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MicroRNAs in Cancer: challenges and opportunities in early detection, disease monitoring, and therapeutic agents

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Abstract

Purpose of review—The goals of this review are to examine the usefulness of miRNAs as diagnostic and prognostic biomarkers for cancer and to evaluate the applicability of miRNAs as cancer therapeutics.

Recent findings—Examination of miRNA milieu from body fluids offers a new alternative for quick, affordable and easy analysis of disease status in patients. Blood-based exosomal miRNAs have increased stability and are an excellent choice for clinical cancer diagnostics and prognostics. Currently, there are many miRNA signatures associated with cancer and progression but there is no consensus among multiple sera and tumor sample studies. Off-target and immunological effects remains an obstacle for use of miRNAs as novel chemotherapeutics in the clinic. Recent developments in nanotechnology and drug delivery systems which target the tumor microenvironment may provide an alternative therapeutic approach with decreased toxicity.

Summary—This review critically evaluates the literature investigating the use of miRNAs as biomarkers and their future as potential therapeutics.

Keywords

microRNA; cancer; biomarker; exosomes; chemotherapy; tumor microenvironment

Introduction

In an increasingly connected world, we are beginning to appreciate the complexity of our increasingly connected cells. Tissues and organs often talk to each other with molecular cues

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Compliance with ethics guidelines

Conflict of interest

Namita Chatterjee, Shushan Rana and Cristina Espinosa-Diez declare that they have no conflicts of interest.

Sudarshan Anand reports a patent 62/300,615 Pharmaceutical compositions comprising microRNA pending, and a patent 9,242,000 Micro-RNAs and micro-RNA inhibitors to modulate blood vessel growth, patterning, tumor growth and malignant disease and method for making and using them issued.

Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

ranging from hormones to lipids to exosomes. Approaches focused on deciphering inter- and intracellular cross talk can uncover disease information, perhaps similar to data mining approaches that use internet searches or social media to understand epidemics [1–3]. Among the molecules that cells use to transmit and regulate information, small RNAs, particularly microRNAs (miRNAs) have emerged as attractive candidates for diagnostic and therapeutic purposes.

It has been more than 20 years since the groups of Ambros and Ruvkun described the first miRNA in *C. elegans* [4, 5]. More than 1800 miRNA precursors and 2500 mature miRNAs have been identified in humans (miRBase v.21) and the biogenesis and function of miRNAs have been well studied [6–8]. In brief, miRNAs are a class of small noncoding RNAs (20–22nt) capable of modulating gene expression. These molecules are initially transcribed by RNA pol II in an unprocessed form called primary miRNA (pri-miRNA), which undergo processing by DROSHA/DGCR8, leaving a hairpin termed the precursor miRNA (pre-miRNA). Exportin 5 then exports the pre-miRNA out of the nucleus. The hairpin molecules are further processed into double stranded miRNA by Dicer, a RNase III endonuclease. The duplex is unwound; one strand is incorporated into the RNA-induced silencing complex (RISC), and then translocated to the complementary mRNA target strand initiating the degradation or translational inhibition of the mRNA.

As expression of miRNAs is often controlled by canonical transcription factors, they can be used as an indicator of the transcriptional status of a cell. The dynamic changes in miRNAs reflect almost real time changes in cellular health and as such, miRNAs can provide valuable information for personalized cancer management. It has been suggested that miRNA profiling will become centralized to many aspects of cancer patient care [9, 10]. Ideally, miRNAs will be used as an integral part of a comprehensive surveillance program to diagnose disease, delineate high-risk individuals, provide insight for personalized treatment regimens, and monitor the efficacy of those treatments while also indicating relapse. Furthermore, a small number of clinical trials are currently investigating the utility of miRNAs alone and in combination with current standard of care treatments for cancer. Here we summarize recent findings regarding the potential of miRNAs as cancer biomarkers for diagnosis and prognosis as well as the value of miRNA therapeutic treatments for cancer patients. We also examine the future implications of miRNAs in cancer pathobiology and describe the current limitations.

miRNAs are effective cancer biomarkers

To be useful in patients, cancer biomarkers have to be quickly and inexpensively acquired using non-invasive or minimally invasive procedures, amenable to a variety of quantification techniques, have high sensitivity and specificity, and robust in a heterogeneous patient population. Biomarkers can be diagnostic (disease vs normal tissue), prognostic (provide survival or progression information) or predictive (gauge response to therapies). Although predominantly studied in blood compartments, miRNAs have been found to be ubiquitous in at least 12 body fluid types including serum, plasma, urine, saliva, etc., providing an alternative for invasive biopsy procedures [11].

While there are a number of approaches to profile and quantify miRNAs, the optimal standard for cancer biomarker discovery in the clinical setting remains to be defined [12, 13]. Numerous miRNA quantitation methods such as qRT-PCR, microarray analysis, and parallel deep sequencing are becoming increasingly affordable and provide quick results for clinicians. However, these methods often offer trade-offs between sensitivity and specificity, and therefore it would be useful to validate any biomarker signatures discovered in one platform, in another platform. Some of these pitfalls and guidelines for designing rigorous miRNA biomarker studies are well described elsewhere [14].

An advantage in utilizing extracellular miRNAs stems from their stability in biofluids likely through association with Argonaute proteins, vesicular encapsulation in exosomes, or binding to high density lipoproteins, which increases resistance from degradation by RNases [15]. With this added protection, extracellular miRNAs have been shown to withstand environmental stress when tested in plasma samples subject to temperature changes and multiple freeze thaw cycles [16–21]. However, it is important to note that some miRNA species appear to be more sensitive to RNases contained in blood where one study demonstrated that certain miRNAs in sera from healthy patients undergo substantial decline, while others remain fairly intact [22].

Exosomal miRNAs may be a better choice for diagnosis

During pathogenesis or in response to stressors, tumor-derived exosomal miRNAs are released into the circulating vasculature, glandular secretions, or waste excretions through a variety of processes [23, 24]. With robust stability in various extracellular environments, baseline activity or change in the cancer-related exosomal miRNAs provides a molecular snapshot of intracellular activity from their tissue of origin. The major advantage of exosomal vs free circulating miRNAs is the stability of exosome bound nucleic acids. A recent study [20] demonstrated that miRNAs contained in exosomes were significantly more stable in comparison to miRNAs isolated from exosomal free plasma samples when stored under different conditions. This study found that storage of plasma samples for up to 5 years at -20°C had no significant impact on miRNA levels when compared to freshly prepared samples. After 2 weeks, significant degradation of samples stored $+4^{\circ}\text{C}$ was noted, but all miRNAs tested were still detectable suggesting short-term storage at $+4^{\circ}\text{C}$ is a viable option. In addition, exosomally-derived miRNAs stored at -20°C with 2 cycles of freeze/thaw were less degraded than plasma-derived miRNAs [20]. Other studies have also reported that miRNAs are intact in samples stored for 6–12 months at -80°C [25]. However, specific miRNAs were degraded faster until 4 years and almost all degraded significantly after 4 years.

Overall, miRNAs from exosomes are considered as diagnostic and prognostic biomarkers obtained through minimally invasive means [26]. Recent clinical studies have shown the potential utility of these novel biomarkers [27–29]. One of the earliest clinical studies on tumor-derived exosomes analyzed their role as a novel diagnostic tool in ovarian cancer. Taylor *et al.* [30] implemented a magnetic cell sorting (MACS) protocol using anti-EpCAM antibody on patient serum to selectively isolate serous papillary ovarian cancer derived exosomes, as these malignant cells abundantly express EpCAM on their surface. Ovarian cancer serum exosomes were compared to serum exosomes from patients with benign

ovarian disease and healthy controls. In general, exosome levels increased incrementally with tumor stage. Subsequent miRNA microarray analyses demonstrated the levels of 8 specific miRNAs were concordantly up-regulated between ovarian cancer cells and ovarian cancer tumor derived exosomes [30].

A similar study was performed in colorectal cancer patients where serum exosomes were isolated via ultracentrifugation techniques [31]. An initial set of tumor-derived serum exosomal miRNAs were harvested from two distinct populations. Serum from one subgroup was collected before and after primary tumor resection, upon diagnosis of liver metastasis, and after resection of liver metastasis. The other subgroup did not have metastatic liver disease with samples limited to before and after primary tumor resection. Microarray analyses revealed a distinct subset of 6 tumor-derived exosomal miRNAs, which were synchronized with liver metastasis development. Among this subset, exosomal miR-19a was further analyzed in a larger cohort of patients and found to be up-regulated compared to normal healthy volunteers. The level of exosomal miR-19a elevation relative to the normal healthy volunteer average was found to correlate with more aggressive disease including nodal involvement, liver metastases, and higher TNM stage [31]. Together these studies are representative of the utility of using exosomal miRNAs as biomarkers across different types of cancers.

How can miRNAs be used for diagnosing cancer?

For those patients with suspected malignancies, evaluation of selected tumor suppressor or oncogenic miRNAs in serum provides a potentially robust alternative to more invasive and painful diagnostic procedures [32, 33]. In the oncologic setting, these miRNA assays can further delineate between aggressive and indolent tumors to tailor personalized treatment regimens. Studies have shown that some miRNAs are located in cancer-associated genomic regions (CAGRs), subject to rearrangements, breakpoints, loss of heterozygosity and deletions; and these miRNA genomic sites will be useful markers for diagnosing cancers associated with these regions [34, 35]. Tumor-specific miRNAs were first discovered in the serum of patients with diffuse large B-cell lymphoma where high levels of miR-21 correlated with improved relapse-free survival [36]. Since then, several studies have identified specific miRNAs associated with distinct tumor types and progression. Below, we provide a few specific examples of promising diagnostic miRNA signatures and a few current miRNA diagnostic clinical trials are shown in Table 1. However, it remains to be empirically demonstrated via both retrospective and eventually, prospective clinical trials that these miRNA signatures are indeed more effective for cancer diagnosis and staging in comparison to current clinical strategies.

Colorectal Carcinoma (CRC)

At present, colonoscopy remains the gold standard for colorectal cancer (CRC) screening. However, this process requires significant allocated time, carries risks associated with evacuative bowel preparation and procedural sedation, and can be expensive. As a result, many patients avoid screening despite the benefits of early detection and thus, less invasive diagnostics are needed. Recently, a number of studies have delineated miRNA signatures to

diagnose, define stage and predict CRC patient outcome [37–39]. Kanaan and colleagues denoted an 8 miRNA plasma signature able to distinguish patients with colorectal adenomas from patients with colorectal neoplasia. A 2 miRNA signature (miR-139-3p and miR-431) was able to differentiate all stages of CRC from normal controls, and a 5 miRNA signature was found to discriminate advanced colorectal adenomas from all stages of CRC [40]. A comparable study recently defined a 4 miRNA diagnostic signature consisting of miR-23a-3p, miR-27a-3p, miR-142-5p and miR-376-3p, which distinguished colon cancer patients from healthy patients with high sensitivity and specificity. This signature of miRNAs exhibited high diagnostic performance when analyzed in early-stage colon cancer patients. In addition, this study developed a prognostic panel based on the expression of miR-23a-3p and miR-376c-3p independent of TNM stage [39]. Lastly, a 7 miRNA expression signature could distinguish between CRC and other cancers and between CRC and colorectal adenomas [41].

Breast Cancer (BC)

Mammography and core needle biopsy are the modern diagnostic standards in breast cancer (BC). Less invasive and higher sensitivity methods of identifying and stratifying BC patients are needed to decrease emotional, physical and financial stress in patients with suspicious breast symptoms. A recent study used microarray analysis to evaluate global miRNA expression in individual samples from 23 BC patients and 9 healthy patients. Eighteen up-regulated miRNAs in BC patients were identified and 9 were further validated in a larger cohort of 46 breast cancer patients and 14 controls. The expression of those miRNAs was higher overall in patients with stage I, II, and III, compared to stage IV, indicating their potential for early detection [42]. Volinia *et al.* studied miRNA dysregulation in normal breast and ductal carcinoma *in situ* samples. They reported down regulation of let-7d, miR-210 and miR-221 in these samples, but detected expression reversal in the transition to invasive carcinoma [43]. In 2014, a miRNA risk scoring system was able to segregate patients to high or low risk of tumor progression in triple negative breast cancer (TNBC) and was further validated in a cohort of 70 patients. The gene signature comprised of miR-18b, miR-103, miR-107, and miR-652 represents one of the first non-invasive prognostic biomarkers for patients with TNBC [44]. The ability to accurately predict prognosis for TNBC is important, as it helps clinicians design the best treatment plan and determine which patients will likely benefit from clinical trials.

Prostate Cancer

Measurement of serum prostate specific antigen (PSA) is the most sensitive and universally adopted test to detect prostate cancer and monitor disease progression. In combination with pathologic Gleason score and disease extent, PSA has been used to stratify patients to the appropriate treatment regimen [45]. However, there remains significant variation to designate these stratifications in PSA interpretation among clinical practitioners. In 2008, pioneering work by Mitchell and colleagues described miR-141 as a blood-borne miRNA prostate cancer biomarker. Along with other studies, miR-141 has been shown to be involved in epithelial-mesenchymal transition in metastatic prostate cancer patients and is able to identify patients with cancer vs healthy controls [21, 46–48]. However, in 2011 Mahn and colleagues identified a signature consisting of miR-26a, miR-195 and Let-7i to distinguish

benign prostate hyperplasia vs prostate cancer patients but were unable to reproduce the results observed by Mitchell *et al.*, [21, 49].

Recent work by Stuopelyte *et al.*, [50] showed that urine may also be an excellent source of tumor-derived circulating diagnostic miRNAs. Initially, this study screened the miRNA expression profiles of a cohort of prostate cancer tissue samples to select potential biomarkers. The levels of 2 miRNAs (miR-148a & miR-375) were further evaluated in urine samples from prostate cancer patients and healthy controls. These 2 miRNAs had significant diagnostic potential in 2 independent cohorts and appeared to improve specificity compared to the PSA test [50]. In summary, it is clear that different groups, even studying the same disease, discover different miRNA signatures in their cohort of patients. While some of this is likely due to technical differences such as profiling method etc, it remains to be seen how these different miRNAs can be integrated and/or consolidated into specific tests that can be evaluated in prospective clinical trials. Evolving/adopting consensus guidelines such as those prescribed by Nair *et al.*, [14] would enable standardization of protocols and likely yield more robust signatures.

Do miRNAs have prognostic/predictive value?

Differential expression of miRNA has recently been shown to have prognostic value for cancer therapy efficacy. Head and neck squamous cell carcinoma (HNSCC) patients with no evidence of recurrent disease within 5 years after surgery and post-operative radiation exhibited significant differential expression of 41 miRNAs compared to similar patients who relapsed within 5 years. Among these miRNAs, miR-196a was found to be highly overexpressed in patients who developed recurrent disease [51]. Further studies screening plasma miRNA in HNSCC patients before and after treatment, in comparison to age matched healthy controls, identified therapy-responsive and highly induced miRNAs (miR-142-3p, -186-5p, -195-5p, -374b-5p, and -574-3p), indicative of decreased progression free survival and/or overall survival [52].

Madhavan *et al.*, identified 16 miRNAs significantly associated with overall survival in BC patients, and were termed as prognostic miRNA panel template (PROMPT). Additionally, 11 of these miRNAs were also associated with progression-free survival. Their prognostic significance was further confirmed in samples from a second cohort of BC patients. miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p were found to be significantly associated with onset of metastasis up to 2 years post clinical diagnosis [53]. Lynam-Lennon *et al.*, demonstrated the role of miR-31 in response to ionizing radiation (IR) in the neoadjuvant setting for esophageal cancer treatment. miR-31 levels in pre-treatment biopsies were significantly increased in patients who had good response to neoadjuvant chemoradiation in the clinical setting. In an additional cohort, patients who were found to have pathologic complete response exhibited higher levels of miR-31 compared to patients with no apparent pathological response [54].

A model for prediction of 3-year overall survival consisting of clinical factors (age, gender, stage), and 2 miRNA candidates (miR-23a-3p, miR-376c-3p) was established, which accurately predicted the outcome of more than 70% of colon cancer patients [39]. Notably,

the added predictive value for staging and survival presents a potentially relevant sub-stratification for treatment decisions. While several of these studies are promising, there are no commercially available miRNA diagnostic tests for cancer. Furthermore, it is unknown if prognostic miRNAs are an improvement over standardized biopsy and histological examinations. Major challenges include validation of signatures in independent cohorts of patients and multi-centric prospective trials that demonstrate significant utility of these signatures in a healthcare setting.

Can miRNAs be used as therapeutic agents in cancer?

miRNA therapeutics, despite great promise in preclinical studies like other RNA interference approaches, face considerable challenges in oncology, particularly with respect to pharmacokinetics, pharmacodynamics and absorption, distribution, metabolism and excretion (ADME) characteristics [55, 56]. Clinical trials are being conducted with miRNAs as a therapeutic and their current status is represented in Table 2. For example, MRX34 (Mirna Therapeutics, Austin, TX) entered clinical testing in 2013 as a single agent in a multi-center Phase 1 clinical trial (NCT01829971), including patients with solid tumors (liver, lung & pancreas) and hematological malignancies (lymphoma & leukemia). Initial results were promising, showing partial and complete responses after 9 cycles with MRX34 treatment in liver cancer patients with lung metastasis. However, in September 2016 the study was halted following multiple immune-related severe adverse events (SAEs) observed in patients. Santaris Pharma (Copenhagen, Denmark) has developed Miravirsin, a LNA-based inhibitor of miR-122 targeting hepatitis C virus (HCV) infection and is well tolerated in patients. However, long-term treatments appear to produce resistance to Miravirsin via *de novo* mutations in the virus (NCT01200420). Mesomir-1, (EnGeneIC Ltd. New York, NY) is a miR-16 mimic in Phase I clinical trials for patients with recurrent malignant pleural mesothelioma (MPM) and non-small cell lung carcinoma (NSCLC) (NCT02369198). Therapy with Mesomir-1 was well tolerated and safe in 5/6 patients, with disease stabilization after 8 weeks with this formulation. miR-16 has also been tested in combination where *in vivo* studies demonstrated that miR-16 sensitizes MPM cells to other chemotherapeutics such as cisplatin and pemetrexed [57].

Is it more beneficial to target miRNA therapies to the tumor microenvironment?

Given the intrinsic challenges in tumor heterogeneity, targeting the tumor microenvironment (TME) has emerged as an important therapeutic opportunity [62]. Indeed, the TME plays an essential role in tumor development, progression, response to therapies and may be more amenable to RNA delivery *in vivo* and *ex vivo* [63, 64]. In fact, recent studies have shown that in specific types of cancer such as CRC, the TME gene expression profiles are better predictors of overall survival rather than tumor cell gene expression profiles [65, 66]. Development of tools taking advantage of the physical and chemical nature of the TME for targeting RNA therapies has become an attractive research focus [67, 68]. For example, Cheng and colleagues utilized a novel hybrid molecule consisting of a peptide nucleic acid miRNA antagonist linked to a low pH-transmembrane structure (pHLIP) that preferentially

targets the inherent acidity of the TME. This approach prevents off target effects in the liver while directly inhibiting the miR-155 oncomiR in a murine model of lymphoma [69]. Similarly, Dahlman and colleagues have recently described novel polymeric nanoparticles (7C1) consisting of low molecular weight polyamines and lipids for delivery of small RNAs (including miRNAs) specifically to endothelial cells [70]. Our group and others have explored the potential use of these nanoparticles for treatment of diseases which rely on proper endothelial cell function, such as cardiovascular disease and cancer, while ultimately avoiding hepatic or immune cell involvement and preventing off-target effects.

Conclusions and future perspectives

Examination of miRNA expression patterns can provide useful insight for management of cancers and other diseases. We envision that in the not too distant future, RNA profiling becomes a key component of diagnostics including status (overall health), screening activities (growing neoplasm or fibrosis or cardiovascular disease), and monitoring of routine cellular functions (apoptosis & autophagy) in tissues. The studies highlighted here outline the potential for using miRNAs in cancer management as diagnostic and/or prognostic signatures, development of miRNA therapeutics, and monitoring treatment responses.

There are limitations that must be addressed prior to full integration of miRNAs in cancer patient care. First, we must standardize miRNA isolation, processing, storage and quantitation methods to eliminate variability among different clinical centers. One potential solution to decrease variability may be the addition of control RNA spike-ins to normalize miRNA isolation techniques and co-purification of PCR inhibitors. Second, we must investigate the effects of endogenous mRNAs (i.e. pseudogenes, sponges) on miRNA expression in biofluids. Currently, it is unknown if mRNAs affect expression levels equally among all miRNA species. Third, it will be relevant to examine the activity of specific and non-specific RNases found in biological fluids; to understand their effects on miRNA expression. Lastly, we must re-imagine and/or re-formulate miRNA therapeutics to prevent off-target immunological effects. These are considerable obstacles to overcome but recent advances in nanotechnology and drug delivery systems provide an excellent impetus for re-evaluation of these therapeutics, particularly with the ability to target RNA treatment to TME.

While this review focuses on the potential of miRNAs, it is important to note that other non-coding RNAs (ncRNAs) such as long non-coding RNAs (lncRNAs)[71, 72], small nucleolar RNAs (snoRNAs)[73], and circular endogenous RNAs (ceRNAs) [74] also play a role in disease development and have potential for use as biomarkers for cancer diagnosis and prognosis. To paraphrase an eloquent review [75], ‘even the lives of the most mundane RNAs are filled with intricacies’. These emerging studies indicate that an intricate network of RNAs can be a powerful window into understanding how cancers develop, progress, respond to treatment regimens and communicate with other host cells. Our challenge is to simply understand enough of these intricacies to tell the tales of cancer cells that remain hidden from our most sensitive imaging devices.

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Table 1

Current clinical studies using miRNAs as diagnostic tools

Title	Disease	Indications	Purpose	Identifier
miRNA expression in renal cell carcinoma (RCC)	RCC	Identify biomarker signature for RCC	Diagnosis	NCT00743054
miRNA machinery in melanoma, melanoma metastases and benign melanocytic naevi	Melanoma	Examine expression profiles of miRNA machinery Profiling miRNA maturation and transport factors, microprocessor complex and RISC subunits	Diagnosis	NCT01444560
Microarray analysis of miRNA expression profiles in cutaneous squamous cell carcinoma (cSCC)	cSCC	Examine miRNA expression profiles of cSCC	Diagnosis	NCT01500954
Clinical significance of hepatic and circulating miRNAs miR-221 and miR-222 in hepatocellular carcinoma (HCC)	HCC	Examine miRNAs expression correlations between tumor tissues and blood from cancer patients	Diagnosis	NCT02928627
miRNA as a diagnostic and prognostic biomarker of hepatocellular carcinoma (HCC)	HCC	Comparison of miRNA expression levels in serum before and after treatment and between serum and tumor tissue	Diagnosis	NCT02448056
miRNAs in patients With neurofibromatosis type 1 (NF-1)	NF-1	Evaluate the presence of miRNAs in archived tumor tissue and blood of patients with NF-1	Diagnosis	NCT01595139
Personalizing Enzalutamide Therapy by Understanding the Relation Between Tumor mRNAs, miRNAs and Treatment Response (ILUMINATE)	Prostate Cancer	Investigate the relationship between the expression levels of preselected miRNAs, tumor-related mRNAs and treatment response in chemotherapy naive patients	Predictive Biomarker	NCT02471469

Table 2

Current clinical trials with miRNA based therapeutics

Clinical trial identifier	Company	Product	miRNA	Phase	Cancer type	Outcome	Reference
NCT01829971	Mirna Therapeutics	MRX34	miR-34 mimic	Cancelled	NSCLC, Liver	Partial or total response in solid tumors. Terminated for multiple immune related severe events	[58, 59]
NCT02369198	EnGeneIC Ltd	Mesomir-1	miR-16 mimic	Phase I	MPM, NSCLC	Well tolerated. Disease stabilization in 5/6 patients	[60]
NCT01200420	Santaris Pharma	Miravirsen	anti-miR-122	Phase II	HCV, HCC	Well tolerated. Decrease in viral load. Long term treatment induces resistance and mutations in HCV virus	[61]