Review

microRNAs in colon cancer: A roadmap for discovery

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Abstract

Cancer omics data are exponentially created and associated with clinical variables, and important findings can be extracted based on bioinformatics approaches which can then be experimentally validated. Many of these findings are related to a specific class of non-coding RNA molecules called microRNAs (miRNAs) (post-transcriptional regulators of mRNA expression). The related research field is quite heterogeneous and bioinformaticians, clinicians, statisticians and biologists, as well as data miners and engineers collaborate to cure stored data and on new impulses coming from the output of the latest Next Generation Sequencing technologies.

Here we review the main research findings on miRNA of the first 10 years in colon cancer research with an emphasis on possible uses in clinical practice. This review intends to provide a road map in the jumble of publications of miRNA in colorectal cancer, focusing on data availability and new ways to generate biologically relevant information out of these huge amounts of data.

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1. Introduction

Research in the past decade has shown that several types of non-coding RNAs (ncRNAs), an RNA molecule that is not translated into a protein but has other important biological functions), including long ncRNAs and notably microRNAs (miRNAs), are involved in cancer development and progression [1]. ncRNAs are classified based on their size and known function. In this review we focus on miRNAs, since they are the most widely studied class of ncRNAs, although many issues about mining the data remain unsolved. We are still far from truly understanding the extent of the involvement of miRNAs in cancer, many results have been produced with the final aim of finding biomarkers for personalized (intended as the P5 concept in its entirety) [2] therapy, but still much must be done in integrating different omics data, validation of results in large cohorts of patients, evaluation of the treatments’ risks, as well as socio-economic plans of what can be really done with the available resources [3]. Colorectal cancer (CRC) is one of the most common malignancies in the western world. Up to 90% of patients can be cured by surgery if the disease is detected at the early stage, but unfortunately it is often diagnosed only at an advanced stage and the prognosis is therefore poor. Synchronous metastases are present in 15–25% of CRC patients and since patients with synchronous colorectal liver metastases comprise at least 25% of patients reported from large resection series, determination of the optimal management of these patients, in terms of classifying subgroup of patients to avoid over- or under-treatment, possibly avoiding the progression of the disease, is of fundamental importance [4], and miRNAs have the potentiality to be the main actor in this scheme, being them regulators of miRNAs, situated in fragile sites [5], involved in cancer biology and having decoy activity [6].

Abbreviations: miRNA, microRNAs; CRC, Colorectal cancer; mCRC, Metastatic colorectal cancer; ncRNA, Non-coding RNA; ISH, In situ hybridization; FFPE, Formalin-fixed paraffin-embedded; NGS, Next Generation Sequencing; MSI-H, Microsatellite instability (high); MSI-L, Microsatellite instability (low); MSS, Microsatellite stability; CIN, Chromosomal instability; HNPPC, Hereditary non-polyposis colorectal cancer; OS, Overall survival; ceRNAs, Competing endogenous RNAs; SRT, Surgically resected tissue; CCCL, Colon cancer cell line

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1.1. miRNA function and colon cancer

miRNAs are short (19-23-nucleotides) RNAs that are processed from hairpin loop structures and control the translation of mRNA into protein. The role of miRNAs in cell physiology and pathology is hard to understand due to their complex relation to biological function:

1. a typical miRNA can control translation of more than one messenger RNA, [7] perhaps as many as a few thousand messenger RNAs [8] and,
2. a single messenger can be controlled post-transcriptionally by more than 1 miRNA [9], perhaps dozens [8,10].

The literature related to miRNAs, has grown exponentially in the last decade, in summary we know that [11,12]:

- miRNAs can act as oncogenes or tumor-suppressor genes.
- miRNAs are involved in tumor progression and metastasis through their role in pathways that contribute to metastasis, including migration, invasion, cell proliferation, epithelial-to-mesenchymal transition (EMT), angiogenesis, and apoptosis.
- miRNAs might be useful as prognostic and predictive markers.

But how are these intermediate results linked together and with other omics data? Have they been analyzed associated to already known subgroups of patients like KRAS mutated, BRAF mutated [13], BRAF-like [14]? Are they particular for CRC or might they be general for cancer? How many studies failed or had discordant results on the same miRNAs when analyzed in different cohorts?

1.2. Public data

The development of miRNA microarrays, RT-PCR platforms and deep sequencing methodologies resulted in the acquisition of a growing number of miRNA profiling studies, and has paved the way to new approaches for biomarker discovery. Some of the published miRNA profiles are publicly available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), miRNA body map (http://www.mirnabodymap.org), TCGA (http://cancergenome.nih.gov) and SMIRNADB (http://www.mirz.unibas.ch).

Two tools offer compact visualizations of uploaded miRNA expression differences:

2. The Gene Expression Atlas (http://www.ebi.ac.uk/gxa/) per organism and per experimental condition.


For CRC, more than 1400 arrays are available in GEO, 873 arrays in ArrayExpress and 187 microRNASeq samples are downloadable from the TCGA database. SMIRNADB contains miRNA and other ncRNA expression data in large and small intestinal samples in the mouse.

1.3. MiRNA profiling

The main methods currently used for miRNA profiling are sequencing, microarray, real-time PCR-based approaches and in situ hybridization (ISH); all of them require standard procedures to be correctly processed and mined. Initially these techniques were applied on fresh-frozen tissue specimen, but recently reproducible profiles of comparable quality have been obtained also using formalin-fixed paraffin-embedded tissue samples (FFPE), making archived tumor tissue collections accessible for study [15,16].

Reis and colleagues reported in 2011 that with FFPE material the Nanostring technology might be preferable over RT-PCR [17] notably on older samples with more highly degraded RNA and DNA [18].

Deep sequencing or Next Generation Sequencing (NGS) platforms have recently emerged as powerful technologies that provide unprecedented insight into biological systems. Thanks to the development of NGS technologies, large portions of the human genome are being re-sequenced in many individuals, opening new opportunities to find out how changes in the genome are associated with disease.

This situation poses a number of new challenges to translational research scientists, especially in regard to requirements for advanced data management and data analysis procedures and for teamwork between clinicians, computer scientists, and molecular biologists.

Therefore, integrative analysis between different omics platforms has become an essential element in the experimental design of studies in the era of NGS genomics [19].

The field of Bioinformatics is undergoing a rapid evolution to create new tools for NGS data visualization, manipulation, and analysis in terms of alignment, assembly, quality control and variations detection.

However, there is a lack of mature high standard data analysis methods although several open-source applications and utilities start providing useful tools for data analysts, for example: Galaxy (http://www.galaxy.psu.edu/, web-based) provides an NGS analysis toolbox for quality control, mapping, SAM tools and several post-processing analyses.

ISH is another extensively applied technique due to its ability to detect and localize specific miRNAs within tissue samples. Among many others, it has been performed while studying the miR-200c function in EMT in metastatic colorectal cancer (mCRC) [20]. After miR-200c expression was found lower in metastasis when compared to normal mucosa, ISH analysis of primary CRC, liver metastasis samples and adjacent hepatocytes suggested what might be the role of miR-200c during cancer progression, notably during the EMT-MET (mesenchymal-to-epithelial transition) switching: modulation of the miR-200c expression could influence the cell invasion and the cell proliferation functions during metastasis development.

2. miRNAs in colon cancer

Since the literature dedicated to miRNAs in colon cancer has grown considerably in the last decade, in Table 1 we report the updated (associations inserted by us are in bold) list of the causal associations between miRNAs and colon cancer taken from http://www.mir2disease.org.

The first definite association of miRNAs with CRC was the realization that in 2003 Human homologues of murine miRNA sequences, miR-143 and miR-145, consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of CRC.

Since then, miRNA alterations have been observed in CRC (for a complete review of miRNAs in CRC diagnosis, prognosis and possible mechanisms of action, see [21]), but in most cases the biological significance of the observation is not fully understood and still there is no clear division between those that are driver events and those that are passenger events without any physiological importance.
miR-21 was found up-regulated in breast, oral, and CRC tumor tissues. Volinia et al. found miR-21, as well as miR-17-5p, miR-191, miR-29b and miR-155 to be up-regulated at least in CRC. Schetter et al. found miR-21 to be positively correlated with higher stage and of prognostic value [25]: expression profiling of colon adenocarcinoma and paired normal tissues was performed on a US training cohort by miRNA microarray and validated in a Chinese cohort by RT-PCR. Associations with tumor status, TNM staging, survival prognosis, and response to adjuvant chemotherapy were evaluated. High miR-21 expression was associated with poor overall survival in both the training and validation cohorts, independent of TNM staging, and was associated with a poor therapeutic outcome (adjuvant chemotherapy). But still little is known about miR-21 function in CRC, the overexpression in CRC samples has been confirmed several times, we also know it is correlated with advanced disease; the involvement of miR-21 in different types of cancer suggests that it may have a general role in tumorigenesis, but still concrete explanations of its role have not been proposed [26].

Another actor, miR-29a, has been found to be up-regulated in CRC versus normal tissue (tested in plasma, primary tumor tissue and paired normal tissue) [27], and in 2012 it was proposed as a potential serum marker for early detection of CRC [28].

In a recent publication, based on fresh frozen and FFPE surgically resected tissues (SRTs), only one miRNA, miR-150, was found to be consistently and progressively deregulated in a collection of adenomas and carcinomas, with lower expression compared to normal tissues. Gao and colleagues reported that Evi1 is a transcriptional suppressor of miR-143: their data pointed to a pathway in which Evi1 suppresses miR-143 gene transcription which in turn leads to elevated levels of K-Ras [24]; the study was performed on fresh frozen surgically resected primary tissue and cell lines.

miR-143 was found to be positively correlated with higher stage and of prognostic value [25]: expression profiling of colon adenocarcinoma and paired normal tissues was performed on a US training cohort by miRNA microarray and validated in a Chinese cohort by RT-PCR. Associations with tumor status, TNM staging, survival prognosis, and response to adjuvant chemotherapy were evaluated. High miR-143 expression was associated with poor overall survival, independent of TNM staging, and was associated with a poor therapeutic outcome (adjuvant chemotherapy). But still little is known about miR-143 function in CRC, the overexpression in CRC samples has been confirmed several times, we also know it is correlated with advanced disease; the involvement of miR-143 in different types of cancer suggests that it may have a general role in tumorigenesis, but still concrete explanations of its role have not been proposed [26].

miR-141 was found to be positively correlated with higher stage and of prognostic value [25]: expression profiling of colon adenocarcinoma and paired normal tissues was performed on a US training cohort by miRNA microarray and validated in a Chinese cohort by RT-PCR. Associations with tumor status, TNM staging, survival prognosis, and response to adjuvant chemotherapy were evaluated. High miR-141 expression was associated with poor overall survival, independent of TNM staging, and was associated with a poor therapeutic outcome (adjuvant chemotherapy). But still little is known about miR-141 function in CRC, the overexpression in CRC samples has been confirmed several times, we also know it is correlated with advanced disease; the involvement of miR-141 in different types of cancer suggests that it may have a general role in tumorigenesis, but still concrete explanations of its role have not been proposed [26].
Microsatellite instability (high) (MSI-H) cases represent about 10–15% of all CRC and are associated with distinctive pathologic features, such as proximal location, poor differentiation, frequent mucinous and medullary phenotype, and marked peritumoral and intratumoral lymphocytic infiltration. MSI carcinomas have a more favorable clinical outcome than MSS (microsatellite stable) tumors and the survival advantage conferred by the MSI phenotype is independent of tumor stage and other clinical and pathological variables [31]. Members of the oncogenic miR-17-92 family (miR-17-5p, miR-20, miR-25, miR-92-1, miR-92-2, miR-93-1 and miR-106a) were found significantly up-regulated in MSS vs. MSI fresh frozen tumor samples [32]; but these findings were only partially confirmed by subsequent studies on FFPE tissues [31].

Concerning the CIN pathway, in which the adenomatous polyposis coli (APC) gene plays a dominant role, Nagel et al. showed that miR-135a and miR-135b directly target the 3’ untranslated region of APC, suppress its expression and induce higher activity in the Wnt signaling pathway although without proof that this is relevant in vivo or during CRC oncogenesis by analyzing colon cancer cell lines (CCCLs), fresh frozen and FFPE CRC tissue and normal epithelium [33]. Nevertheless, miR-135b was also found over-expressed in inflammatory bowel disease, a condition that can lead to CRC. In mouse models, treatment with anti-miR-135b resulted well differentiated in tumors whereas those in the control groups showed low differentiation.

Along with studies of single or few miRNAs, it has become clear in the last years, that if we want to understand miRNAs biology and functional role, they must be studied in combination among them and with other omics data.

For example, the group of Volinia found networks of miRNAs in normal tissues and in their pathologic counterparts (more than 3000 tissue samples were studied) which defined independently regulated miRNAs, and target genes of uncoordinated miRNAs involved in cancer-specific pathways [34]. Software Applications that can help scientists to study the interactions between genomics data and the pathways in which they are involved include MetaCore (http://www.genego.com/metacore.php), Ingenuity (http://www.ingenuity.com/), and Cytoscape (http://www.cytoscape.org/).

For our understanding of the role of miRNAs in cancer, it is essential to move on from reports of mere associations to knowledge of how single miRNAs are functionally related to each other and connected to the expression of proteins in the cell. For example, several methods have become available for identifying miRNA target sites (see Table 2), but the mere presence of a miRNA-binding site is insufficient for predicting target regulation. Regulation of targets by miRNAs is subject to various levels of control. The finding that any messenger RNA and RNA molecule can sequester miRNA molecules acting as “competing endogenous RNAs”: ceRNAs has shown that targets can reciprocally manage the function of miRNAs. This interconnected regulation of miRNAs and target genes needs to be further clarified in order to understand to what extent miRNAs regulate genes.

This represents a way forward in the myriad of data, the integration of miRNA and target genes to identify “network” deregulation is still in its infancy, and there are few examples where we begin to unravel the possible role that miRNAs in tumour cells in tumorigenesis and tumour progression and metastasis.

Many are the open questions: it remains to be understood how miRNAs differentiate low from high microsatellite instability, how they are expressed in mutational subgroups like KRAS, BRAF and BRAF-like mutated patients. Among these subgroups, are miRNAs specific of a particular mutation? Each study should be ideally lead by a consortium of partners allowing miRNAs to be studied together with mutational profiles, clinical-pathological variables, miRNAs expression, verification of published subgroups and signatures to avoid cases like those reported by Roepman et al., in NSCLC where 8 previously published gene expression signatures were almost disjoint [35]. At least two large independent cohorts should be always available for discovery and validation. For example, Chen and colleagues [36] studied the role of miR-143 as target of KRAS in CRC, the study provides the first evidences that miR-143 is significant in suppressing CRC cell growth through inhibition of KRAS translation, but how miR-143 able to distinguish between KRAS mutated and WT patients is? Only 13 paired samples were analyzed, what happens if we analyze a larger cohort, are there other sub-groups of patients? And if we have the opportunity to extract normal tissue from different sites, what are we going to discover [37]? Furthermore, if we consider that for FDA omic signatures are medical devices [38], should not we, as research community, have a register of negative results? In this framework, it is clear how the importance of consortiums, large heterogeneous team works, research networks like TCGA (http://cancergenome.nih.gov/) along with projects like p-medicine (http://www.p-medicine.eu) and VPH-NoE (http://www.vph-noe.eu/), that contribute to the integrative omics study, data sharing, data mining, privacy, access and ethical issues, toward research driven by interdisciplinary discussions, are going to lead the research field, not only in terms of transparency and scientific results but also in term of better prospective treatment, health and well-being for patients as well as decision support for clinicians.

3. miRNAs as candidate biomarkers for targeted therapies?

The ability to detect miRNAs in body fluids, such as serum and plasma, along with their role in cancer progression raised the question if they could be used as convenient diagnostic biomarkers and for monitoring therapy response. Wu and colleagues found that a mutation downstream of let-7e resulted in a significant reduction of its expression in vivo, suggesting that screening for genetic variations in miRNA genes in human cancers has potential for identification of molecular diagnostic and therapeutic targets [39]. They screened sequence variations in ~300 miRNAs from ~150 patients (mainly from human colon and prostate cancer patients) and in 20 human tumor cell lines. Interestingly, they found that a germ line mutation located downstream of the pre-let-7e miRNA led to a significant reduction of its expression. This underpins the potential use of circulating miRNAs as a biomarker for cancer detection although we would think that somatic mutations detected in blood might be indicating presence of a cancer, but population polymorphisms (germ line mutations) maybe indicate a heritable risk but not yet presence of cancer. Along these lines, miR-144 has been found as a potential diagnostic biomarker for CRC in the feces [40].

There are cases in which the analysis’s focus is on only one miRNA, especially when it is observed as a specific candidate that characterizes a sub-group of CRC patients, examples are the roles of let-7a and miR-143 that were found to depend on KRAS mutation [41–43].

Anti-EGFR target therapy has been extensively used in all mCRC patients until unresponsiveness of KRAS mutated patients to this therapy was discovered [44].

Since then, retrospective studies on patients KRAS-mutated treated with anti-EGFR have been very useful in understanding potential mechanisms that allow identifying KRAS-mutated patients that can still benefit from the anti-EGFR therapy. There is a first indication that among patients with mCRC (refractory to irinotecan) those with tumors KRAS mutated with a T > G base change in the let-7a KRAS mRNA binding site (rs61764370) might have higher chances of survival (overall and progression free) when let-7a is highly expressed [42].
Furthermore, KRAS mutation is an indicator of only part of the unresponsive patients to EGFR-targeted therapy (approximately ~35–45%), and miRNAs became good candidates in the characterization of other patients that are KRAS wild type but still unresponsive to the anti-EGFR therapy [45].

miR-143 low expression has been shown to be predictive of poor prognosis (using cancer–specific survival as end-point) among patients that are KRAS wild type but unresponsive to EGFR targeted therapy, suggesting that miR-143 might be a prognostic biomarker in this subgroup [43].

In other cases, patterns (modules) of miRNAs distinguish among classes of samples, like in a recent paper, where samples of non-neoplastic mucosa, low- and high-grade dysplasia in adenoma and invasive adenocarcinoma of the colon (FFPE) have been hybridized in microarrays to highlight modules of miRNAs that systematically change their regulation during tumor development and progression [46].

When studying the miRNAs functional involvement in cancer is also important to distinguish between mature sequences of the same step loop miRNA. For example, a study of the regulation of the expression of the miRNAs miR-28-3p and miR-28-5p has shown that they are diverse not only in the miRNAs they target but also in the regulation of their expression. miR-28-5p and miR-28-3p are down regulated in CRC whereas miR-28-5p altered the expression of CCND1 and HOXB3 and miR-28-3p bound NM23-H1. Overexpression of miR-28-5p reduced CRC cell proliferation, migration and invasion in vitro, whereas miR-28-3p increased CRC cell migration and invasion in vitro [47].

In unraveling these complex functional relations, robust methodology for measuring miRNAs and normalization of qRT-PCR data are essential [48].

### 3.1. Pros and cons

#### 3.1.1. Diagnostic, prognostic and predictive applications

miRNAs potentially constitute effective diagnostic markers for cancer. Individual expression levels of particular miRNAs might be associated with risk of cancer relapse. In this regard, the stability of particular miRNAs in FFPE tissues, the need of little tissue (allowing assessment in small biopsies) and their presence in body fluids is a potential advantage [15]. However, standardized methods for predicting and sequencing miRNAs and miRNA targets remain to be developed. The biomedical and bioinformatics research community is working to fill in these gaps in miRNA research that remain so that the findings can be translated into clinical practice.

If miRNAs are intended to be used as biomarkers, therapeutic targets or therapeutic agents, consistency of results and methodological considerations will become increasingly important. The significance of these findings and potential roles as molecular classifiers or clinical biomarkers will require very well-supported validation in larger cohort studies.

#### 3.1.2. Therapeutic applications

Given the role that certain miRNAs might have in driving disease, intervention on their expression may represent a rationale for new treatment modalities. Proof of principle has been provided in vitro for miR-30b/c, miR-221 and miR-222 which are involved in the modulation of gefitinib-induced apoptosis in Non-Small Cell Lung Cancer [49].

miRNAs need only partial sequence match to a target mRNA to repress gene expression. However, they do share the same gene-silencing machinery to silence target gene expression. Therapeutic approaches based on miRNA and siRNA have intrinsic similarities and differences. Therefore, the potential clinical benefits of modulating miRNAs can be explored from parallel studies of siRNA in cancer therapies, but with caution due to the intrinsic differences [50]. While the risk of toxicity might be reduced when we will have a better understanding of the feedback mechanisms in the miRNA cellular processing, potential toxicity from off-target effects and immune activation during treatment (with miRNAs) appear to be relevant.

Therefore, when designing miRNA approaches, we need to use the most potent miRNA candidate at lowest concentration possible to interfere with tumor growth.

The fact that one miRNA usually regulates multiple genes adds a unique layer of complexity to miRNA therapy, which might render it difficult to control in practice. The use of tumor-specific delivery agents, such as tumor-specific nanoparticles or viral vectors, as has been demonstrated to work for siRNA in humans, may obviate at
least concerns regarding specific delivery. In patients with solid cancers, siRNA has been intravenously administered using targeted nanoparticles. Post treatment analysis showed that siRNA treatment remains effective after several cycles of administration which provides the first example of dose-dependent accumulation of targeted nanoparticle in human tumors. These data demonstrate that RNAi treatment is in principle feasible in patients using systemically delivered siRNA, and that siRNA can be used as a gene-specific therapeutic approach. With a better understanding of the role of miRNAs in tumor progression and a more sophisticated design of miRNA-modulating molecules, miRNA-mediated therapy will likely start providing new therapeutic options to be tested in clinical trials. It seems reasonable to postulate for the not so distant future that the analysis of cancer genome sequences and the use of omics based biomarkers will become important tools in the conduct of clinical trials and ultimately find their ways into daily clinical practice.

4. Conclusions

miRNAs have several properties that could make them effective diagnostic markers for cancer, for example, measuring a patient’s expression levels of specific miRNAs could help a clinician decide whether the patient is at risk for developing cancer or whether the patient’s tumor has metastasized. The stability of miRNAs in FFPE tissues and body fluids is advantageous for biomarker discovery and validation; furthermore miRNAs can be extracted from small biopsy specimens. In addition, miRNAs are potential therapeutic agents for personal cancer management. The research community is working toward switching from potentiality to reality.

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