strand (the mature miRNA, also called guide strand) is actively loaded into the RNA-induced silencing complex (RISC or miRISC), and the other passenger strand (the star-strand miRNA*) is degraded [9, 11]. The miRNA/RISC complex plays a role in the miRNA function, in the repression of gene expression through the interaction of miRNAs to specific target mRNAs.

In the RISC complex, the single strand miRNA interacts with Ago2, a protein belonging to the Argonaute family, which represents the catalytic component of RISC complex [24]. Ago2 is responsible for mRNA degradation or translational suppression [25]. Ago2 protein also undergoes regulation, depending on the cell state and external stimuli: site specific hydroxylation or phosphorylation of Ago2 is responsible for localization of Ago2 to processing bodies (P-bodies) [26], distinct areas in somatic cells cytoplasm containing storage of untranslated miRNA and enzymatic proteins responsible of regulation of mRNA expression [27].

MiRNA molecules can strongly influence gene expression by interacting with target miRNA at its 3'UTR region through miRNA "seed" region, a region of less than 10 nucleotides at their 5' end [28].

Depending on the degree of complementarity of this miRNA-mRNA binding, the riboproteic complex can alternatively cleave mRNA, leading to its degradation, in case of high specificity, or simply suppress mRNA translation in case of less affinity binding between the two molecules [29].

Studies on the biogenesis of the miRNA pathways have shown that the miRNAs expression is regulated at three different levels, including transcription, processing and subcellular localization [30]. The regulation of pri-miRNA transcription is one of the most relevant mechanisms modulating miRNA abundance, by the action of several factors including transcription factors, silencers, enhancers,
and epigenetic modification in miRNA promoters [31]. Moreover, post-transcriptional factors can also modulate the miRNAs expression, such as p53, histone deacetylase I, and cytokines [32].

The study of miRNAs expression patterns appears of key interest as miRNAs show different and altered expression levels during disease. The huge potential role of these small molecules offers a new perspective to the knowledge of the complex gene regulatory networks, especially in cancer research.

### ROLE OF miRNAs IN COLON CARCINOGENESIS

Undoubtedly, a role for miRNAs has been indicated in CRC oncogenesis and progression, invasion, metastasis and angiogenesis [33, 34]. MiRNAs appear to interfere with several genetic mutations involving oncogenic and tumor suppressor genes residing in the colonic epithelium [33, 35]. In principle, it is currently believed that miRNAs which are overexpressed in tumor cells might function as inhibitors of different tumor suppressor genes. Mechanisms involve amplification, translocation, pleomorphism or mutation in miRNA transcribing genes and nuclear over production of pri-miRNAs. By contrast, those miRNAs which are silenced in tumors might play a role in downregulating oncogenes in normal tissues. In this case mechanisms involve mutation, deletion, promoter methylation or any abnormalities in the miRNA biogenesis leading to over expression of oncogenic miRNAs [33].

Whereas initial studies found downregulated miRNAs' expression in tumors [36], later studies found the opposite, since global miRNAs expression pattern in CRC was more over-expressed than under-expressed [37, 38]. This is in line with the observation that human miRNAs in CRC are more represented in regions of genomic copy number gain, and less represented in regions of copy number loss [39].

Fig. 1. Steps involved in the biogenesis and functions of miRNA. 1) Transcription of miRNAs starts in the nucleus from the intragenic or intergenic region. RNA polymerase II is involved to generate pri-miRNAs. 2) The Drosha ribonuclease enzyme-dependent process (box “d”) leads to intra-nuclear transcription of the pre-miRNA. 3) Pre-miRNA is transported to the cytoplasm by the protein exportin-5 (box “e5”). 4) Pre-miRNA is processed by the ribonuclease enzyme Dicer. 5) Generation of the mature double stranded miRNA (miRNA/miRNA* complex) by Dicer processing. 6) Generation of mature miRNA (guide strand). The other half of the complex is named passenger strand (miRNA*) which is usually degraded. 7) Mature miRNA may start a transcription regulation function in the nucleus, which is RISC (RNA-induced silencing complex) independent. 8) Formation of miRNA/RISC complex by active loading of miRNA with RISC and activation of RISC-dependent functions of miRNA. 8a) Pairing of miRNA/RISC with mRNA molecules is achieved at 3' untranslated region causing translational repression (imperfect pairing) or degradation (perfect pairing) of mRNA; 8b) Opening of the reading of mRNA frame of the 5' UTR sequences (ORF) by miRNA/RISC pairs resulting in degradation; 8c) Targeting of mRNA at 5' UTR by miRNA/RISC pairs resulting in translational activation. 9) Another RISC independent function involves the interaction of target miRNA binding proteins by mature miRNA, resulting in decoy activity. Adapted from Aslam et al. J. Translational Med., Biomed Central, 2012 [51]
miRNAs, including miR-143, might play a role during CRC cell proliferation by acting on v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog oncogene (K-RAS). In 30%-60% of the cases, a mutation of K-RAS occurs, and this change facilitates cell proliferation [59]. K-RAS belongs to the family of Ras proteins (guanine nucleotide-binding proteins) that are involved in wide variety of signaling pathways modulating different biological processes, including cell proliferation, cell adhesion, cytoskeletal integrity and apoptosis [60].

Studies on the human colon adenocarcinoma (LoVo) cell line found an inverse correlation between K-RAS protein and miR-143. LoVo cells transfected with the miR-143 mimic exhibited significant inhibition of K-RAS expression, while LoVo cells treated with miR-143 inhibitor exhibited increased K-RAS protein level and cell proliferation. Thus miR-143 acts as suppressor in CRC cell growth by inhibiting K-RAS [47]. MiR-18a* is another miRNA that targets on K-RAS, by acting as CRC suppressor. When miR-18a* is repressed, K-RAS expression, cell proliferation and anchorage-independent growth of human cancer cells increase. This has been shown in squamous carcinoma A431 cells, colon carcinoma HT-29 cells and fetal hepatic WRL-66 cells [48].

Another mechanism by which some miRNAs (i.e., miR-192 and miR-215) may modulate the carcinogenesis is via p53-miRNA. This is clearly shown in the study by Song et al, in which different human colon cancer cell lines HCT-116 (wt-p53), HCT-116 (null-p53), RKO (wt-p53) and HT-29 (mut-p53) were transfected with miR-192, miR-24 precursors or non-specific control miRNA [61]. Such miRNAs interact with the 3'-UTR region of the miRNA of dihydrofolate reductase (DHFR), the enzyme mostly implicated in intracellular folate metabolism [62]. MiR-192 over-expression was associated with decreased expression of DHFR protein and suppression in cellular proliferation (mostly in HCT-116 (wt-p53) cells and RKO (wt-p53) cells). Also, miR-215 targets DHFR, and induces G1 and G2-M cell cycle arrest by acting in a manner-dependent on functional p53 as miR-192 [63].

In another study, human colon cancer HCT 116 cells harboring wt-p53 were treated with adriamycin (a DNA damaging agent), and exhibited highly up-regulated miRNA-34. MiRNA-34 transiently was introduced into HCT 116 and RKO cells were associated with altered cell proliferation and induced senescence-like phenotypes, likely acting as suppressors (Fig. 2). Moreover, miR-34 induced upregulation of the p53 pathway and downregulation of the pathway of E2F, a group of genes that codifies a family of transcription factors involved in cell cycle regulation and synthesis of DNA in mammalian cells [64]. Tumor cell proliferation might be due to the aberrant methylation that inactivates tumor suppressor genes during tumorigenesis. In this respect, DNA analysis of miRNA-345 showed high methylation levels in tumor versus normal tissues. Furthermore, in 51.6% of CRC, miRNA-345 expression was downregulated compared with normal tissues and associated with altered cell proliferation and induced senescence-like phenotypes, likely acting as suppressors (Fig. 2). Moreover, miR-34 induced upregulation of the p53 pathway and downregulation of the pathway of E2F, a group of genes that codifies a family of transcription factors involved in cell cycle regulation and synthesis of DNA in mammalian cells [64]. Tumor cell proliferation might be due to the aberrant methylation that inactivates tumor suppressor genes during tumorigenesis. In this respect, DNA analysis of miRNA-345 showed high methylation levels in tumor versus normal tissues. Furthermore, in 51.6% of CRC, miRNA-345 expression was downregulated compared with normal tissues and associated with metastasis process, confirming a role for miRNA-345 as a growth inhibitor in CRC development [65].

The mechanism by which the neoplastic cells can pass through the basement membrane and reach the blood/lymph vessels is poorly understood, so far. However, several studies have shown a possible role of miRNA-21 in invasiveness by...
down-regulation of tumor suppressor programmed cell death 4 (Pdcd4), which in turn inhibits invasion/intravasation/metastasis [66]. Moreover, miRNA-21 is more expressed in metastatic than in non-metastatic CRC, and associated with lymph node metastasis [67]. The invasion/intravasation/metastasis mechanism is also facilitated by several adhesion molecules, including VCAM-1 (vascular cell adhesion molecule 1). An inverse correlation between miR-126 and VCAM-1 expression has been shown, demonstrating the inhibiting and regulating action of this miRNA on adhesion molecule expression and control of vascular inflammation [68]. Finally, miRNAs are involved in pathways which facilitate the metastatic process. Several miRNA levels were abnormal in cancer stem cells compared to their non-stem counterpart. MiRNA-328, miRNA-26b were downregulated and significantly associated with the invasiveness and metastasis of CRC cells [69]. The evaluation of the mentioned studies provides strong evidence that changes in miRNA expression usually occur in the process of carcinogenesis, highlighting the involvement of miRNA in the most common biologic pathways of CRC. Since miRNAs are dysregulated and differently expressed in tumors compared to non-tumoral tissues, they might represent potential diagnostic biomarkers and therapeutic targets [47].

ROLE OF miRNAs AS CRC BIOMARKERS

MiRNAs seem to meet all the requirements of ideal biomarkers (Table I). MiRNAs are present in plasma and feces at detectable levels [70], and are stable, since miRNAs are not degraded by endogenous ribonucleases. MiRNAs can be sampled in a non-invasive manner, showing a good sensitivity (from 62% to 89%) and specificity (from 41% to 89%) [71-74], and are able to distinguish controls from CRC patients. Thus, miRNAs are being validated as biomarkers for screening and diagnosis, with potential therapeutic reflections.

**Plasma miRNA**

The discovery of circulating miRNAs into exosomes and microvesicles in peripheral blood [75], and their stability have led to emerging interest in carcinogenesis. Chen et al detected 69 different plasma microRNAs in CRC patients compared to controls; many were common to lung cancer, but 14 miRNAs were uniquely expressed in CRC patients [76]. Ng et al reported elevated miR-17-3p and miR-92a levels in plasma and tissues of CRC patients with a reduction after tumor removal. Notably, miR-92a could distinguish between CRC and other gastrointestinal tumors [74]. According to Huang et al [72], miR-92a and miR-29a were able to distinguish CRC from adenoma, and controls. Pu et al [73] tested the usefulness of miR-221 in whole plasma in 103 CRC and 37 controls, and reported a sensitivity and specificity of 86% and 41%, respectively. MiR-221 could distinguish between CRC and controls, was a significant prognostic factor for poor overall survival in CRC patients, and at immunohistochemistry correlated with p53 expression.

**Faecal miRNA**

MiRNAs are abundantly present in stool samples. Continuous cell exfoliation in the gastrointestinal tract makes the study of miRNAs possible in CRC and adenomas. Endogenous miRNA is packed in microvesicles and exosomes, which are protected by RNase activity. This feature confers miRNAs a high stability, and evidences a major difference with mRNA and protein, which are rapidly degraded. A number of studies have focused on the expression of fecal miRNAs in different stages of CRC. MiR-21 and miR-106a were overexpressed in patients with CRC compared with patients with adenomas or control subjects [77]. Instead, miR-143 and miR-145 were down-regulated in CRC patients compared to healthy subjects [78]. MiR-92a level was higher in patients with polyps than in controls. Notably, miR-92a displayed a higher sensitivity for
distal than proximal CRC, for advanced adenoma compared with minor polyps, and decreased considerably after tumor or polyps removal [79].

MiR-144* was overexpressed in CRC patients compared with controls, displaying a sensitivity and specificity of 74% and 87%, respectively [80]. Koga et al analyzed miR-17-92 cluster and miR-135 in exfoliated colonocytes isolated by feces of CRC patients and controls. Their expression was significantly higher in CRC patients than in healthy subjects with an overall sensitivity and specificity of 74.1% and 79.0%, respectively [81].

### ROLE OF miRNAs IN PROGNOSIS OF CRC

Although many studies have evaluated the potential diagnostic role of miRNAs in CRC, few have investigated their role in prognosis. In a study by Pu et al [73], plasma miR-221 expression was associated with poor overall survival and p53 expression in CRC patients. Cheng et al [71] showed that circulating miR-141 was associated with stage IV disease and predicted a poor survival. Huang et al [72] showed that miR-29a level was higher in the serum of liver metastatic CRC patients, than in those without metastases, could distinguish between the two groups, and was correlated with nodal invasion. In a study by Chen et al [82], miR-148a and miR-152 were only correlated with advanced pT stage in CRC. Vickers et al [83] found elevated expression of let7a in metastatic disease compared to normal mucosa or non-metastatic disease. In a study by Lou et al, miR-625 was significantly down-regulated in CRC and its decreased expression was positively correlated with metastasis in lymph nodes and liver, with poor overall survival, and unfavorable prognosis [84]. Interestingly, the analysis of 5 miRNAs (miR-20a, miR-21, miR-106a, miR-181b, and miR-203) in paired normal and CRC archival tissues collected from black and white subjects revealed that their prognostic value might be different according to patient race/ethnicity and stage of disease [85]. More specifically, high expression of miR-203 was associated with poor survival of whites with stage IV CRC, and poor survival of blacks with stages I and II CRC. Increased miR-21 expression was correlated with poor prognosis for stage IV in white patients. High miR-181b expression was correlated with poor survival of only black subjects with stage III CRC.

The correlation between miRNAs expression levels with the presence of metastasis and/or advanced stages increases the clinical significance of miRNAs, attributing them a potential prognostic value.

### ROLE OF miRNAs IN THERAPY OF CRC

MiRNAs have features which make them suitable for gene therapy, as miRNAs expression is frequently deregulated in cancer cells, and targeting of miRNA expression might influence cancer phenotype. MiRNA pathways manipulation might involve inhibition of neoplastic growth and invasion, apoptosis, metastasis and angiogenesis. Major problems in miRNA-dependent gene therapy in oncology, however, remain delivery of molecules to specific target tissues, use of viral and non-viral strategies, multi-genic targeting by miRNAs and subsequent side effects, and growth of toxic phenotypes in targeted cells [51].

The evidence that oncogenic miRNAs are upregulated in neoplasms suggest that optimized antisense oligonucleotides, acting as competitive antimiR inhibitors of mature miRNA, might be used to block oncogenic miRNAs expression [86]. When antimiR were employed to block oncogenic miR-20a, miR-21, miR-31 and miR-95, miR-675 using several cell lines (SW480, SW620, RKO, HCT-116/LoVo, HT29), reduced cell proliferation, transformation, migration, and metastasis were demonstrated. Furthermore, enhanced sensitivity to chemotherapy was noticed [66, 87].
Another possibility is the use of locked nucleic acid constructs (LNA constructs) in which the ribose ring is locked by a methylene bridge. Beside their use in chronic hepatitis C [88], LNA oligonucleotides display hybridization affinity directed to complementary single-stranded RNA and DNA (single- or double stranded) [89]. In CRC, the potential translational value of the study of Valeri et al [90] needs to be mentioned in SW480, HCT-116 and RKO colon adenocarcinoma cell lines in which LNA anti-mir-21 was knocked down.

Some miRNAs have a tumor suppressor role, which might be down-regulated in CRC cells. Restoration of expression of such tumor-suppressor miRNAs might represent another therapeutic target. This approach might benefit from the use of synthetic oligonucleotides mature miRNA mimics, miRNA precursors or pre-miRNA mimics, to be introduced in different CRC cell lines, including DLD-1, SW480, RKO, CLY, HT-29, LoVo, CLY, Colo320, SV1116, SV620, HCT116 [46, 91-93]. Restoration of silenced miRNAs in CRC cell lines produced beneficial effects including reduced cell growth and colony formation, reduced migration and invasion, and increased sensitivity to therapeutic agents.

For example, using miR-34a mimic in DLD-1 lines, cell growth decreased and sensitivity to 5-Fluorouracil (5-FU) was enhanced [91]. These findings underscore the importance of gene therapies involving miR-34a which acts as tumor suppressor factor [94]. Restoration of miR-34a with the mimic or pre-miR-34a (where reduction appears to be linked to p53 loss in cancer cells) led to reduced cell growth, migration and invasion [91].

In a similar way, cell proliferation was reduced when using a miR-143 precursor in SW480 CRC cell line [93]. Similar results were obtained with miR-145 and let-7a precursor [46, 92].

Additional approaches to block oncogenic miRNAs

Whether oncogenic miRNAs will be satisfactorily silenced by additional approaches, is still a matter in CRC research. Methods might include the use of miRNA sponge constructs (transcripts containing multiple tandem-binding sites to a miRNA of interest) [95] or miRNA masking antisense oligonucleotides (single-stranded 2′-O-methyl-modified antisense oligonucleotides, fully complementary to predicted miRNA binding sites in the 3′ UTR of the target miRNA) [96]. Inhibitors of miRNA expression by appropriate screening of drugs is another possibility which has been tested with diazobenzene-1 on upregulated miR-21 by in HeLa cells [97]. Modulation of miRNA biogenesis leading to either increased or decreased miRNA processing (depending on the specific miRNA) might represent another therapeutic choice in the future. This approach is based on the assumption that a dysregulation of miRNAs levels might influence the CRC pathways [98]. Another possibility is the stem cell targeted miRNA therapy, since miRNAs appear to be involved in maintenance and differentiation of normal and cancer stem cells, e.g. involving miR-17-92 cluster and the target E2F1 governing colonic carcinogenesis and human colon embryogenic development [99]. Mutations in proteins involved in biogenesis of miRNA might impact self-renewal and differentiation process [100]; this might represent another target of therapeutic options.

CONCLUSIONS

The knowledge of mechanisms involved in the control of gene expression has gained great popularity since the discovery of miRNAs.

The discovery of miRNAs has led to active researches focusing on their role in cancer and several crucial pathways involving angiogenesis, cancer-stem-cell biology, epithelial–mesenchymal transition, formation of metastasis, and drug resistance. Concerning CRC, miRNAs might soon represent novel prognostic and diagnostic tools. MiRNA might prove useful as therapeutic tools, as well. In this respect, emerging novel therapies will be based on increasing or decreasing miRNAs expression, involving also gene therapy and stem cell approach. Before miRNAs will become available in the clinical setting, however, a number of large prospective studies are necessary to check for best sensitivity and specificity, fluids or tissue to be analyzed, and whether a single or cluster of miRNAs are to be employed. Thus, beside the diagnostic value of different miRNAs, treatment and management of CRC might benefit from identification of targets for miRNA and ways to deliver miRNAs together with more tailored treatment of cancer.

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Conflicts of interest: None to declare.

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