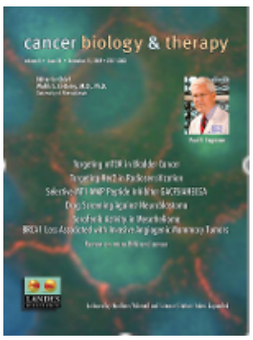

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miRNAs and cancer

New research developments and potential clinical applications

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Key words: miRNA, cancer, detection, quantification, microarray, biomarker, clinic

miRNAs are small non-protein coding transcripts that regulate gene expression post-transcriptionally by binding to the 3' UTRs (untranslated regions) of messenger RNAs (mRNAs). The number of newly discovered miRNAs and our understanding of their biological roles continue to grow. In addition to their roles in important biological processes such as development, differentiation, proliferation and cell death, deregulated expression of miRNAs has been implicated in a wide range of pathologies, especially in cancer. We now understand that miRNA expression is often deregulated in cancer cells and that a vast number of genes, including tumor suppressor genes and oncogenes, are regulated by these small RNAs. The small size of miRNAs and sequence similarity of miRNA family members pose some challenges in routine molecular detection and quantification techniques. Therefore, methods are being modified to specifically and sensitively detect miRNAs in cancer cells. Our current knowledge and the ever increasing pace of new discoveries clearly show that miRNAs are quite important in normal and in cancer cells in surprisingly diverse aspects. The better we understand how miRNAs contribute to cancer, the more likely we will be able to exploit them as tumor classifiers, biomarkers and, potentially, as unique targets for therapeutic applications.

Introduction

Despite many laudable advances in diagnostic, prognostic and therapeutic applications, cancer still remains a major problem worldwide. While better and more efficient diagnostic, prognostic and therapeutic approaches are being developed everyday to decrease mortality and morbidity, miRNAs are also emerging as intriguing and potentially powerful candidates in the arsenal to combat cancer. miRNAs are small non-protein coding transcripts of 16–29 nucleotide-long RNAs that regulate gene expression post-transcriptionally by binding to the 3' UTRs of target mRNAs. Since the discovery of these small non-coding RNAs, our understanding of miRNAs immensely evolved. Here, we try to present an overview of some of the recent developments in the field of miRNAs, as relevant to oncology, including approaches used for both research and clinical purposes.

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miRNA Genes and their Expression

miRNA genes can be expressed individually or within clusters, and can be found in introns of protein coding genes as well as within repetitive regions and transposable elements.[1] It is widely accepted that the vast majority of miRNAs are transcribed by RNA polymerase II and have 5' caps and poly (A) tails. miRNA biogenesis (**Fig. 1**) starts with a several kb long primary miRNA (pri-miRNA) that is later cleaved by the nuclear RNase III enzyme, Drosha and DGCR8 (DiGeorge syndrome critical region gene-8) to form a 60–70 nucleotide long precursor miRNA (pre-miRNA) with a 3' overhang of two nucleotides.[2] After Drosha cleavage of pri-miRNA into pre-miRNA, pre-miRNA is transported to the cytoplasm through Exportin-5, the nuclear export protein, together with Ran-GTP.[3] In the cytoplasm, a mature miRNA duplex is cleaved from the pre-miRNA by the cytoplasmic RNase III, Dicer, and its RNA binding partner TRBP, HIV-1 trans-activating responsive element (TAR) RNA binding protein. This double stranded RNA then assembles into a multi-protein complex called RISC (RNA-induced silencing complex).[2] The target mRNA and the mature miRNA strand ('guide' strand, miRNA) are then brought together with RISC while the 'passenger' strand (a.k.a. miRNA*) is removed.[4] Selection of the miRNA or the miRNA* is thought to be due to the thermodynamic stability of the strands and the less stable strand on its 5' end is usually integrated into RISC.[5] However, both strands can be functional and be integrated into RISC in a tissue specific manner.[6]

Our understanding of miRNA processing seems rather limited as evidence shows that Drosha cleavage can also occur during transcription of both independently transcribed and intron-encoded miRNAs.[7] Other, as of yet poorly understood, factors seem to have roles in the downstream processing steps as well. For example, the processing of pri-let-7 in embryonic cells can be post-transcriptionally blocked by a negative regulator, Lin28, a developmentally regulated RNA binding protein.[8] Moreover, Lin28 also promotes cellular transformation and therefore is regarded

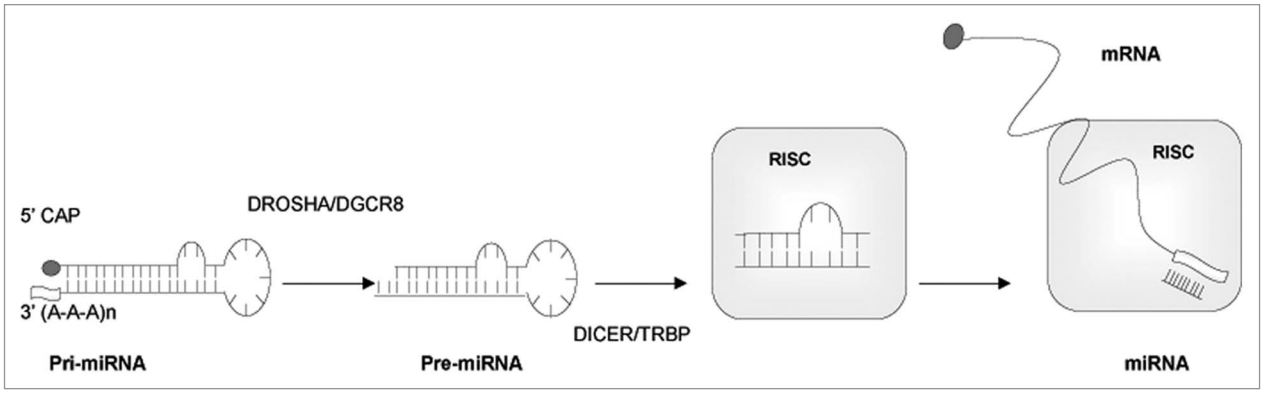

Figure 1. miRNA biogenesis. A several kb long pri-miRNA with a 5'cap and 3'poly (A) tail, is cleaved by nuclear RNase III Drosha and DGCR8 into a 60–70 nucleotide long pre-miRNA. Dicer, and its RNA binding partner TRBP cleave the pre-miRNA into the mature miRNA duplex (20–25 nucleotides), which assembles into the RISC (RNA-induced silencing complex). The target mRNA and the mature miRNA strand ('guide' strand) are brought together within RISC, the 'passenger' strand is removed.

as a proto-oncogene.[9] Recently, the 5'→3' exoribonuclease, XRN-2, was shown to regulate mature miRNA turnover in the absence of its mRNA target in *C. elegans*.[10] Whether these proteins are global regulators of all miRNAs remains to be determined.

The complementary interaction between target mRNA and miRNA causes a decrease of the target protein by either degradation of the mRNA or translational repression. Deadenylation of mRNA due to the binding microRNA causes degradation of mRNA[11] and to some extent, translational repression.[12] miRNA mediated translation repression can happen at either the initiation or elongation stages of translation[13] or through the inhibition of 60S ribosomal subunit joining to the 40S initiation complex.[14] Ribosomal drop-off is another mechanism that can lead to repressed translation.[15]

While the current widely accepted view of miRNA regulated gene expression is based on post-transcriptional downregulation of target genes, intriguing recent findings suggest that this may be incomplete and limited. One such surprising finding is that some miRNAs are detected in the nucleus rather than the cytoplasm where post-transcriptional silencing takes place. In support of this observation, cis or trans activity of miRNAs on gene promoters is now being questioned. For example, miR-320 is encoded in the promoter region of *POLR3D* [polymerase (RNA) III (DNA directed) polypeptide D] and acts in cis to negatively regulate *POLR3D* expression.[16] An opposite example comes from miR-373. It binds in trans to the promoter regions of *CDH1* (E-Cadherin) and *CSDC2* (cold shock domain containing promoter 2) and causes transcriptional upregulation of these genes in the PC-3 prostate cancer cell line.[17] Moreover, the promoter of an mRNA also seems to be a determinant of how it will be regulated by miRNAs (i.e., inhibition of protein synthesis at the initiation or post-initiation steps). Evidence suggests that identical mRNAs controlled by different promoters (e.g., SV40 or TK) are indeed inhibited at different stages of translation.[18]

As miRNAs' new roles in gene expression are being revealed, mRNA isoforms with varying 3' UTRs due to alternative cleavage and poly-adenylation sites are shown to be differentially regulated by miRNAs. Primary murine $CD4^+$ T lymphocytes were shown to express shorter 3' UTRs for selected mRNAs during immune cell activation. As more upstream poly-adenylation signals were preferred, shorter UTR containing mRNAs escape from miRNA regulation.[19] Bioinformatics tools predict the presence of alternative poly-adenylation sites for as many as 49% of human, 31% of mouse and 28% of rat genes.[20] Alternative miRNA binding to different length 3' UTRs, therefore, promises to be a very important regulator of gene expression. Given this and the fact that many genes implicated in cancer are postulated to drive oncogenesis based on altered levels of expression, one can speculate that miRNAs may play important roles in the overexpression of oncogenes.

Following the initial discovery of miRNAs in *C. elegans* in 1993, miRNAs have attracted a lot of attention, thanks to a dramatic rate of new miRNA discovery as well as an ever-growing understanding of their intriguing functions and implications in human diseases. Mounting evidence makes it clear that miRNAs are essential for various important cellular mechanisms including metabolism, development, differentiation, cell proliferation and apoptosis and are highly relevant to disease processes.[21]

miRNAs and Cancer

Following the initial identification of a link between miRNAs and cancer, many miRNA genes within regions of genomic instability and chromosomal fragile sites are shown to have abnormal DNA copy numbers in cancer cells.[22-24] Although we are gaining global insight into deregulated miRNA expression in different tumors and/or tumor subtypes, our understanding of individual miRNA functions and their effects on downstream pathways is also expanding. Recent findings showed that specific miRNAs have significant roles during the early and late stages of neoplastic transformation, invasion and metastatic processes. For example, miR-21, on 17q23, which is overexpressed in breast cancers, glioblastomas, pancreatic cancers, hepatocellular cancers, cholangiocarcinomas, ovarian and colorectal cancers, targets *PTEN* and *PDCD4*, which have roles in cell survival and transformation processes (reviewed in ref. 25). Overexpression of

miR-21 further stimulates invasion, intravasation and metastasis through targeting mRNAs such as *SERPINB5, TPM1, RECK, TIMP3* (reviewed in ref. 26). miR-10b, which is highly expressed in metastatic breast cancer cells (almost 50% more than non-metastatic breast cancers), regulates the migration and invasion of otherwise non-invasive breast cancer cells.[27] Similarly, miR-373 and miR-520c were found to stimulate cancer cell migration and invasion both in vitro and in vivo by targeting CD44, a receptor for hyaluronan found in the extracellular matrix.[28] miR-146a and miR-146b, which target IRAK1 and TRAF6, cause the down-regulation of NFκB, thereby inhibiting the invasion and migration of breast cancer cells.[29] While the list of individual miRNAs involved at the different steps of tumorigenesis expand, a miRNA microarray study of 43 matched primary tumors and their corresponding lymph node metastases samples (colon, bladder, breast and lung) resulted with 32 differentially expressed miRNAs.[30] As an early step during metastasis, the epithelial to mesenchymal transition (ET) also seems to be tightly regulated by miRNAs, mainly the miR-200 family.[31]

In addition to the identification of miRNA targets that have roles in tumorigenesis, important oncogenes or tumor suppressors have also been shown to control miRNA expression in different cancers. For example, downregulated let-7 targets the widely recognized oncogene, *RAS*,[32] while transcription of miR-34a and miR-34b/c are found to be induced by TP53 (reviewed in ref. 33), and the oncogenic miR-17-92 cluster is activated by MYC.[34]

With well-established roles in tumorigenesis, deregulated epigenetic mechanisms also seem to regulate miRNA expression. Several studies suggested that some cancers showed hypermethylation of miRNA promoters and thus decreased expression of miRNAs. A cancer specific CpG hypermethylation signature was demonstrated for miR-148a, miR-34b/c and miR-9 in three lymph node metastatic cell lines.[35] miR-143 was proposed to be a tumor suppressor based on its potential role in epigenetic mechanisms in colorectal cancer by targeting DNA methyltranferase 3A (DNMT3A).[36]

Such findings point out how crucial it is to unravel the consequences of deregulated miRNA expression in cancer cells. Considering the number of mRNAs that a miRNA can potentially target, deregulated expression of a miRNA may indeed affect multiple pathways in a cell. Therefore, the molecular tools used in laboratories are being modified so as to detect miRNAs with greater sensitivity and specificity as well as to understand the pathways they may regulate during tumorigenesis. In the following sections, an overview of different techniques to detect and/or quantify miRNAs and how they contributed to oncology will be provided.

miRNA Detection

Hybridization based methods. Modified northern blotting protocols are used to specifically capture small RNA on urea-acrylamide gels rather than agarose gels by using LNA (locked nucleic acid) probes to increase sensitivity and specificity.[37] Generally, high throughput miRNA profiling is needed and because cDNA microarrays have provided valuable information in cancer research, conventional microarray technologies are being modified to handle the small sizes of miRNAs. LNA based probes have proven to be effective in microarray studies as well, as they provide thermal stability for hybridization. For the labeling of target miRNAs; there are different applications such as guanine labeling of miRNAs, T4 RNA ligase labeling, poly (A) polymerase labeling, chemical labeling, random primed reverse transcription based labeling (reviewed in ref. 38). Although microarrays provide high throughput results, fabrication costs could be high. Moreover, the number of newly identified miRNAs is increasing almost every day, which is a challenge for designing updated platforms. Yet, microarrays provided significant information for miRNA expression profiling.

Studies have shown tissue specific expression patterns of miRNAs and suggested their discriminatory power for the normal vs. tumor cells. Volinia et al. identified a set of differential miRNA expression profiles in which the miRNAs were largely overexpressed for matched normal and tumor samples (breast, lung, stomach, prostate, colon and pancreas) using miRNA microarrays.[39] Such studies further confirmed the significance of miRNA expression differences among tumor and normal tissues and their potential power to be used as diagnostic tools. Recently, a 5-miRNA signature (normalized by U6 expression) was determined for predicting survival in 112 non-small cell lung carcinoma patients.[40] Similarly, miRNA expression profiles were shown to be more effective as predictors of overall survival than a previously described 50-gene prognostic signature for squamous cell lung carcinomas.[41]

On the other hand, in situ hybridization (ISH) with LNA probes provides the advantage of observing spatial organization of miRNA expression so that a better understanding of miRNA function may be inferred. Whole-mount ISH can give spatiotemporal information about which miRNAs have important roles in the development in certain tissues. ISH with formalin-fixed, paraffin-embedded (FFPE) samples also gives valuable information about spatial expression levels of miRNAs in tumor and matched normal tissues. As more sensitive ISH techniques are being developed for miRNAs, studies have already generated information about where miRNAs are expressed in normal and cancerous cells and tissues. One such study identified a group of differentially expressed miRNAs by LNA microarray and these miRNAs were further examined by ISH in FFPE breast tumors.[42] miR-145 and miR-205 expression in the myoepithelial/basal cells in normal mammary tissue was found to be decreased in the matching tumor samples. Similarly, let-7a expression was decreased in tumors whereas miR-21 expression was high.[42] miR-21, present in the luminal cells, was found to be progressively increasing in flat epithelial atypia, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) FFPE samples compared to normal cells.[43] Such applications may prove to be clinically useful for the rapid classification of tumors.

In addition to these, a comparative hybridization experiment was also developed where a Renilla luciferase labeled oligo probe that had the same sequence as the miRNA competed with the miRNA itself to bind to an immobilized biotinylated anti-miRNA probe.[44] miR-21 levels in MCF7 and MCF-10A cells

Table 1. A comparison of most commonly used miRNA detection and quantification techniques

Technique	Applications/Advantages	Disadvantages
Northern Blotting	• Urea-polyacrylamide gel and LNA probes are used • Precursor and mature miRNAs can be detected at the same time	• Labor intensive • High RNA concentrations needed • Not high-throughput
Microarrays	• High throughput • High sensitivity due to LNA probes	• Available platforms may not represent all miRNAs due to high miRNA discovery rate • High fabrication rates • Sensitivity issues for detecting closely related miRNA family members
In situ hybridization	• Spatiotemporal miRNA expression can be detected	• Does not provide size information • Proper fixation of samples required • Not high-throughput
Bead based systems	• Hybridization happens in solution • High specificity	• Requires both PCR and hybridization • Flow cytometer is needed
Real-time PCR	• Stem loop RT-PCR primers provide specificity for mature miRNA detection	• Not high-throughput
RAKE	• miRNA homologs can be specifically detected	• Requires both PCR and hybridization

were detected this way with a limit of 1 fmol and confirmed higher expression of miR-21 in MCF7 cells.[44]

Recent advances in next-generation sequencing technologies are also applicable to miRNA quantification and novel miRNA identification. Using Illumina sequencing technology, expression levels of 334 known miRNAs were determined in embryonic stem cells before and after differentiation. Moreover, 104 novel miRNA genes were identified using this approach.[45] While such technologies provide high speed and sensitivity, data analysis using appropriate bioinformatics tools is gaining importance. A few examples of tools specifically developed for miRNA analysis by the next-generation of sequencing results are miRDeep[46] and miRanalyzer.[47]

Bead based systems. A bead based hybridization and flow cytometry method was developed to detect miRNA expression profiles of 334 different tissue samples including tumors.[48] miRNA specific oligonucleotide-capture probes were coupled to beads labeled with different fluorescent dyes. Following adapter ligations and PCR amplification of miRNAs using biotinylated primers, hybridized beads were stained with streptavidin-phycoerythrin. Flow cytometric analysis of beads then gave miRNA identity (due to bead color) and miRNA abundance (due to phycoerythrin intensity) information. This hybridization was suggested to be advantageous to microarray based systems in terms of specificity, which becomes an important issue for the specific detection of different members of a miRNA family. Using this system, Lu et al. demonstrated that miRNA expression profiles clustered tumors according to their developmental lineage and differentiation states.[48] The authors pointed out that when compared to matching normal tissues; some miRNAs were globally under-represented. Another study using the same system for 93 primary breast tumors identified a miRNA signature that was specific enough to differentiate tumor subtypes.[49]

Amplification based methods. Amplification based methods for miRNA detection and/or quantification are attractive as it is then possible to start with lower sample concentrations compared to hybridization based approaches. miRNAs can be quantified by real time RT-PCR (qRT-PCR) after a stem-loop primer is used to generate the cDNA by reverse transcription.[50] Stem-loop based Taqman assays and SYBR Green based qRT-PCR systems with LNA primers are sensitive enough to discriminate between miRNA family members and are widely being used by researchers. There are also high-throughput miRNA quantification systems (384-well reaction plates),[50] some of which require very small sample sizes. Another approach is RAKE (RNA primed-array-based Klenow enzyme assay) where miRNAs bound to their probes act as primers to amplify a fragment by Klenow with incorporated tagged nucleotides for detection purposes.[51] This method can discriminate nucleotide mismatches at the 3' end where most differences are observed between miRNA homologs.

A summary of the above mentioned commonly used techniques and their advantages and disadvantages are provided in **Table 1**.

miRNA Target Identification

The fact that a single miRNA can target multiple mRNAs with various degrees of complementarity makes target mRNA prediction, identification and validation issues quite intricate and challenging. Various computer based tools are widely used to determine potential miRNA targets. Each tool has specific strengths and weaknesses regarding sensitivity and specificity of identifying targets. These tools and predictions are usually based on sequence similarity, conservation and structural properties. Such predictions have proved to be helpful for determining a potential mRNA target to experimentally validate the interaction; but many false positive and false negative results make it quite difficult to select the real biologic targets of the miRNAs. To help address these prediction problems, a RISC immunoprecipitated mRNA pool followed by microarray analysis for miR-124a was

developed to physically identify mRNA targets of miRNAs. The immunoprecipitated mRNAs were enriched in miR-124a seed complementary sequences in their 3' UTRs.[52]

While most of the miRNA detection/validation experiments focus on mRNA levels, it is obvious that miRNA effects would be most visible at the protein level. In such a study, SILAC (stable isotope labeling with amino acids in culture) and quantitative mass spectroscopy were used to reveal protein level effects of miR-1 in HeLa cells.[53] While revealing altered protein levels, this approach also showed that that the miR responsive proteins' mRNAs harbored "seed-matched" sites in their 3' UTRs. Another proteomics approach to miRNA target identification was to silence miR-21 in MCF7 cells and identify differentially expressed proteins by quantitative tandem mass spectroscopy.[54] In this study, 58 putative protein targets of miR-21 were identified, many of which had stable mRNA levels in the miR-21 positive and silenced MCF7 cells.[54]

miRNAs as Cancer Biomarkers

miRNAs are emerging as potential targets for biomarker discovery in clinical samples partly because, somewhat surprisingly, a large number of RNase resistant miRNAs have been detected in body fluids; mainly serum and plasma. For example, miR-21 levels were high in lymphoma patients' serums correlating with their relapse-free survival.[55] Another miRNA, miR-210, a hypoxia induced miRNA, was overexpressed in different tumors and was found in the serum of lymphoma patients, thus it may be a biomarker candidate for hypoxic malignancies.[56] Similarly, serum levels of miR-141 were also distinguishable between prostate cancer patients and healthy controls.[57] miRNA expression profiling using an oligonucleotide microarray with low serum volumes were also shown to be discriminatory between normal and prostate, colon, ovarian, breast and lung cancer patient samples.[58] In parallel to previous findings, miR-21, miR-210, miR-155 and miR-196a expression profiles in plasma were able to differentiate pancreatic adenocarcinoma patients from healthy controls.[59] In addition to plasma and serum, saliva also harbors miRNAs. miR-125a and miR-200a levels were in lower quantities in the saliva of oral squamous cell carcinoma patients compared to control subjects.[60] miRNAs are also promising effective biomarkers for tracing the tissue of origin of cancers of unknown primary origins.[61]

miRNAs and their relationship with prognosis and/or patient responses to therapy are already being exploited by many studies. In such a study of 241 hepatacellular carcinoma patients, miR-26 levels appeared low in patients who had shorter overall survival but a better response to interferon therapy.[62]

While all these exciting results indicate that miRNAs may function as reliable biomarkers, a larger number of patients need to be screened before they are proven to be so. Hopefully such developments in biomarker discovery will lead to the detection of cancers at very early stages and thus increase the survival rates. Therefore, the development of tools to detect the expression of certain miRNAs in body fluids and/or pathological samples is crucial and is already underway.

Current Developments and Future Directions

Given the deregulated expression of miRNAs in cancer cells and their significant roles in tumorigenesis, miRNAs are being tested for their potential in medical therapeutics. Modified anti-sense oligos have been shown to effectively and specifically bind to mature miRNAs and prevent miRNA functions. Therefore, targeting miRNAs by anti-sense oligos may have many implications for research and therapeutic applications. For example, the use of an anti-sense LNA/2'-O-methyl oligonucleotide mix-mer or Peptide Nucleic Acid (PNA) oligomer was able to specifically block miR-122 in liver cells.[63] However, an unspecific immune response and/or unspecific changes in gene expression due to external miRNA introduction into cells are major problems that may complicate the success of a therapeutic application. Khan et al. report that the introduction of small RNAs into cells can create a competition between endogenous and exogenous RNAs for RISC (and possibly other machinery) binding.[64] Therefore, genes that are normally regulated by miRNAs may be affected due to the saturation of the RISC. In that case, deregulated expression of unintended targets and associated potential toxicities will have to be investigated both in vitro and in vivo. Aside from these caveats, any miRNA can bind to numerous mRNAs, therefore introducing or silencing an miRNA in cells should be carefully investigated. Another challenge we have to address is the issue of polymorphisms that could be important during miRNA biogenesis steps and miRNA-mRNA interactions. Therefore, it will be quite exciting to establish the relevance of polymorphisms with miRNA expression and/or function and to screen large sets of patients for association studies.

In summary, since their discovery, miRNAs have gained well deserved attention. miRNA expression patterns, their mRNA targets and functions demonstrated important roles both in normal and cancerous cells. Moreover, miRNAs have already proved to have key roles in various aspects of tumorigenesis. As our understanding of miRNAs improves, we will be able to use this information to develop more effective diagnostic, prognostic and therapeutic tools.

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