MicroRNAome Genome: A Treasure for Cancer Diagnosis and Therapy

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The interplay between abnormalities in genes coding for proteins and noncoding microRNAs (miRNAs) has been among the most exciting yet unexpected discoveries in oncology over the last decade. The complexity of this network has redefined cancer research as miRNAs, produced from what was once considered "genomic trash," have shown to be crucial for cancer initiation, progression, and dissemination. Naturally occurring miRNAs are very short transcripts that never produce a protein or amino acid chain, but act by regulating protein expression during cellular processes such as growth, development, and differentiation at the transcriptional, posttranscriptional, and/or translational level. In this review article, miRNAs are presented as ubiquitous players involved in all cancer hallmarks. The authors also describe the most used methods to detect their expression, which have revealed the identity of hundreds of miRNAs dysregulated in cancer cells or tumor microenvironment cells. Furthermore, the role of miRNAs as hormones and as reliable cancer biomarkers and predictors of treatment response is discussed. Along with this, the authors explore current strategies in designing miRNA-targeting therapeutics, as well as the associated challenges that research envisions to overcome. Finally, a new wave in molecular oncology translational research is introduced: the study of long noncoding RNAs. **CA Cancer J Clin 2014;64:311-336. © 2014 American Cancer Society.**

Keywords: microRNA, diagnosis, therapy, cancer.

Introduction

MicroRNAs Are Strangers in the Genomic Galaxy

The central dogma of molecular biology is an explanation of the flow of genetic information within a biological system and it is summarized in the fact that "DNA makes RNA, which encodes protein." However, over the past few years, DNA segments have been shown to produce RNA transcripts that do not encode proteins. These transcripts were named noncoding RNAs (ncRNAs), and they were considered to be part of a "dark" unexplored segment of the human genome. MicroRNAs (miRNAs) are a class of small ncRNAs 19 to 25 nucleotides (nt) in length that can regulate gene expression by various mechanisms that have still not been fully investigated. They represent the most explored side of the "dark" matter of the genome,[1-3] and the full complement of known (cloned) miRNAs present in a genome is named microRNAome (for a glossary of terms, see Table 1).

Initially, the DNA segments containing miRNA-coding genes are transcribed by an RNA polymerase II or III (RNA Pol II and III) to initiate their biogenesis. The primary transcript (pri-miRNAs) can be hundreds or thousands of nt long, but it is further processed, forming a ~100-nt precursor transcript that folds upon itself (Fig. 1).[4-6] The precursor sequence is then exported to the cytoplasm, where it undergoes a series of catalytic steps prior to achieving maturation. In the cytoplasm, mature single-stranded miRNAs are integrated into a number of proteins that compose the RNA-induced silencing complex (RISC), and thereafter they interact by sequence complementarity with the messenger RNA (mRNA) (Fig. 1).[4-6,7]

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DISCLOSURES: George A. Calin, MD, PhD, is The Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. Work in Dr. Calin's laboratory is supported in part by National Institutes of Health/National Cancer Institute grants 1UH2TR00943-01 and 1 R01 CA182905-01; Developmental Research Awards in prostate cancer, multiple myeloma, leukemia (P50 CA100632), and head and neck (P50 CA097007) Specialized Programs of Research Excellence (SPORE) grants; a Sister Institution Network Fund (SINF) University of Texas MD Anderson Cancer Center DKFZ grant in chronic lymphocytic leukemia (CLL); a SINF grant in colon cancer; a Kidney Cancer Pilot Project; The University of Texas MD Anderson Cancer Center Duncan Family Institute for Cancer Prevention and Risk Assessment; The Blanton-Davis Ovarian Cancer-2013 Sprint for Life Research Award; the Laura and John Arnold Foundation; the RGK Foundation and the Estate of C.G. Johnson, Jr; and by the CLL Global Research Foundation. Dr. Berindan-Neagoe's work was financed by a POSCCE 709/2010 grant.

doi: 10.3322/caac.21244. Available online at cacancerjournal.com

TABLE 1. Glossary of Terms Used in the MicroRNA World

ASO: An antisense oligonucleotide is a single-stranded, chemically modified DNA-like molecule that is 17 to 22 nucleotides in length and designed to be complementary to a selected messenger RNA (mRNA) or noncoding RNA and thereby specifically inhibits expression of that gene.
Exome sequencing (targeted exome capture): An efficient strategy to selectively sequence the coding regions of the genome.
Messenger RNA (mRNA): The form of RNA that mediates the transfer of genetic information from the DNA in the cell nucleus to ribosomes in the cytoplasm, in which it serves as a template for protein synthesis.
MicroRNAome: The full complement of known (cloned) microRNAs (miRNAs) present in a genome. Due to multiple cloning efforts, it is growing constantly and by May 2014 contains 2578 mature human miRNAs (release 20 of miRBase available at mirbase.org/index.shtml).
Noncoding RNAs (ncRNAs): Any RNA molecule that is not translated into a protein.
Oncogenic miRNA: A miRNA that when expressed at higher levels than normal initiates or favors the development of a tumor.
Open reading frame (ORF): A section of an mRNA that begins with an initiation (methionine ATG) codon and ends with a nonsense codon. ORFs all have the potential to encode a protein or polypeptide; however, many may not actually do so.
Pol II: RNA polymerase II (also called RNAP II) catalyzes the transcription of DNA to synthesize precursors of miRNAs and most small nuclear RNAs.
Pol III: RNA polymerase III (also called RNAP III) transcribes DNA to synthesize ribosomals RNA, transfer RNAs, and other small RNAs. The genes transcribed by RNA Pol III fall in the category of "housekeeping" genes whose expression is required by all cell types and most environmental conditions.
Pseudogene: A copy of a gene that usually lacks introns and other essential DNA sequences necessary for function. A pseudogene has been mutated into an inactive form over the course of evolution, but contains the majority of interactor sites with miRNAs as the original functional gene.
Sense/antisense: Refers to the strand of a nucleic acid that directly specifies the product or refers to the strand of a double-stranded molecule that does not directly encode the product but is complementary to it (antisense).
Single nucleotide polymorphism (SNP): A variation at a single position in a DNA sequence among individuals. If a SNP occurs within a gene, then the gene has more than one allele.
The Cancer Genome Atlas (TCGA): A project started in 2005 to catalogue genetic alterations responsible for cancer, using gene expression profiling, copy number variation profiling, single nucleotide polymorphism genotyping, genome-wide DNA methylation profiling, miRNA profiling, and exome sequencing.
Transcription: The process by which RNA is synthesized from a DNA template.
Translation: The process of ribosome-mediated production of a protein by which the primary structure of the protein is determined by the codon nucleotide sequence of an mRNA.
Tumor suppressor miRNA: A miRNA that when expressed normally blocks the initiation or development of a tumor; at lower levels of expression, this braking effect disappears and the tumor can develop. The same miRNA can behave as an oncogene in one type of tissue and as a tumor suppressor in another type.
Untranslated region (UTR): 5′UTR is the portion of an mRNA from the 5′end to the position of the first codon used in translation. The 3′ UTR is the portion of an mRNA from the 3′end of the mRNA to the position of the last codon used in translation.

As part of the new ncRNA-based dogma, miRNAs have been known to bind to mRNA at the 3′-untranslated region (UTR) and cause the downregulation of protein-coding genes (named targets) in the cytoplasm. They do so by inhibiting the ability of the ribosome to "translate" the mRNA. Alternatively, miRNAs can increase mRNA degradation, also reducing the possibility of the mRNA being translated into proteins.[8] The level of complementarity between miRNA and the target mRNA may determine the mechanism by which the translation of mRNA to protein is blocked. Perfect or near-perfect complementarity has been found to induce mRNA degradation by RISC, and partial complementarity has been found to repress mRNA translation by blocking ribosomal access to the mRNA.[9] Due to the fact that each miRNA has hundreds or thousands of mRNA targets, a broad segment of the protein-coding genome is under their control. The regulation of miRNA-coding genes is of critical interest because they might be involved in any type of pathophysiological process and/or pathway, such as B-cell survival (miR-15a and miR-16-1), B-cell lineage fate (miR-181), brain patterning (miR-430), pancreatic cell insulin secretion (miR-375), adipocyte development (miR-145), cell proliferation control (miR-125b and let-7), or cell survival (let-7 family).[10]

The understanding of the mechanisms of action of miRNAs has significantly expanded in the last few years, with discoveries demonstrating unexpected complexities of their regulative manner, such as promoter binding, protein binding, or direct interaction with other ncRNAs (Fig. 2). miRNAs can be relocalized in the nucleus; for example, human miR-29b has been found predominantly in the nucleus, demonstrating that despite their small size, specific miRNAs contain specific nucleotide sequences that control their subcellular localization.[11] This localization supports the already proven hypothesis that miRNAs can alternatively regulate transcriptional processes at a DNA level. For example, human miR-373 binds to the E-cadherin (CDH1) promoter, thereby inducing its expression.[12] Futhermore, miRNAs target other genetic regions at the mRNA level besides the 3′-UTRs, such as 5′-UTR and coding regions.[13-15] Finally, besides mRNAs, miRNAs can target

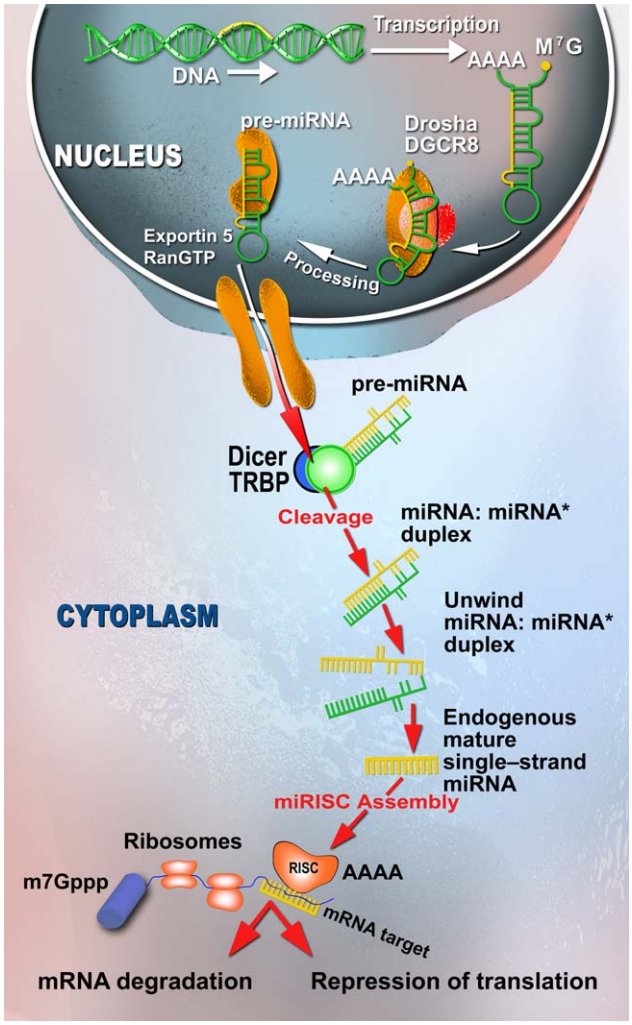

FIGURE 1. MicroRNA Processing. RNA polymerase II is responsible for the initial transcription of the microRNA (miRNA) gene into a long, capped, and polyadenylated (poly-A) precursor, called primary miRNA (pri-miRNA).[4,5] A double-stranded RNA-specific ribonuclease, Drosha, in conjunction with its binding partner DGCR8 (DiGeorge syndrome critical region gene 8 or Pasha), further processes pri-miRNA into a 70- to 100-nucleotide hairpin RNA precursor (pre-miRNA).[6] Pre-miRNA is translocated from the nucleus to the cytoplasm by Exportin-5/RanGTP, and cleaved into an 18- to 24-nucleotide duplex by a ribonucleoprotein complex composed of ribonuclease III (Dicer) and TRBP (human immunodeficiency virus-1 transactivating response RNA-binding protein). Finally, the duplex interacts with the RNA-induced silencing complex (RISC), which includes proteins of the Argonaute family (Ago1 to Ago4 in humans). One strand of the miRNA duplex remains stably associated with RISC and becomes the mature miRNA, which primarily, but not exclusively, guides the RISC complex to the 3′-untranslated region of target messenger RNAs (mRNA). The other strand named "star" has also been found to be functional. Although the interaction between miRNA and mRNA usually results in translation inhibition, some cleavage of target mRNAs has also been observed.

different types of ncRNAs, some of them being highly conserved among species such as the ultraconserved genes[4] or poorly conserved such as the pseudogenes (see Table 1 for the definition of terms).[5]

In the past few years, miRNAs have also been found to favor protein expression (in addition to downregulating it). MiR-369-3p was shown to interact with adenylate uridylate-rich elements in the tumor necrosis factor-alpha (TNF-alpha) mRNA, consequently recruiting proteins that increased the process of translation during cell cycle arrest.[6] Similarly, miR-328 was revealed to increase translation by interacting with hnRNPE2, a translational regulator, leading to the release of CCAAT/enhancer-binding protein alpha mRNA. Conversely, it decreased the translation of PIM1 kinase by binding specifically to its mRNA. Both mechanisms are active during the acute transformation of the chronic phase of chronic myeloid leukemia (CML) and are independent of patients' response to imatinib. Together, these data reveal that miRNAs possess the ability to control the fate of protein-coding genes by attaching through base pairing complementary to mRNA sequences or by interfering with regulatory proteins directly.[16]

MiRNAs also work as secreted molecules that trigger a receptor-mediated response in a different cell or tissue. They have the ability to be released into the extracellular environment within exosomes (cell-derived vesicles originated by the inward budding in the plasma membrane generating multivesicular bodies) that are present in many and perhaps all biological fluids. In this way, they can act as "hormones."[17] For example, it has been shown that macrophages influence breast cancer cell invasion through the exosome-mediated delivery of oncogenic miR-223; moreover, pretreatment of mice with tumor-derived exosomes accelerates lung metastasis formation.[18]

Similarly, exosomes have been shown to modulate tumor microenvironments by releasing miRNAs in a coordinated manner. In this regard, exosomes derived from hypoxic cultures of a leukemic cell line in vitro were found to carry and release miR-210 among other angiogenic miRNAs, increasing tube formation by endothelial cells.[19] Along this line, it was also shown that miR-21 and miR-29a can be transported through exosomes and can act as direct agonists of Toll-like receptors (TLRs). By binding as ligands to receptors of the TLR family in immune cells, these miRNAs were shown to trigger a TLR-mediated prometastatic inflammatory response (such as secretion of interleukins) that could favor tumor growth and metastasis.[20,21] Understanding such newly recognized ways in which the miRNAs are working is important for both scientists and physicians as novel therapeutic approaches can be designed, such as blocking of miR-328 interaction with hnRNP E2 in patients with CML or blocking the miR-21 and miR-29a agonistic effects on TLRs in patients with metastatic diseases.

MiRNAs as Ubiquitous Players in Cancer

MiRNA alterations have been identified in many human diseases such as autoimmune and cardiac disorders, schizophrenia, and cancer (where they have been found to be highly dysregulated).[22] MiRNAs have been found to be differentially expressed between all types of analyzed human tumors and normal tissues, including benign and

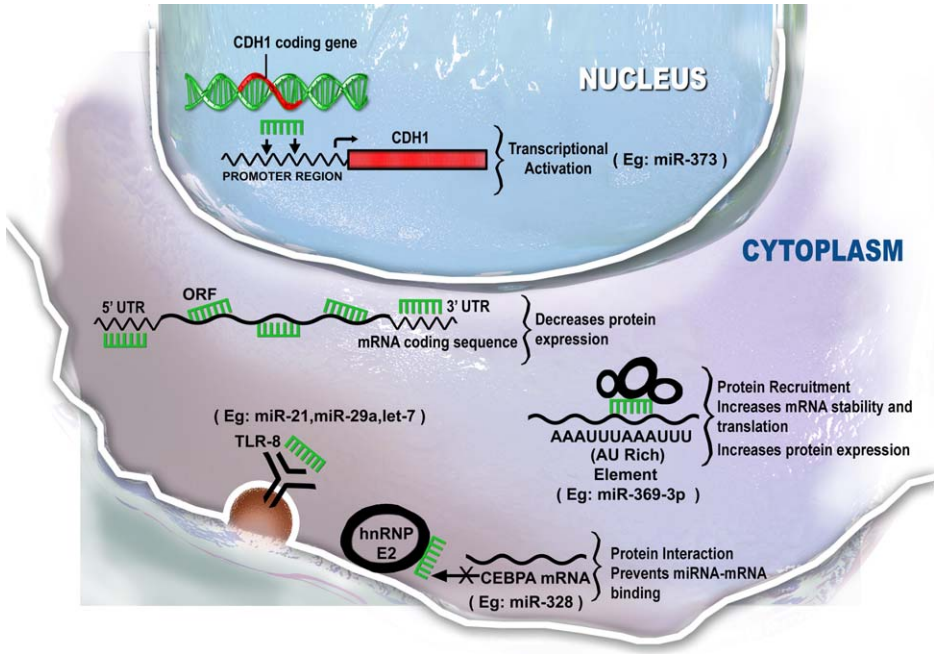

FIGURE 2. Mechanisms of Action of MicroRNAs. MicroRNAs can work through a variety of mechanisms, including (A) direct binding by complementarity to multiple regions at the messenger RNA (mRNA) including the 3′-untranslated region (UTR), 5′UTR, and coding regions and decreasing protein expression (majority of miRNAs); (B) positive regulation of translation (miR-369-3p) through the increased recruitment of processing proteins; (C) direct interaction with promoter sequences (miR-373); (D) direct agonism of receptors such as Toll-like receptors (miR-21, miR-29a, and let-7); and (E) direct interaction with protein and enhancing protein expression through a decoy mechanism (miR-328). For gene abbreviations please see ncbi.nlm.nih.gov/gene.

malignant tumors[23] such as leukemias, lymphomas, lung cancers, breast cancers, colorectal cancers (CRCs), papillary thyroid carcinomas, glioblastomas and other brain tumors, hepatocellular carcinomas, pancreatic tumors, cervical cancers, prostate cancers, kidney and bladder cancers, or pituitary adenomas.[23,24]

MiRNAs can act as oncogenes and/or tumor suppressors. MiRNAs have been proven to work as oncogenes (Table 1), such as miR-21 or miR-155, both of which are among the most frequently overexpressed miRNAs in human cancers.[23] For example, in a transgenic miR-155 mouse model, the rodents initially exhibited a preleukemic pre-B-cell proliferation evident in the spleen and bone marrow, followed by frank B-cell malignancy.[25] Overexpression of miR-21 in mice similarly leads to a pre-B-cell malignant lymphoid-like phenotype, demonstrating that this gene is also a genuine oncogene. When miR-21 was inactivated in vivo, the tumors regressed completely within a few days, partly as a result of enhanced apoptosis.[26]

MiRNAs can act also as tumor suppressors, such as the miR-15a/16-1 cluster, which is frequently deleted in chronic lymphocytic leukemia (CLL)[27] and prostate cancers.[28] The induced deletion of these miRNAs in a knockout mouse model causes the development of indolent B-cell, autonomous, clonal lymphoproliferative disorders, recapitulating the spectrum of CLL-associated phenotypes observed in humans. The miR-15a/16-1-deletion has been demonstrated to accelerate the proliferation of both human and mouse B cells by modulating the expression of genes controlling cell cycle progression.[29]

In some instances, the same miRNA can have dual activities, thereby acting as an oncogene in one specific cell type and as a tumor suppressor in another. For example, miR-221 is overexpressed in liver cancers, in which it targets the *PTEN* tumor suppressor, and in this way it promoted liver tumorigenicity in a miR-221 mouse transgenic model.[30] Similarly, in CRC, this same miRNA promotes cell invasion and metastasis by targeting RECK (which normally inhibits invasion and metastasis).[31] However, in other tumor types such as gastrointestinal stromal tumors, miR-221 is downregulated and the consequent derepression of c-KIT and ETV1 (target oncogenes) promotes this malignancy.[32]

The genetic basis for abnormal miRNA expression in cancer cells is complex. The widespread disruption of miRNA expression in malignant cells is only beginning to be understood, and a variety of abnormalities could contribute to the miRNAome expression profile in each tumor. Transcription factors are involved in the regulation of specific miRNAs, such as the miR-34 family and miR-15/16 cluster modulated by TP53,[33,34] the miR-17-92 cluster regulated by MYC,[34,35] or miR-210 regulated by hypoxia-inducible factor-1 alpha (HIF-1α).[36] The aberrant expression causes abnormal levels of mature and/or precursor miRNAs in comparison with the corresponding levels in normal tissues.[37] At least 3 different mechanisms (that could act independently or together) have been described to

cause this: the location of miRNA-coding genes at cancer-associated genomic regions frequently deleted or amplified[38]; epigenetic regulation of miRNA expression by methylation (which adds methyl groups to the cytosine or adenine DNA nucleotides, thereby suppressing genetic expression) or histone modification (which are proteins that package and order the DNA)[39]; and finally, abnormalities in miRNA processing genes or proteins (required for miRNA biogenesis and maturation).[40,41]

Germline and Somatic Mutations in miRNAs

Variations in the sequences of miRNAs located in the mature, precursor, or primary transcript may contribute to cancer predisposition and initiation.[42] For example, germline (passed from parental germ cell) or somatic (acquired by the somatic cell) mutations of some miRNA genes were found in patients with CLL. In the initial analyses of sequence variations in miRNAs, it was reported that in 2 patients diagnosed with CLL, a nucleotide substitution (C for T) was associated with lower levels of mature miR-16 (a tumor suppressor miRNA).[43] This mutation proved to affect the SRp20 site, which is an RNA-splicing protein implicated in processing the primary miR-16 transcript, and this was hypothesized to be contributing to the development of CLL.[44] Findings such as this point out that some patients with CLL may have a genetic predisposition toward this type of cancer. Furthermore, a mouse model also supported the role of certain miRNAs in the pathogenesis of CLL. Similarly, these mice harbor a point mutation (one nt) adjacent to miR-16, which results in its reduced overall expression.[45]

On a separate note, single nucleotide polymorphisms (SNP, see Table 1 for definition) in the protein-coding mRNAs targeted by miRNAs have shown to influence cancer risk as well. For example, let-7, a tumor suppressor miRNA known to target the mRNA of the *KRAS* oncogene, has been proven to be unable to bind to a *KRAS* SNP variant found to be disproportionately enriched in non-small cell lung carcinoma (NSCLC) (present in 18%-20% of cases).[46] This same *KRAS* variant was also associated with an increased risk of developing epithelial ovarian cancer, a finding that was consistent among 3 independent cohorts and 2 case-control studies.[46] Moreover, it was present in 61% of patients with a history of hereditary breast and ovarian cancer who were previously genetically uninformative (*BRCA1-2* negative).[46] This suggests that the SNP variant may be a new independent cancer risk biomarker for hereditary breast and ovarian cancer families. To date, most studies of miRNA binding-site SNPs have followed the case-control study design, and therefore they are centered on cancer risks. Consequently, the majority of our knowledge to date centers on cancer risk. For more information regarding miRNA binding-site SNPs considered to be cancer risk biomarkers, see the recent review by Preskill and Weidhaas.[46]

MiRNAs as Hormones

A large amount of data have accumulated over the last few years describing the participation of miRNAs and microenvironment cells in malignant processes.[47,48] Similar to hormones, miRNAs are released by a donor cell as "free" molecules or in secreted vesicles, and delivered from bodily fluids into receptor cells located in other areas.[17] For example, the tumor suppressor miR-143 was found to be released by normal epithelial prostate cells, inducing growth inhibition of prostate cancer cells in vitro and in vivo.[49] Similarly, intercellular transfer of miR-142 and miR-223 from immune cells to malignant cells (hepatocellular carcinoma cells) inhibited proliferation and destabilized microtubule regulation in vitro.[50] In addition, in a breast cancer study, miR-210 was shown to be released from tumor cells to endothelial cells, thereby promoting angiogenesis and metastasis through an exosomal-mediated transfer.[51] MiR-210 was also proven to be trafficked through exosomes in a leukemia cell line (under hypoxic conditions).[52] Along with other oncogenic miRNAs, the delivery of miR-210 resulted in increased angiogenesis through the promotion of tube formation by endothelial cells in vitro.[19]

MiRNAs Are Involved in All Cancer Hallmarks

The hallmarks of cancer comprise the biological capabilities acquired during the stepwise process of developing human tumors (Fig. 3). MiRNAs have the potential to influence these hallmarks, and the recognition of the applicability of these interactions will increasingly influence the development of new therapeutic alternatives for patients with cancer.[53,54] Herein are some representative examples of miRNAs that act as regulators of tumor biology; more detailed information on the roles of miRNAs in cancer can be found in several reviews.[22,24,40,55,56]

Self-Sufficiency in Growth Signals

Activation of the *RAS* oncogene is a common event that allows tumor cells to escape growth factor dependency and become "oncogene addicted." All 3 *RAS* genes (*K-RAS, NRAS,* and *H-RAS*) have been proven to be directly modulated by the tumor suppressor miRNA family of let-7's in a posttranscriptional manner.[57] Alternatively, let-7 also targets high mobility group A2 (HMGA2), a pleiotropic transcription factor. The characteristic downregulation of let-7 accompanying tumor development results in an increased expression of the *RAS* oncogenes along with their downstream effects. It also derepresses *HMGA2*, thereby

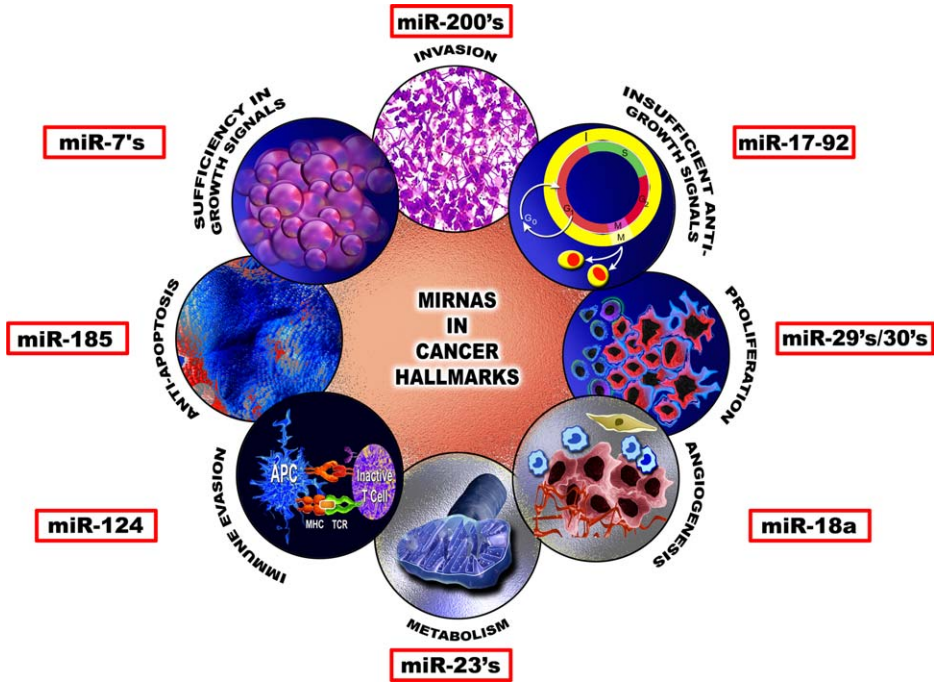

FIGURE 3. Examples of MicroRNAs Involved in the Cancer Hallmarks. The 8 biological capabilities acquired during the multistep development of human tumors include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction. For each, one representative example of a microRNA is presented.

facilitating anchorage-independent growth in cell cultures,[58] as well as cell proliferation and differentiation.[59] Evidently, both of these mechanisms have significant relevance regarding tumorigenesis and cancer development. Furthermore, reconstituting the levels of let-7 in an experimental approach demonstrated that this precursor miRNA family can inhibit cell proliferation, resulting in tumor regression as shown in lung cancer models in mice.[60]

Insensitivity to Antigrowth Signals

E2F is a group of genes that encode a family of transcription factors that tightly regulate cell cycle progression and DNA synthesis. Three of these, E2F1, E2F2, and E2F3a, are known as the cell cycle "activators," and they represent attractive factors to target in cancer because they contribute to uncontrolled cell growth. Several miRNAs have been demonstrated to have the potential to modulate the translation of the mRNAs of these transcription factors. For example, miR-20a, miR-17-5p, miR-93, and miR-106b have been shown to negatively regulate E2F1.[61,62] Moreover, the miR-17-92 cluster has been shown to decrease the levels of E2F1-3.[63] Therefore, in this sense, it is likely that the downregulation of these miRNAs in different types of cancer could favor a proliferative transcriptional network contributing to tumorigenesis. Thus, reconstituting the basal levels of expression of these miRNAs (in E2Fs-dependent tumors) could serve as a future therapeutic alternative with clinical relevance. Some of these miRNAs are part of positive or negative feedback mechanisms, and thus the end result of modulating their levels still remains to be explored.

Finally, in separate studies, the transcription factor FOXO1 (tumor suppressor that controls proliferation and regulates apoptosis) was found to be decreased in classic Hodgkin lymphoma. In classic Hodgkin lymphoma cell lines, the levels of FOXO1 were proven to be repressed by 3 upregulated microRNAs: miR-96, miR-182, and miR-183.[64] This repression highly increased proliferation, and at the same time inhibited apoptosis in vitro.

Evasion From Apoptosis

Apoptosis is a physiological self-destructive cellular mechanism that leads to the removal of unwanted cells. MiR-25 was identified as being elevated in cholangiocarcinoma cell lines as well as patient samples, and its increased levels were shown to contribute to the evasion of TNF-related apoptosis. In experiments that reduced levels of miR-25 (in vitro), cells in culture were sensitized to apoptotic death, thereby implicating the miRNA in the control of tumor cell apoptosis.[65]

The cancer-associated genomic region 1p36 (frequently lost or rearranged in many types of leukemias) contains a crucial tumor suppressor, miR-34a. In neuroblastoma, loss of miR-34a synergizes with *MYCN* oncogene amplification, and miR-34a has been shown to be a *MYCN*-negative regulator.[66] In addition, miR-34a is known to induce cell cycle arrest and subsequent caspase-dependent

apoptosis by repressing the antiapoptotic protein Bcl2[66] and the transcription inducer of cell cycle progression E2F3.[67] The overexpression of this same miRNA exhibits a transcriptome expression similar to that observed with p53 (a widely known tumor suppressor) induction, being highly enriched for genes regulating apoptosis, cell cycle progression, DNA repair, and angiogenesis.[68-70] This, along with other observations, points to the fact that the loss of this miR-34a sidesteps typical apoptotic pathways. Recently, another miRNA was also found to influence pathways leading to apoptotic evasion. In a gastric cancer study, a novel mechanism whereby miR-185 directly targets apoptosis repressor with caspase recruitment domain (ARC) was revealed. The role of this miRNA was studied in vitro and further validated in a gastric tumor xenograft model.[71]

Limitless Replicative Potential

Cellular senescence is a physiological withdrawal from the cell cycle in response to a variety of stressful stimuli and involves telomerase (an enzyme that prevents the loss of important DNA from chromosome ends) deregulation. MiRNAs have been linked to premature senescence, and their relevance has been addressed through the generation of a genetic miRNA screening library. The miR-29 and miR-30 miRNA families are upregulated during induced replicative senescence and their high levels influence the repression of the *MYBL2* oncogene inhibiting cellular DNA synthesis.[72] Moreover, miR-373 and miR-372 were identified as being capable of allowing proliferation and tumorigenesis of primary human cells harboring oncogenic *RAS* and wild-type *TP53* (a functional tumor suppressor).[73] These miRNAs neutralized p53-mediated cyclin-dependent kinase inhibition, possibly through direct inhibition of the expression of a tumor suppressor called *LATS2*. This evidence definitely implicates both of the miRNAs as being oncogenic, particularly in the development of human testicular germ cell tumors.

Angiogenesis

Tumor cells activate an "angiogenic switch," producing high amounts of proangiogenic factors that promote neovascularization. The most important one, vascular endothelial growth factor (VEGF), is highly expressed in most tumors, both solid and hematologic, and has been proven to be induced by hypoxia. During tumor progression, hypoxia has been found to contribute to the modulation of miRNA expression, in part by a direct transcriptional activation of specific miRNAs (such as miR-26, miR-107, and miR-210) triggered by HIF-1.[36] These miRNAs have dual functions: on the one hand, they participate in angiogenesis but conversely, they potentiate the ability of cells to engage in antiapoptotic mechanisms to sustain survival. For example, miR-27a restrains the zinc finger gene *ZBTB10*, a negative regulator of the Specificities Protein (SP) transcription factors, and through this repression it induces a SP-dependent transcription of genes related to both survival and angiogenesis (ie, survivin, VEGF, and VEGF receptor [VEGFR]).[74] Furthermore, miR-210, through direct modulation of the tyrosine kinase receptor ligand Ephrin A, represents a component of the circuitry controlling endothelial cell chemotaxis and tubulogenesis.[75] In addition, the downregulation of miR-18a has also been recently linked to angiogenesis. MiR-18a is known to inhibit the phosphorylation of 2 substrates of the mammalian target of rapamycin pathway. This results in an inactivation of the pathway and a consequent downregulation of factors that stimulate blood vessel production, such as HIF-1α and VEGF.[76]

Invasion and Metastasis

The process of metastasis begins with the acquisition of invasive properties that allow cells to detach from the primary tumor, enter the blood or lymphatic vasculature, and spread to distant organs. Upregulation of miR-10b has been demonstrated to promote invasion and metastasis by targeting HOXD10, a homeobox transcription factor that promotes or maintains a differentiated phenotype in epithelial cells.[77,78] Recently, a new miRNA that is involved in the migration and metastases of lung cancer cells was identified.[79] In lung cancer-positive transgenic mice, researchers found that miR-136, miR-376a, and miR-31 were prominently overexpressed. Among these, the antagonization of miR-31 suppressed tumor growth, suggesting causation. This same group also identified LATS2 (a tumor suppressor protein important for cytoskeleton function) and PPP2R2A (a phosphatase that catalyzes the removal of phosphate groups and is therefore considered an "inactivator protein") mRNAs as targets of miR-31 in mouse and human lung cancer models. In the attempt to identify miRNAs associated with lymph node metastasis in tissue samples from patients with lung adenocarcinoma, genome-wide next-generation miRNA sequencing and a training validation approach identified and validated miR-31 upregulation as being significantly associated with the presence of lymph node metastasis as well as poor patient survival. In addition, miR-31 was shown to be able to modulate the migratory, invasive, and proliferative behavior of lung adenocarcinoma cell lines in culture by stimulating the oncogenic ERK1/2 signaling pathway.[80]

Reprogramming Energy Metabolism

Malignant cells tailor metabolic pathways to meet their energy requirements.[81] Glutamine and glucose are the 2 major nutrients that fuel cellular metabolism and the pathways using these nutrients are often altered in malignant tumors cancer.[82] Various studies have exposed the fine

interplay between metabolic pathways orchestrated by protein-coding genes and by miRNAs. For example, glutamine metabolism (glutaminolysis) was shown to be modulated by the *MYC* oncogene via miR-23a/b in prostate cancer and also in B-cell lymphoma.[83] In addition, it can also be modulated by a p65-mediated activation that downregulates miR-23a levels.[84] Finally, glycolysis has been proven to be modulated by a series of different miRNAs, including miR-378-star[85] and miR-143.[86]

Evading Immune Destruction

Signal transducer and activator of transcription 3 (STAT3) regulates a key pathway mediating immunosuppression in the tumor microenvironment. Recently, the role of miRNAs in tumor-mediated immunosuppression began to be discovered. MiR-124 was found to be strongly downregulated in all grades and pathologic types of gliomas in comparison with normal brain tissue, and it was identified as an important modulator of STAT3 signaling.[87] Upregulation of miR-124 in glioma cancer stem cells has been shown to inhibit the STAT3 pathway. This resulted in the reversed induction of forkhead box P3 regulatory T cells (Treg) and it also reversed the glioma cancer stem cells-mediated immunosuppression of T-cell proliferation. Furthermore, the systemic administration of miR-124 by intratumoral or intravenous injection demonstrated to have antiglioma therapeutic effects in engineered murine glioblastoma models. The resulting effects indicated that miR-124 depends on the presence of a T cell-mediated antitumor immune response.[87]

Methods to Detect the Expression of miRNAs

Multiple platforms are available to identify and quantify miRNAs. An ideal method using miRNAs as biomarkers for human disease should be easy to perform and would not require expensive reagents or equipment; furthermore, it would be specific enough to detect only the miRNA of interest without detecting closely related miRNAs. It should also be sensitive enough to provide a quantitative expression analysis, even with low amounts of starting material from small clinical samples, and it would need to possess the ability to process multiple samples in parallel.[88,89] The gold standard meeting these requirements for miRNA detection in clinical laboratories is quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). MiRNA microarrays are more expensive than qRT-PCR and usually are used for the discovery step of biomarker identification (Table 2).[90-96] All these techniques, however, use miRNAs extracted from a patient tumor sample that includes malignant cells as well as nonneoplastic stromal and inflammatory cells. If the detection aims to specifically analyze miRNA in malignant cells, it is advisable to use cell sorting with flow cytometry for hematologic malignancies or laser capture microdissection for solid tumors. Another option is to perform in situ hybridization (ISH)[97] because the miRNA of interest can be detected within the different types of cells that compose the tumor (malignant vs microenvironment), and this approach provides additional information regarding the subcellular localization of the miRNA (Table 2).[90-96] The newest method to discover and measure miRNA expression is next-generation RNA sequencing. This technique is highly sensitive, highly specific, can be used for high-throughput analysis, and also for "de novo" discovering of miRNAs. RNA sequencing generates a massive amount of complex data that need to be analyzed by a well-trained bioinformatician. For this reason, as well as the high costs of a single RNA sequencing sample, this method is not yet appropriate for diagnostic purposes, but it can still be considered an alternative to investigate diseases such as cancer.

Understanding the Reproducibility of Profiling Data

There are some technical challenges that make it difficult to compare results from similar profiling platforms used in different places. For example, the different primer design for measurements by qRT-PCR and microarrays is an important factor.[98,99] Another factor is the use of different protocols for sample preparation: some studies use an enriched small RNA fraction whereas others use total RNA.[100] Furthermore, technical and biological variability has been assessed by a range of different normalization processes, mostly based on the expression of reference genes. Specifically for cell-free miRNA analysis, no known extracellular reference RNA is currently suitable for a proper normalization.[98] Commonly used reference genes such as U6 small nuclear RNA (*RNU6B*) and 5S ribosomal RNA were found to be less stably expressed than others or degraded in serum samples.[101] In addition, the significant differences in the choice of reference genes to use represent a major obstacle in comparing expression levels between normal tissue and tumors. Finally, the assessment of sample quantity and quality is more challenging for miRNAs than for mRNAs of protein-coding genes, for which the sizes and relative abundance of ribosomal RNAs can be used to check RNA integrity.

MicroRNAs: The Mix of Cancer Biomarkers and Hormones

Characteristics of a Good Biomarker

The measurement of miRNAs in bodily fluids including plasma and serum may represent a gold mine of noninvasive biomarkers for cancer.[17,102] An "ideal" biomarker should have a unique expression profile in the diseased compared with healthy tissues and should show highly increased or decreased expression levels in the diseased organ or tissue

TABLE 2. Established Profiling Methods to Quantify miRNAs

	ADVANTAGES	LIMITATIONS	REFERENCES
qRT-PCR	• Semi-high throughput • Good quantification • Amplification enables superior sensitivity (fM)	• Difficult to distinguish single nucleotide differences • Not for discovery of ncRNAs	90, 91
Microarray	• Very high throughput • Good ratio between cost and generated information • Easy to perform data analyses • Results are often validated with qRT-PCR	• In some cases fair specificity • Medium sensitivity (pM) • Sensitivity and specificity can be improved by LNA modification of the probes • Limited for quantification • Not useful for discovery of ncRNAs	92, 93
In situ hybridization	• Locate miRNA in tissue and cell compartments • miRNA and target identification on the same slide	• Low throughput • Invasive sample collection • Limited sensitivity • Very limited quantification	94, 95
RNA sequencing	• High throughput due to barcoding • High sensitivity(<fM) • High specificity • Can be used for discovery of novel ncRNAs	• Large amount of complex data that need to be analyzed • High cost	92, 96

fM indicates femtomole; pM, picomole; LNA, locked nucleic acids; miRNA, microRNA; ncRNA, noncoding RNA; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction.

compared with nondiseased material. Moreover, the biomarker should be reliable in detecting disease initiation or development before clinical symptoms appear with the goals of enabling early detection, persistence of minimal residual disease, or relapse after treatment. Furthermore, ideal biomarkers should be accessible through noninvasive methods; should have a long half-life in clinical samples; and should be rapidly detectable by simple, accurate, and inexpensive methods.[103] MiRNAs are very stable; even in bodily fluids such as plasma, serum, urine, and saliva,[17] their expression is specific to tissues or organs and their expression level can be easily measured by methods such as quantitative PCR and miRNA microarrays.[98] Consistently, it has been shown that serum miRNAs remain stable after being subjected to severe conditions that would normally degrade most mRNAs, such as very low or high pH levels, boiling, extended storage, and 10 freeze-thaw cycles. These features make them very suitable as biomarkers.

MiRNA profiling of tumor versus normal tissues shows significant changes and defines common altered miRNAs in human cancers. MiRNA profiling by various methods has allowed for the identification of signatures associated with the diagnosis, staging, disease progression, prognosis, and response to treatment of human tumors (Table 3).[80,104-119] MiRNA expression profiles now allow for the classification of human cancers.[23] To date, every type of tumor analyzed by miRNA profiling has shown significantly different miRNA profiles (for mature and/or precursor miRNAs) compared with normal cells from the same tissue type (Table 3).[80,104-119] For example, it was shown that profiling a few hundred miRNAs has a significantly better predictive power of diagnosing a cancer of unknown primary site (CUP) compared with profiling several tens of thousands of mRNAs.[120]

MiRNAs in Bodily Fluids as Biomarkers of Therapeutic Response, Residual Disease, and Overall Survival in Patients With Cancer

Clinical studies have demonstrated the potential of using miRNAs as predictors of a cancer's sensitivity to radiotherapy and anticancer agents (Table 3).[80,104-119] For example, serum miR-21 levels were found to be higher in patients with hormone-refractory prostate cancer whose disease was resistant to docetaxel-based chemotherapy when compared with those with chemosensitive disease.[121] In a separate study regarding lung adenocarcinoma and squamous cell carcinoma, 11 serum miRNAs proved to be able to separate patients into those with longer survival versus those with shorter survival. Furthermore, the levels of 4 of these miRNAs were significantly associated with overall survival (OS) and this same signature was consistently found to be an independent predictor of OS.[122] Likewise, upregulated and downregulated miRNAs have been detected in sensitive or resistant cell lines, in order to predict patient response to anticancer agents. One of these studies identified a miRNA chemosensitivity profile from a set of 59 human cancer cell lines derived from diverse tissues (NCI-60 cell lines). Downregulation of miR-34, miR-17, and let-7a was correlated with sensitivity to drugs commonly used as cancer treatments, such as 5-fluorouacil, doxorubicin, and

TABLE 3. Examples of Malignant Cells or Body Fluid MicroRNAs Profiles With Clinical Significance for Patients With Cancer

CANCER TYPE	NO. OF PATIENTS	miRNA	ROLE	REFERENCE
Multiple cancers	1809 patients: 7 different types of cancers: breast cancer, primary head and neck squamous cell carcinoma, renal cancer, soft tissue sarcoma, pediatric osteosarcoma, bladder cancer, and glioblastoma	miR-210	Predictive effect on survival of patients with studied cancer types as indexed by disease-free survival, progression-free survival, and relapse-free survival	104
Multiple cancers	174 patients and 39 controls: 50 breast cancers, 30 gastric cancers, 31 lung cancers, 31 esophageal cancers, and 31 colorectal cancers	miR-21	Potential broad-spectrum serum-based diagnostic marker for the detection of solid tumors	105
Breast cancer	120 patients and 40 controls	6 circulating miRNAs: miR-10b, miR-17, miR-34a, miR-93, miR-155, and miR-373	Known to be relevant for tumor development and progression; serum concentrations of deregulated miRNAs may be linked to a particular biology of breast cancer favoring progression and metastatic spread	106
Colon cancer	102 patients and 54 controls	miR-141	Highly correlated with TNM stage in patients with colorectal cancer; elevated levels associated with liver metastasis in patients with colorectal cancer	107
Colon cancer	100 patients with colorectal cancer, 37 with adenomas, and 59 controls	miR-29a	Association with TNM stage in patients with colorectal cancer	108
Colon cancer	103 patients with colorectal cancer and 37 controls	miR-221	Potential noninvasive molecular marker for the diagnosis of colorectal cancer	109
Colon cancer	Phase 1: 12 patients with stage I and stage IV colorectal cancer Phase 2: 182 patient with colorectal cancer and 24 controls Phase 3: 156 matched tumor tissues from the phase 2 colorectal cancer cohort plus an independent set of 20 matched primary colorectal cancers with corresponding liver metastases	miR-200c	Independent predictor of lymph node metastasis and tumor recurrence, emerging as an independent prognostic marker for colorectal cancer	110
Colon cancer	Exosome-enriched serum samples from 88 patients with primary colorectal cancer and 11 healthy controls and 29 paired samples from patients after tumor resection	let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, and miR-1246	The serum exosomal levels of 7 miRNAs were significantly higher in patients with primary colorectal cancer, even those with early-stage disease, compared with healthy controls, and were significantly downregulated after surgical resection of tumors	111
Gastric cancer	124 patients with noncardia gastric cancer and 36 patients with cardia adenocarcinoma and 160 controls	miR-16, miR-25, miR-92a, miR-451, and miR-486-5p	Detection of the early-stage gastric cancer	112
Gastric cancer	104 patients and 65 controls	miR-18a	Diagnostic power (high sensitivity and specificity)	113
HCC	101 patients, 89 controls, and 48 patients with hepatitis B	miR-21, miR-122, and miR-223	Elevated in patients with HCC or chronic hepatitis, with strong potential to serve as novel biomarkers for liver injury but not specifically for HCC	114
Lung cancer	Training set of 64 patients with adenocarcinoma; The Cancer Genome Atlas data set: 223 patients with adenocarcinoma	miR-31	Predictor of survival in a multivariate Cox regression model even when checking for cancer staging; exploratory in silico analysis indicated that low expression of miR-31 is associated with excellent survival for patients with T2N0 disease	80

TABLE 3. *Continued*

CANCER TYPE	NO. OF PATIENTS	miRNA	ROLE	REFERENCE
Lung cancer	25 paired NSCLC paracancerous tissues and serum, 103 control sera, and 201 patients with NSCLC	miR-19a	High serum miR-19a expression may be an independent poor prognostic factor for survival in patients with NSCLC	115
Lung cancer	30 NSCLC patients and 75 individuals without tumor pathology (eg, inflammatory-interstitial diseases, infections, nontumor lung nodules, hemotypsis and other diseases)	Exosomes isolated in plasma and BAL	In plasma, a higher percentage of miRNAs with increased levels compared with tumor BAL or in nontumor plasma; the data reveal differences between BAL and plasma exosome amount and miRNA content	116
Glioblastoma	Phase 1:122 patients with untreated WHO grade 3 to 4 disease and 123 control serum samples Phase 2: 55 WHO grade 2, 15 WHO grade 1, 11 astrogliosis, 42 other primary brain tumors serum samples, and 8 WHO grade 2 to 4 astrocytoma tumor tissues	miR-15b*, miR-23a, miR-133a, miR-150*, miR-197, miR-497, and miR-548b-5p, and the 7-miRNA panel	The 7-serum miRNA panel demonstrated a high sensitivity and specificity for the prediction of malignant astrocytomas; a marked difference in serum miRNA profile was observed between high-grade astrocytomas and normal controls	117
Prostate cancer	Prebiopsy serum samples of 133 enrolled patients from 3 study centers	miR-26a-1 and miR-141	The analysis of circulating miRNAs does not appear to help identify patients with cancer undergoing prostate biopsy; however, their levels may be useful to identify patients with high-risk prostate cancer	118
Ovarian cancer	360 patients with epithelial ovarian cancer and 200 controls from 2 institutions	miR-205, let-7f	Plasma miR-205 and let-7f are biomarkers for ovarian cancer detection that complement CA 125; let-7f may be predictive of ovarian cancer prognosis	119

BAL indicates bronchoalveolar lavage; HCC, hepatocellular carcinoma; miRNA, microRNA; NSCLC, non-small cell lung cancer; WHO, World Health Organization.

cyclophosphamide, respectively.[123] These findings suggest that several miRNA-based models are efficient diagnostic tools, and they can furthermore be considered as useful when predicting patterns of resistance/sensitivity to drugs used as cancer treatment.

MiRNA Profiling Represents a New and Useful Clinical Tool

The identification of noninvasive biomarkers still represents the most promising strategy for the early detection and accurate diagnosis of many malignancies. Due to their tissue-specific profiles and stability in bodily fluids, miRNAs rank among the top candidates of biomarkers. Because of the exponential increase in the number of publications related to miRNA profiling in human tumors, we will highlight the significance of recent studies reporting miRNA signatures published mainly within the last 3 years.[92] Many in-depth reviews cover publications from the last decade, including profiling topics in multiple types of cancers[96,124]such as lung cancers,[125] breast cancers,[103] gastroesophageal cancers,[126] ovarian cancers,[127,128] hepatocellular carcinomas,[129] CLL,[130] or B-cell lymphomas.[131] A compilation of recent publications reporting diagnostic, prognostic, or therapeutic associations in studies with over 100 patients is presented in Table 3.[80,104-119] Such reports offer the scientific basis for the rational design of prospective large-scale studies needed to confirm the multiple biological and clinical associations.

Lung Cancer

In both early-stage and late-stage lung cancer, there is an urgent need to find reliable discriminators for disease diagnosis and prognosis. A large study of 165 lung adenocarcinomas and 125 squamous cell carcinomas of the lung analyzed by miRNA array identified a set of 5 miRNAs (let-7b, miR-103, miR-107, miR-181a, and miR-191) whose combination was able to distinguish adenocarcinoma from squamous cell carcinoma ($P_{global}<.0001$).[132] In addition, among male smokers with early-stage squamous cell carcinoma of the lung, the higher expression of 5 miRNAs (miR-25, miR-34a, miR-34c-5p, miR-191 and let-7e) was predictive of a low mortality risk (hazard ratios [HRs] ranged between 0.38-0.51; *P* values ranged between .0001-.011).[132] These data suggest that miRNA signatures can discriminate between cancer histotypes and correlate with the most important clinical parameter (eg, the mortality risk).

In 3 independent cohorts from different countries (United States [89 patients], Norway [37 patients], and Japan [191 patients]), higher expression of miR-21 was

found to be significantly associated with cancer-related mortality (HR, 2.06; 95% confidence interval [95% CI], 1.13-3.75 [P = .018]; HR, 2.78; 95% CI, 1.22-6.31 [P =0.014]; and HR, 2.82; 95% CI, 1.57-5.07 [P<.0005], respectively).[133] The higher levels were detected in more advanced-stage tumors, consistent with the findings that miR-21 is involved in disease progression. Importantly, multivariable Cox regression analysis for just those cases with TMN stage I disease showed that higher miR-21 expression levels were associated with poor cancer-specific mortality in the cohorts from the US and Norway (HR, 2.16; 95% CI, 1.11-4.21 [P = .025]) and worse relapse-free survival in the Japanese cohort (HR, 3.40; 95% CI, 1.57-7.36 [P = .001]) independently of all clinical covariates, suggesting the use of miR-21 as an early-stage prognostic biomarker for patients with TMN stage I disease at high risk of metastases.[133]

An interesting study evaluated the potential of a previously identified 24-plasma miRNA signature classifier (MSC)[134] for use as a noninvasive screening tool for lung cancer in the Multicenter Italian Lung Detection cohort of current and former smokers. Plasma samples from 69 patients with lung cancer and 870 disease-free individuals (652 of whom had been screened with low-dose computed tomography [LDCT] and 287 from an unscreened observation group) were analyzed by qRT-PCR. All the patients were categorized as belonging to one of the 3 cancer risk groups (low, intermediate, and high) on the basis of predefined cutoff points of positivity for 4 different expression ratio signatures of 24 miRNAs, defined as risk of disease, risk of aggressive disease, presence of disease, and presence of aggressive disease. The study confirmed the diagnostic, prognostic, and predictive value of this MSC. The diagnostic performance of MSC for lung cancer detection was 87% sensitivity (SE), 81% specificity (SP), 27% positive predictive value (PPV), and 99% negative predictive value (NPV) within the group of 69 lung cancer patients when compared with disease-free patients overall (n=870), 88% SE, 80% SP, 31% PPV, and 99% NPV within the LDCT arm (patients who developed lung cancer versus healthy individuals), and 82% SE, 83% SP, 16% PPV, and 99% NPV within the observational arm as specified above. For all patients, MSC had an NPV of 99% and 99.86%, respectively, for disease detection and disease-related death, whereas LDCT had a 79% SE and 81% SP with a false-positive rate of 19.4%. The combination of both MSC and LDCT provided a 5-fold reduction (compared with LDCT alone) in the false-positive rate to 3.7%, which means that MSC might complement LDCT screening, especially among individuals with a low MSC and good prognosis, thereby avoiding unnecessary invasive follow-up and optimizing health care costs.[135] Similarly, a combined 17-miRNA risk score derived from serum samples of patients with advanced NSCLC (n=391) was found to accurately identify those at the highest risk of death. In particular, individuals with a high-risk score had a 2.5-fold increased risk of death, corresponding to a 7.8-month decrease in median survival (P < .0000001) when compared with those with a low-risk score (95% CI, 1.8-3.4 [P < .0000001]).[136] Thus, consistent with the data reported above, circulating miRNAs represent diagnostic and prognostic biomarkers that could be efficiently implemented in the clinical setting for lung cancer management.

Breast Cancer

Early-stage breast cancer is curable by surgery and by a combination of hormonal treatment, chemotherapy, and radiation. However, late relapses are frequent events after many years. MiRNA-based tests could potentially improve the prediction of OS as well as treatment response. An integrated miRNA/gene signature was recently reported in a group of 466 patients with breast cancer from The Cancer Genome Atlas (TCGA) (Table 1).[137] The final signature, including 30 mRNAs and 7 miRNAs (miR-93, miR-103, miR-148b, miR-328, miR-484, miR-874, and miR-1307), proved to significantly predict OS in a multivariable model independent of other clinical pathological characteristics of the tumors, and showed the highest prognostic value of distant relapse-free survival in patients with early stage I and II tumors (receiver operating characteristic [ROC] area under the curve [AUC], 0.77; P<.001). Furthermore, the validation of this signature on 8 independent breast cancer cohorts, comprising a total of 2399 patients, demonstrated its superior performance for risk stratification with respect to other RNA predictors, including the mRNAs used in the MammaPrint (Agendia, Irvine, Calif) and Oncotype DX (Genomic Health, Redwood City, Calif) assays.[137] Separately, in a series of 173 triple-negative breast cancers, a 4-miRNA signature (miR-27a, miR-30e, miR-155, and miR-493) allowed the subdivision of triple-negative breast cancers not only into core basal (epidermal growth factor receptor and/or CK5 or CK 6 positive) or 5-negative (when all markers are negative) subgroups (75% SE and 56% SP; AUC, 0.74), but also into groups with high and low risks of death.[138] In particular, the median OS times for the high-risk versus low-risk miRNA signature groups were 75.5 months versus 82 months (HR, 2.46; 95% CI, 1.43-4.12 [P= .001]). This panel also showed the ability to predict outcomes of treatment with the 2 most common chemotherapy regimens used in patients with triple-negative breast cancer (ie, anthracyclines or anthracyclines plus taxanes).

Recently, in an attempt to identify circulating miRNA signatures in patients with breast cancer, a microarray analysis of fresh-frozen preoperative sera as well as normal and breast cancer paired tissue samples was performed in a large

cohort of Asian-Chinese patients with breast cancer.[139] The miRNA expression profiles between sera and matched tumor samples were largely dissimilar, thereby confirming the high tissue specificity of miRNAs and their selective release from the primary tumor. Notably, among the resulting 23 breast cancer-associated serum miRNAs, the ROC curves derived from the combinations of the overexpressed miR-1, miR-92a, miR-133a, and miR-133b exhibited AUCs of 0.90 to 0.91, confirming that miRNA models are robust diagnostic tools to apply in the clinic.

In an independent study of 48 white patients with estrogen receptor-positive early stage breast cancer (24 of whom were lymph node positive and 24 of whom were lymph node negative), a novel circulating 9-miRNAs profile provided for the first time the ability to distinguish the serum of patients with breast cancer from that of healthy controls (24 age-matched women).[140] This signature included both overexpressed (miR-15a, miR-18a, miR-107, and miR-425) and underexpressed (miR-133a, miR-139-5p, miR-143, miR-145, and miR-365) miRNAs. The miRNA profile validation on a cohort of 111 serum samples as well as independent data from 3 publicly available databases further confirmed the discriminatory power with an AUC of 0.665 (95% CI, 0.562-0.768, [ROC test]) and a *P* value of .012. Using a probability cutoff of 0.48, the SE was 83.3%, the SP was 41.2%, the PPV was 62.5%, and the NPV was 67.4%.

If compared, in the studies reported above, only one miRNA was held in common between the 2 patient populations analyzed.[139,140] Thus, there is evidently a high variability of miRNA expression in bodily fluids, which underlines the need to conduct large consortium studies that include samples from as many parts of the world as possible.

Colon Cancer

Genetic profiling has been found to be somewhat useful when determining prognosis for patients with intermediate-stage disease, although to date it is not widely used.[141] With regard to miRNA profiling, a microarray study of 2 colon adenocarcinoma patient cohorts (84 from a Maryland test cohort and 113 from a Hong Kong independent validation cohort) compared tumors with adjacent nontumorous tissue.[142] In both cohorts, high miR-21 levels were found to be predictive of poor survival in both the training cohort (HR, 2.5; 95% CI, 1.2-5.2) and the validation cohort (HR, 2.4; 95% CI, 1.4-3.9), independently of clinical stage. Moreover, in patients with stage II and III disease treated with 5-fluorouracil-based adjuvant chemotherapy (either intravenous 5-fluorouracil or oral drugs including tegafur with uracil, levamisole or leucovorin), increased expression of miR-21 correlated with poor response and worse OS (HR, 4.3; 95% CI, 1.1-16.4 [$P = .03$] and HR, 3.5; 95% CI, 1.1-11.6 [$P = .04$] for the Maryland and Hong Kong populations, respectively). miR-21 was also found to be overexpressed both in tissue samples of CRC (46 patients; $P < 0001$) and related liver metastases (30 patients; $P < .0001$) compared with normal paired tissues and correlation with a worse disease-free interval was noted in the CRC sample set ($P = .0026$).[143] Finally, 2 other miRNAs have been proposed to be good predictive markers for therapeutic response in patients with CRC: miR-126[144] and miR-150.[145] miR-126 was detected by in situ hybridization (ISH) assay in formalin-fixed paraffin embedded tissue from primary tumors, and the expression levels indicated as area per image (μm^2) by using image analysis. Higher levels of miR-126 were predictive of a good response to first-line treatment with capecitabine and oxaliplatin in patients with metastatic CRC; the median miR-126 expression level was significantly higher in the responding patients, with an (area of the ISH signal) of 3629 μm^2 (95% CI, 2566 μm^2–4846 μm^2) compared with the nonresponding patients (area per image of 1670 μm^2; 95% CI, 1436 μm^2–2041 μm^2 [$P = 5 \times 10^{-6}$]). Accordingly, lower expression levels of miR-126 were predictive of a worse progression-free survival and OS, along with the association with the number of metastatic sites. Specifically, the median progression-free survival for patients whose tumors demonstrate high miR-126 expression levels was 11.5 months (95% CI, 9.0 months-12.7 months) compared with 6.0 months (95% CI, 4.8 months-6.9 months) for patients with low expression levels. The median OS in the group with high miR-126 expression was 26.2 months (95% CI, 21.8 months-32.8 months) compared with 16.8 months (95% CI, 13.8 months-19.1 months) in the group with low miR-126 expression. Conversely, low expression levels of miR-150 were found to be associated with worse prognosis (HR 0.57; 95% CI, 0.33-0.97 [$P = .037$]) and poor therapeutic outcome in patients with stage II and III CRC after fluoracil-based adjuvant therapy with or without leucovorin, levamisole or cisplatin (289 patients in cohort 1: HR, 0.44; 95% CI, 0.20-0.93 [$P = .032$] and 185 patients in cohort 2: HR, 0.38; 95% CI, 0.19-0.79 [$P = .009$]).

To date, the early detection of adenomas is based on invasive screening approaches (such as colonoscopy and biopsies) and on noninvasive approaches (such as fecal occult blood tests or stool DNA tests based on mutations and methylation) with limited diagnostic accuracy. Thus, there is a great need for new noninvasive methods for routine clinical investigation. Recently, researchers embarked on a pilot study with the challenge of identifying specific biomarkers to detect precancerous lesions, colorectal adenomas, and CRCs.[146] The results of the investigation lead to a panel of 8 plasma miRNAs that distinguished patients with colorectal adenoma (n=16) from controls (n=26) with high accuracy (AUC, 0.868 [95% CI, 0.76-0.98]; 88% SE and 64% SP), and a panel of 3 plasma miRNAs that

could distinguish patients with stage IV CRC (n=15) from controls (AUC, 0.868 [95% CI, 0.76-0.98]; 93% SE and 74% SP).[147] A further 6 miRNA profiles of upregulated miRNAs (miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335) was achieved when analyzing a total of 196 plasma samples from 123 patients newly diagnosed with sporadic colorectal neoplasia (63 with CRC and 60 with advanced adenoma) and 73 healthy individuals (controls). The panel showed a high accuracy in discriminating patients with CRC from healthy controls (AUC ranging from 0.80 [95% CI, 0.71-0.89] to 0.70 [95% CI, 0.59-0.80]), whereas only miR-18a was confirmed to be significantly upregulated in patients with advanced adenomas (AUC, 0.64; 95% CI, 0.52-0.75).[147]

As underlined above for breast cancer, the last 2 studies reported for colon cancer exclusively share the clinical relevance of miR-15b. This again highlights the importance of performing wider trials to confirm these data in larger cohorts and patient populations of different ethnicities.

Ovarian Cancer

Because the majority of patients with ovarian cancer are diagnosed with advanced-stage disease, identifying miRNA biomarkers capable of detecting early-stage disease would have great clinical value. Currently, evidence from microarray-based studies is being limited because of the small number of patients, thus the certainty of the results hinge on this fact. Nevertheless, some exhibit great potential to address some clinical issues including characterization of different histologic and genetic subtypes, the identification of markers for diagnosis and screening, prediction of clinical outcome, and better individualization of therapies.[148] Recently, miRNA expression analysis performed in a large multicenter cohort of 198 patients (86 patients as a training set and 112 patients as validation set) demonstrated that the downregulation of 3 miRNAs (miR-217, miR-484 and miR-642) was predictive of resistance to platinum-based chemotherapy when responding and nonresponding individuals were compared (P=.046, P=.0007 and P=.04, respectively).[149] In this context, additional studies on miR-484 demonstrated its capability to interfere with the VEGFB and VEGFR2 pathways, thus indicating new putative targets for therapeutic approaches on tumor vasculature.[149]

Separate studies[150,151] showed the clinical relevance of miR-200c in ovarian cancer. Leskela et al showed that women with International Federation of Gynecology and Obstetrics stages III and IV serous ovarian carcinoma (n=57) without a complete response to a regimen of paclitaxel and carboplatin have tumors with significantly lower miR-200c levels when compared with those who achieved a complete response (HR, 1.43; 95% CI, 1.02-1.99 [P=.037]).[150] Downregulation of miR-200c was also identified as being associated with OS (HR, 0.094; 95% CI, 0.012-0.766 [P= .0272]) and progression-free survival (HR, 0.035; 95% CI, 0.004-0·311 [P= .0026]) in a multivariable analysis of 144 samples of stage I epithelial ovarian cancer.[151] Finally, the integrated analysis of miRNA and transcriptome profiles from a TCGA ovarian cancer data set revealed that the expression levels of the miR-200 family members are able to clearly differentiate between 2 clinically relevant subtypes (ie, mesenchymal and epithelial phenotypes) of serous ovarian cancer.[152]

Gliomas

MiRNAs seem to have promising potential in gliomas, in which they might play an informative role in discriminating malignant phenotypes as well as predicting the outcome of surgical resection. Expression profiles of 261 gliomas from the TCGA data set identified 121 miRNAs that were able to segregate tumor samples into 5 genetically and prognostically different subclasses.[153] Another comprehensive analysis in 160 glioma samples from Chinese patients (63 with World Health Organization (WHO) grade 2 disease, 33 with WHO grade 3 disease, and 64 patients with glioblastoma multiforme [GBM]) revealed that the combination of 21 miRNAs (so-called "hub miRNAs" because of their regulating more than 30 target mRNAs) was able to predict the survival of patients with all types of gliomas (HR, 1.097 [95% CI, 1.068-1.127]; correlation coefficient [R^2], 0.093 [$P = 5.64 \times 10^{-12}$] and HR, 1.076 [95% CI, 1.044-1.109]; R^2, 0.074 [$P = 1.12 \times 10^{-6}$] from univariable and multivariable analyses, respectively). Among the 21 relevant miRNAs, miR-524-5p (HR, 0.849 [95% CI, 0.721-0.999] R^2, −0.164 [P= .044]) and miR-628-5p (HR, 0.679 [95% CI, 0.470-0.982]; R^2, −0.387 [P = .036]) expression levels represented protective factors, whereas miR-938 (HR, 1.113 [95% CI, 1.029-1.204]; R^2, 0.107 [P= .007]), miR-595 (HR, 1.200 [95% CI, 1.059-1.360]; R^2, 0.183 [P= .004]), and miR-346 (HR, 1.631 [95% CI, 1.043-2.552]; R^2, 0.489 [P= .029]) expression levels represented risk factors.[154]

The possibility of detecting miRNAs in bodily fluids such as serum and/or cerebrospinal fluid (CSF) represents a novel, minimally invasive, experimental procedure that could possibly identify and characterize gliomas without requiring surgical intervention. For example, pilot studies on a limited number of patients have shown that miR-21 and miR-10b[155-157] are significantly increased in the CSF of patients with GBM or those with brain metastases from breast and lung cancer when compared with CSF from patients whose tumors are in remission and those with non-neoplastic conditions. It is interesting to note that because miR-10b is generally expressed in extracranial tissues but is undetectable in the brain and CSF of patients without cancer, probably because of the blood-brain membrane, its

specific abundance in the CSF from patients with GBM and brain metastases could be referred to as a specific signature of brain pathology.[157] Conversely, members of the miR-200 family (miR-200a, miR-200b, miR-200c, and miR-141) are found to be highly elevated in the CSF of patients with brain metastases but not in those with GBM ($P<.0001$) or other primary brain tumors, suggesting they could serve as specific markers of metastatic brain tumors.[157] In addition, through a genome-wide serum miRNA analysis by next-generation sequencing, serum samples from 122 patients with untreated astrocytomas and 122 normal controls were evaluated, and a panel of 7 miRNAs (miR-15b*, miR-23a, miR-133a, miR-150*, miR-197, miR-497, and miR-548b-5p) were found to be significantly decreased ($P<.001$) between patients with tumor grades in the range of WHO grade 2 to 4 and the control group.[117] The AUC for the combination of the 7 miRNAs was 0.972 (95% CI, 0.954-0.990) for patients with malignant astrocytomas (all grades) and controls and with an optimal cutoff of 5.6085, the SE was 88% and the SP was 97.87%. Moreover, a marked increase in the levels of expression of the same group of miRNAs was detected after patients underwent tumor resection ($P<.001$).[117]

A study of exosomes isolated from patient sera demonstrated that the expression levels of RNU6-1, miR-320, and miR-574-3p were able to effectively discriminate patients with GBM from healthy individuals, suggesting their significance as a novel potential diagnostic tool.[158] In particular, ROC curve analyses revealed that the expression levels of either RNU6-1 alone or in combination with miR-320 and miR-574-3p were useful and robust biomarkers for differentiating patients with GBM (n=50) from healthy controls (n=30), with an AUC of 0.722 (95% CI, 0.60-0.84 [$P=.0007$]) for RNU6-1 and 0.775 (95% CI, 0.65-0.90 [$P=.0001$]) for the 3 markers together. Importantly, at a cutoff value of 0.372 for RNU6-1, the SE was 66% and the SP was 68%, whereas at a cutoff value of 0.374 for the miRNA signatures, the SE was 70% and the SP was 71%.

Taken together, these data suggest alternative noninvasive approaches for the prediction of malignant progression and detection of residual disease in these types of tumors.

Chronic Lymphocytic Leukemia

CLL has become the paradigmatic disease of miRNA involvement in cancer as the downregulation of the miR-15a/miR-16 cluster in the majority of patients with CLL was the first report linking miRNAs to cancer.[27] A unique miRNA signature composed of 13 genes including miR-15a, miR-16, and miR-155 as well as members of the miR-181 family possesses the ability to distinguish aggressive from indolent CLL.[43] More recently, a scoring method combining miR-21 expression with the routine cytogenetic procedures of fluorescence in situ hybridization (FISH) and Karyotype (KARYO) (21 FK score: miR-21 qRT-PCR, FISH, Karyotype) was developed in order to stratify patients with CLL according to survival.[159] When compared with the classic prognostic factors, including B2M, ZAP-70, IgVH, and $CD19^+CD38^+$, the 21FK score was found to be the only rating able to distinguish patients with a good prognosis (low miR-21 expression, low score) from those with poor prognosis (high miR-21 expression, high score) both in a homogenously selected cohort of 104 patients with 17 DEL CLL and nonhomogeneous sets of 80 cases with either one chromosomal abnormality (13qDEL, 11qDEL, T12, or 17pDEL) or a normal karyotype. Indeed, from the multivariable analysis using the 21FK score, IgVH, and ZAP-70, the 21FK score was demonstrated to be the only significant variable in both sets of patients (HR, 3.514; 95% CI, 1.409-8.764 [$P=.007$] and HR, 5.217; 95% CI, 1.408-19.327 [$P=.013$], respectively). In the same study, when patients with 17p deletions were compared with those with normal karyotypes, miR-21, miR-34a, miR-155, and miR-181b were found to be differentially expressed as well. In particular, miR-181b was specifically downregulated in patients whose disease was refractory to their treatment regimen, with a mean treatment-free survival of 5.35 months versus 13.80 months for patients with high miR-181b expression (HR, 2.77; 95% CI, 1.295-5.91 [$P=.006$]).[159]

An interesting study successfully demonstrated the potential of using circulating miRNAs for both the detection and stratification of patients with CLL.[160] In a cohort of CLL plasma samples, a number of detectable circulating miRNAs including miR-17, miR-19b, miR-92a, miR-150, miR-223, miR-320, and miR-484 were found to be highly expressed when compared with normal control plasma. Among them, several miRNAs showed distinct profiles between CLL and other hematological malignancies such as hairy cell leukemia or multiple myeloma. Circulating miR-20a (AUC, 0.920) and miR-195 (AUC, 0.951), as well as the miR-29a, miR-195, and miR-222 combination pattern (AUC, 0.982) were found to be the best classifiers for clearly distinguishing patients with CLL from healthy individuals.

Finally, with the aim of investigating the mechanisms underlying the transition from a clinical premalignant lesion named monoclonal B lymphocytosis (MBL) to CLL, Ferrajoli et al evaluated the differential expression of previously mentioned miR-155 in 224 samples from 2 independent validated cohorts of patients with CLL and MBL.[161] MiR-155 expression was higher in purified B cells from patients with MBL when compared with normal B cells ($P<.0001$) and patients with CLL ($P<.0001$). Increased miR-155 plasma levels, measured before treatment, were found to be associated with poor OS ($P=.01$)

and subsequent resistance to therapy with either fludarabine, cyclophosphamide, or rituximab ($P = .02$) or lenalidomide ($P = .03$) in nonresponding patients compared with individuals with complete responses. These data support the ability of miR-155 to predict prognosis and response to treatment independent of the type of therapy administered.[161]

Acute Myelogenous Leukemia

The profiling findings with acute myelogenous leukemia (AML) have highlighted the existence of a complex network comprising gene mutations, along with aberrantly expressed genes and miRNAs. All of them collectively define the AML phenotype associated with clinically aggressive disease. High levels of miR-10a-5p in patients with NPM1-mutant AML were found to be associated with complete remission after chemotherapy with idarubicin and cytarabine (odds ratio [OR], 1.33; $P = .019$).[162] When *NPM1* mutation status and miR-10a-5p were included in a multivariable model, they demonstrated a significant interaction correlating with complete remission status ($P = .01$), suggesting a synergistic effect enabling the tumor to acquire sensitivity to treatment.[162] Furthermore, another study including a large cohort of patients with complex karyotype AML with known *TP53* status demonstrated that low levels of miR-34a expression such as *TP53* alterations were predictive of response to chemotherapy (OR, 0.78; 95% CI, 0.42-1.44 [$P = .42$]) and poor clinical outcome (OS: HR, 1.47; 95% CI, 1.06-2.03 [$P = .02$] and relapse-free survival: HR, 1.90; 95% CI, 1.14-3.18 [$P = .01$]).[163] As in patients with CLL, high levels of miR-155 have also been shown to be significantly correlated with a shorter OS (HR, 1.62; 95% CI, 1.25-2.09 [$P < .001$]) and a complete remission rate of less than 50% (OR, 0.46; 95% CI, 0.27-0.81 [$P = .007$]), independent of other strong clinical and molecular predictors.[164]

Recently, a global screening of 670 miRNAs in 238 patients with intermediate-risk cytogenetic AML demonstrated that high levels of miR-644 and miR-196b were otherwise independent miRNAs associated with shorter OS (OR, 0.51; 95% CI, 0.51-0.85 [$P = .004$]; and OR, 0.42; 95% CI, 0.19-0.94 [$P = .036$], respectively) and, in addition, increased levels of miR-135a and miR-409-3p were correlated with a higher risk of relapse at 5 years (OR, 0.23; 95% CI, 0.0863-0.624 *[*$P = .003$]; and OR, 0.62; 95% CI, 0.3762-1.033 [$P = .067$], respectively).[165] This 4-miRNA scoring scale generated an independent prognostic score allowing a finite stratification of patients with AML and intermediate-risk cytogenetic findings into different prognostic categories and facilitating the proper assignment of postremission therapy.[165]

Regarding circulating miRNAs in patients with AML, less is known about their potential clinical application. Advancements have been made in the past year by analyzing 140 patients newly diagnosed with AML and 135 healthy adult donors. New data set analyses have led to the identification of a 6-miRNA panel consisting of miR-10a-5p, miR-93-5p, miR-129-5p, miR-155-5p, miR-181b-5p, and miR-320d, which are able to differentiate between patients with AML and healthy controls (AUC range, 0.8129-0.9531).[166] Even more, high levels of miR-181b-5p in patient sera were found to be significantly associated with a worse OS ($P = .002$).[166] Overall, these data demonstrated that the expression patterns of circulating miRNAs might be systematically altered in patients with AML and detecting their levels could provide clinically useful diagnostic and prognostic classifiers.

Acute Lymphoblastic Leukemia

One of the first large-scale miRNA profiling analyses in a total of 72 cases of acute lymphoblastic leukemia (ALL), 98 cases of AML, and 13 normal bone marrow controls identified a signature of 27 differentially expressed miRNAs that were able to separate patients with ALL from those with AML. Six miRNAs (miR-128a, miR-128b, miR-130b, miR-151*, j-miR-5, a newly identified miR, and miR-210) were expressed at a significantly higher level in patients with ALL compared with those with AML.[167] More importantly, 4 miRNAs (miR-128a, miR-128b, let-7b, and miR-223) revealed the strongest diagnostic performance: the combination of any 2 or 3 of these miRNAs were found to separate ALL from AML cases with an overall diagnostic accuracy of 97% to 99%.[167]

When referring to ALL, the pediatric population becomes one of clinical significance because ALL is the most common malignancy diagnosed in children, representing over 25% of all pediatric cancers. In pediatric ALL bone marrow samples, it was shown that miR-100, miR-196b, and let-7e are downregulated, whereas miR-128a and miR-181b are upregulated when compared with normal pediatric control samples.[168] The subsequent correlation analysis with the clinical and biological features showed that miR-196b expression was significantly elevated ($P = .01$) in the T-cell ALL phenotype and miR-100 expression was elevated in patients with a white blood cell count less than 50,000/mm^3 at diagnosis, as well as in the presence of t(12;21) ($P = .04$) and the absence of a hyperdiploid karyotype ($P = .04$).[168] Furthermore, patients with ALL with reduced miR-335 expression are likely to have a poorer 5-year OS rate (74.2%) compared with patients with high miR-335 expression (OS rate of 96.9%; $P = .009$). ROC curve analysis using the expression level of miR-335 resulted in a highly significant AUC of 0.73 ($P = .003$). In vitro studies suggested that the poor prognosis would be linked to the development of resistance to glucocorticoid therapy mediated by the derepression of MAP1K.[169] Indeed, after restoring

miR-335 levels, cancer cells proved to acquire sensitivity to a prednisolone-based therapy because of the miR-335-mediated direct inhibition of MAPK1. These findings provide suggestions for the development of new strategies that might work, for example, against the glucocorticoid resistance arising in treated patients with ALL.[169]

Multiple Myeloma

Because of the inherent heterogeneity of multiple myeloma (MM), several groups have attempted to classify the disease based on gene expression profiling. One of the largest profiling studies was performed on 163 bone marrow samples from patients with MM and revealed distinct miRNA signatures associated with the translocation/cyclin D-classified MM subgroups. This study demonstrated the miRNA pattern correlation with clinical outcome in the different translocation/cyclin D prognostic groups of patients, including both the unfavorable 4p16 and MAF (v-maf musculoaponeurotic fibrosarcoma oncogene homolog [avian]) and the favorable 11q13 groups. In particular, the specific upregulation of the miR-99b/let-7e/miR-125a cluster on 19q was detected in the 4p16 group, and miR-150, miR-155, and miR-34a were upregulated in the MAF group. Upregulation of miR-1275 and downregulation of miR-138 were observed in the 11q13 cases. Furthermore, the upregulation of 3 clusters of miRNAs (miR-503-424, miR-17HG, and miR-106A-363) were found to be significantly associated with decreased OS. More importantly, after a stepwise selection in a multivariable analysis, miR-886-5p and miR-17 expression levels were used to construct a miRNA-based risk predictor that was able to stratify patients into 3 risk groups (median OS times of 19.4 months, 40.6 months, and 65.3 months, respectively; $P = .001$). This scale was demonstrated to ameliorate the classification of patients according to OS by improving the predictive power of the conventional International Staging System/FISH approach ($P = .0004$). Indeed, the expression value of this miRNA risk estimator identified a separate class of patients with a significantly worse prognosis within a group defined as "low risk" by the International Staging System /FISH classification system.[170]

Dysregulated patterns of circulating serum miRNAs in comparison with healthy controls have also been identified in patients with MM. When using a multivariate logistical regression model on a set of 103 newly diagnosed patients, 18 patients in relapse, 57 patients with monoclonal gammopathy of undetermined significance, and 30 healthy donors, a combination of 2 miRNAs (miR-34a and let-7e) proved to be able to distinguish patients with MM and monoclonal gammopathy of undetermined significance from healthy donors (AUC, 0.898 [80.6% SE and 86.7% SP]; and AUC, 0.976 [91.1% SE and 97.6% SP], respectively).[171] In same study, lower levels of miR-744 and let-7e were found to be associated with shorter OS and remission: the 1-year mortality rate for miR-744 and let-7e was 41% (95% CI, 28.8%-57.9%) and 34.6% (95% CI, 23.4%-49.2%), respectively, in the low miR-expressing patients and 3.3% (95% CI, 0.8%-12.7%) and 3.9% (95% CI, 1.0%-14.8%), respectively, in the patients with high expression. The Cox model also showed the prognostic impact on time to disease progression for both miR-744 (HR, 0.690; 95% CI, 0.584-0.817 [$P < .0001$]) and let-7e (HR, 0.552; 95% CI, 0.424-0.718 [$P<0001$]), overall suggesting they might be useful as a determinant of patient survival rates.[171] It was also proven that miR-29a is upregulated in the serum of patients with MM, and that this dysregulation could potently discriminate patients with MM from healthy donors with a SE of 88% and an SP of 70%.[172]

Carcinoma of an Unknown Primary

Metastatic CUP represents one of the 10 most frequent cancer diagnoses worldwide (3%-5% of newly diagnosed cancer).[173] Patients with CUP present with metastases (late-stage disease) without an identified primary tumor. MiRNAs are of particular importance in the identification of the site of origin for CUP because their expression levels and profiles reflect with great fidelity the tissue origin, and also because they are highly stable in formalin-fixed paraffin-embedded (FFPE) tissue blocks (the most available specimen type in pathology). MiRNA profiling may be helpful in further discriminating the histological origin of the tumor, as well as its optimal treatment. In an attempt to classify tumors based on their tissue-specific miRNA signatures, miRNA expression profiling was performed in 400 paraffin-embedded and fresh-frozen samples from 22 different tumor tissues and metastases by a custom-made microarray.[174] The authors built an miRNA-based classifier including a total of 48 differentially expressed miRNAs, which was demonstrated to successfully classify most of the samples with a high confidence (accuracy of 89%).[174] This 48-miRNA panel was further validated on a cohort of 204 FFPE tumor samples comprising 25 different classes as accurately stratified by the array. Indeed, for 124 of 204 the samples (60%), the sensitivity of the array was 90% (111 of 124 of the classifications agreed with the reference diagnosis), and it exceeded 90% for most tissue types. Specificity (negative agreement) in this group ranged from 95% to 100% and averaged above 99%.[175] A new enriched miRNA-based assay (second-generation array) comprising a higher number of miRNAs (64) and able to discriminate a greater number (48) of tumor types has been developed.[176] As reported in a cohort of 84 FFPE samples from patients with CUP, data generated by this assay have been shown to agree with the clinical diagnosis at the time of presentation and after patient management in 70% and 89% of patients, respectively; moreover, it agreed with the

final clinical diagnosis reached with supplemental immunohistochemical stains in 92% of patients, indicating a 22% improvement in agreement from diagnosis at presentation to the final clinical diagnosis.[177] In conclusion, these novel miRNA-based approaches demonstrate high accuracy in identifying the final clinical diagnosis in patients with CUP, thereby providing a useful tool for the better management and rational therapeutic intervention of this particularly challenging tumor category.

MiRNAs as Targets for Anticancer Drug Therapy

RNA Inhibition With the Use of MiRNAs

By blocking the function of a mRNA, miRNAs could represent a valid option for treating patients with specific cancers in the near future.[178-180] MiRNAs can be separated into 2 groups: tumor suppressive or oncogenic, depending on their mRNA targets. Both groups are very well suited for therapeutic purposes, and they are represented by patients with a negatively correlated expression between a specific miRNA and its experimentally proven target(s) in the tumors. With the objectives of recovering tumor suppressor proteins and inhibiting oncogenic ones, the strategy would involve restoring the miRNAs that are downregulated and silencing the miRNAs that are upregulated (Fig. 4). There are 2 major advantages to using miRNAs (compared with other gene-silencing therapies explained in detail in Table 4)[181-195]: first, miRNAs are "natural" molecules produced in human cells and second, they can target multiple genes from the same pathway and therefore their action can occur at multiple levels in the same pathway, thereby significantly reducing the development of resistance because multiple mutations in multiple genes are needed. For example, miR-15a and miR-16-1, both of which have reduced expression in patients with CLL, have 2 antiapoptotic targets: the mRNAs encoded by the oncogenes for *BCL2* and *MCL1*.[196] Therefore, a personalized therapy based on the identification of patients with reduced miR-15a/miR-16-1 cluster expression and BCL2 and MCL1 protein overexpression in CD5/CD19-positive B lymphocytes can be envisioned. Note how these types of therapies are very specific for patients having these molecular characteristics in the same cells. Patients with low levels of miR-15a/miR-16 but normal levels of *BCL2* or *MCL1* or conversely with normal levels of miRNAs but abnormal levels of these antiapoptotic oncogenes should be excluded from clinical trials targeting miR15a/miR-16.

Strategies for MiRNA Targeting

Strategies for miRNA targeting are based on either restoring miRNA levels or blocking miRNA function with oligonucleotide-based strategies (Table 4).[181-195] A targeted approach to replenish the expression levels of particular miRNAs is restoring the level and function of one or a limited number of miRNAs, usually located within a cluster (such as miR-15a and miR-16-1 at 13q14.3), either with miRNA mimics or with miRNAs encoded in expression vectors. MiRNA mimic molecules are double-stranded sequences with 100% similarity to the endogenous miRNA, which can be delivered by nanoparticles and thereby are expressed in the cells. This approach is particularly attractive because nanoparticles can be coated with antibodies that recognize tumor-specific antigens, therefore allowing a tumor-specific delivery of the miRNA of interest. A recent study reported the delivery of miR-34a using nanoparticles coated with a neuroblastoma-specific antidisialoganglioside GD_2 antibody. With this design, the therapy resulted in the inhibition of neuroblastoma tumor growth in a murine orthotopic xenograft model.[197] Moreover, MRX34, a liposome-formulated mimic of the tumor suppressor miR-34, has been developed in a clinical phase 1 trial for patients with advanced or metastatic liver cancer. Preclinical studies have already shown that tail vein injection of MRX34 reduced tumor growth and significantly enhanced survival with a favorable safety profile in orthotopic mouse models of hepatocellular carcinoma.[198]

Another mechanism has been targeting new blood vessel formation in tumors. Positively charged liposomes have been investigated for the efficient delivery of drugs to tumor blood vessels. The negative charge of the proteoglycans on the endothelial cell membranes can bind and help cells internalize cationic liposome due to their electrostatic interaction.[199] This approach has been proven to be effective in the delivery of miRNA mimics, such as was observed in a NSCLC xenograft murine model. MiR-29b has been shown to be significantly reduced in patients with NSCLC, consequently increasing the expression of its oncogenic target cyclin-dependent kinase 6. Through cationic lipoplexes (LPs)-based carriers, miR-29b was successfully delivered, resulting in inhibited tumor growth by 60% compared with a LP-miR-negative control.[200] These results demonstrated that cationic LPs target tumor vasculature in a specific manner, and thereby achieve a greater therapeutic potential for lung cancer treatment.

Current strategies for inhibitory targeting of microRNAs are mainly based on antisense oligonucleotides (so-called anti-miRNAs), comprised of locked nucleic acids (LNA) along with tiny LNA anti-miRNA constructs, antagomirs, and miRNA sponges (Table 4).[181-195] Miravirsen (SPC3649), an LNA against miR-122, is the first miRNA target therapy tested in a clinical trial for the treatment of hepatitis C virus infection. The recently completed phase 2a trial showed that SPC3649 exhibited robust antiviral activity in a dose-dependent manner.[201] More impressively, 4 of 9 patients treated at the highest dose (7 mg/kg) with SPC3649 reached undetectable levels of hepatitis C virus

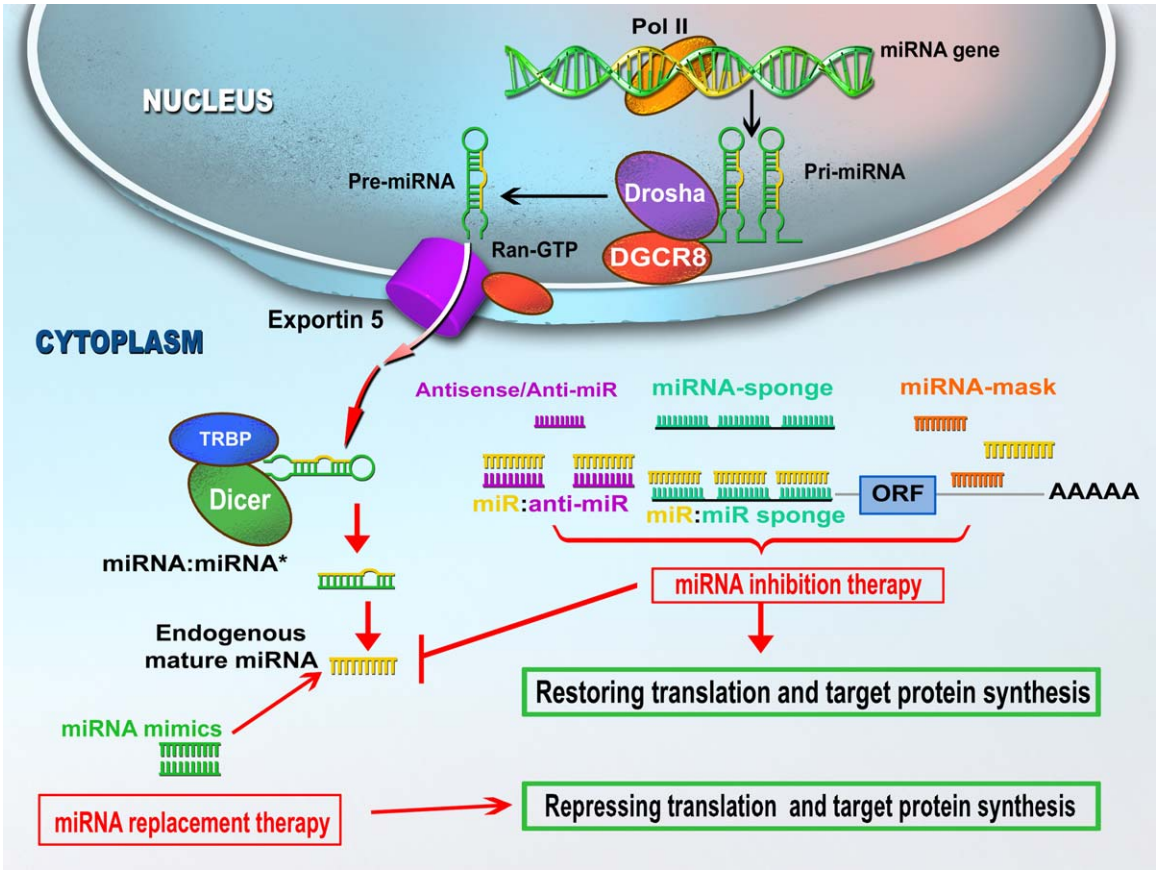

FIGURE 4. MicroRNAs as Targets for Anticancer Drug Therapy. In order to restore tumor suppressor protein levels, oncomirs can be targeted with therapies such as antagomirs or locked nucleic acids anti-microRNAs. When aiming to target oncogenic proteins, the levels of tumor suppressor miRNAs can be restored by using miRNA mimics. TRBP indicates human immunodeficiency virus-1 transactivating response RNA-binding protein; RISC, RNA-induced silencing complex.

RNA (consequently decreasing the possibility of developing hepatocellular carcinoma).[202] The effectiveness of antimiR-122 treatment proved the plausibility of the LNA-based therapeutics and encourages the development of other specific miRNA-targeting therapeutic strategies.

Combination Approaches With MiRNA Therapeutics

The introduction of combination chemotherapy by Freireich and Frei in the early 1960s contrary to the initial opinion in the medical community, not only did not harm children with acute lymphocytic leukemia but moreover represented a huge step forward, and was responsible for the excellent outcome of this otherwise 100% lethal disease.[203] Therefore, we emphasize the need to use combination therapies of various miRNA agents in "cocktails" to be delivered to patients along with chemotherapeutics (therapies with miRNA mimetic and anti-miRNA agents). With regard to CLL for example, there are 2 hypotheses that drive our multimodal approach, in which we could achieve the inhibition of mRNAs and therefore protein expression.[130] First, a "sandwich RNA inhibition strategy" comprising the use of multiple agents could focus on a major molecular alteration clearly linked to CLL pathogenesis. Given the fact that for this disease the overexpression of antiapoptotic factor BCL2 has been correlated with poor patient outcome, we could target this protein by designing regimens using a cocktail of anti-BCL2 antisense oligonucleotides and miR-15a and miR-16 (which are already known to target BCL2 mRNA). Published studies have already proven that oblimersen sodium (an antisense oligonucleotide compound designed to specifically bind to human BCL2 mRNA) has relative efficacy in treating patients with relapsed or refractory CLL[204]; therefore, our expectations for this multimodal approach appear to be promising for this indolent disease. Second, "the multiplex RNA-inhibition strategy" targets various molecular defects that are part of the same pathway (such as apoptosis). In this case, multiple synthetic miRNAs targeting overexpressed regulators of apoptosis such as BCL2 (miR-15a and miR-16) and MCL1 (the miR-29 family) may be more efficient at consistently and robustly reducing the expression levels of these proteins versus a single therapeutic agent.

TABLE 4. The Principal Types of RNA Therapeutic Drugs

Antisense oligonucleotides (ASOs)
Definition: A single-stranded chemically modified DNA-like molecule 17 to 22 nucleotides in length designed to be complementary to a selected messenger RNA (mRNA).
Mechanism of action: Specifically inhibits expression of that gene mainly through formation of an mRNA-ASO duplex by sequence complementarity, leading to cleavage of the mRNA of target gene.
Example: NCT00030641 [181]
Oligonucleotide: Oblimersen, phase 2/3
Condition: Lung cancer: stage IIIB or IV relapsed or refractory disease
Molecular target: BCL-2
Rationale: Oblimersen may increase the effectiveness of docetaxel by making the tumor cells more sensitive to the drug.
Purpose: Randomized phase 2/3 trial to compare the effectiveness of docetaxel with or without oblimersen in treating patients who have relapsed or refractory non-small cell lung cancer that has been previously treated.
Other examples:
NCT01563302: A Phase 1/2 Study of ISIS 481464, an Antisense Oligonucleotide Inhibitor of STAT3, Administered to Patients With Advanced Cancers. [182]
NCT01780545: The Borealis-2 Clinical Trial: A Randomized Phase 2 Study Comparing Docetaxel Alone to Docetaxel in Combination With OGX-427 in Patients With Relapsed or Refractory Metastatic Urothelial Carcinoma After Receiving a Platinum-containing Regimen: Hoosier Oncology Group GU12-160. [183]
NCT01839604: A Phase I/Ib, Open-Label, Multicentre Study to Assess the Safety, Tolerability, Pharmacokinetics and Preliminary Anti-tumour Activity of AZD9150 in Patients With Advanced/Metastatic Hepatocellular Carcinoma. [184]

Ribozymes or DNAzymes
Definition: A ribozyme, or RNA enzyme, is an RNA molecule that can catalyze a chemical reaction. A DNAzyme, or deoxyribozyme, is a catalytic DNA that is site specifically cleaving the target RNA.
Mechanism of action: Consists of 3 steps, cyclically repeated: base pairing to a complementary target sequence followed by site-specific cleavage of the substrate and finally release of the cleavage products.
Example: NCT00021021 [185]
Oligonucleotide: Angiozyme phase 2
Condition: Kidney cancer
Molecular target: Vascular endothelial growth factor
Rationale: RPI.4610 may stop the growth of metastatic kidney cancer by stopping blood flow to the tumor.
Purpose: Phase 2 trial to study the effectiveness of RPI.4610 in treating patients who have metastatic kidney cancer.
Other examples:
NCT01449942: Phase I/II Study of EBV-LMP1 Targeted DNAzyme in Nasopharyngeal Carcinoma. [186]

Small interfering RNAs (siRNAs)
Definition: A double-strand RNA homologous to an mRNA of a target gene.
Mechanism of action: The siRNAs are incorporated into a multiprotein RNA-induced silencing complex, leaving the antisense strand to guide this complex to its homologous mRNA target for endonucleolytic cleavage of mRNA.
Example: NCT01591356 [187]
Oligonucleotide: siRNA-EphA2-DOPC phase 1
Condition: Advanced cancers
Molecular target: EphA2
Rationale: siRNA targeting EphA2 may decrease tumor growth in cancer relapse.
Purpose: The goal of this clinical research study is to learn about the safety of siRNA-EphA2-DOPC when given to patients with advanced, relapsed cancer. Researchers also want to learn the highest tolerable dose of this drug that can be given. siRNA-EphA2-DOPC is designed to shut down the activity of a gene that causes tumor growth.
Other examples:
NCT00672542: Phase I Study of Active Immunotherapy of Metastatic Melanoma With Mature Autologous Dendritic Cells Transfected With Tumor Antigen RNA and Small Inhibitory RNAs to Alter Proteasomal Antigen Processing. [188]
NCT02110563: Phase I, Multicenter, Cohort Dose Escalation Trial to Determine the Safety, Tolerance, and Maximum Tolerated Dose of DCR-MYC, a Lipid Nano particle (LNP)-Formulated Small Inhibitory RNA (siRNA) Oligonucleotide Targeting MYC, in Patients With Refractory Locally Advanced or Metastatic Solid Tumor Malignancies, Multiple Myeloma, or Lymphoma. [189]
NCT00257647: Use of SV40 siRNA Vectors to Treat CML. [190]

MicroRNA mimics
Definition: A microRNA (miRNA) mimic is a small single-strand 19 to 24 nucleotide RNA with an identical sequence to the miRNA of interest (to be reexpressed).
Mechanism of action: Mimic the effects of an endogenous miRNA with consequent inhibition of protein production by either transcriptional inhibition or translational block or both.
Example: NCT01829971 [191]
Oligonucleotide: MRX34 (miR-34 mimic) phase 1
Condition: Primary liver cancer, solid tumors, lymphoma, AML, ALL, CLL, CML (in accelerated or blast phase)
Molecular target: MYC, MET, BCL2, β-catenin.
Rationale: MRX34 will block tumor development and progression by targeting a variety of cancer related pathways.
Purpose: This is a study to evaluate the safety of MRX34 in patients with primary liver cancer or those with liver metastasis from other cancers. The drug is given intravenously, twice per week for 3 weeks in a row and then 1 week off. In addition, patients with hematologic malignancies will be evaluated on a treatment schedule of 5 days in a row with 2 weeks off.

ASOs/AMOs, LNAs anti-miRNAs, and antagomirs
Definition: Small single-stranded oligonucleotides specifically designed to block the function of miRNA.
The ASOs/AMOs are single-stranded, chemically modified DNA-like molecules that are 17 to 22 nucleotides in length and designed to be complementary to a selected miRNA and specifically inhibit its expression.
The LNAs anti-miRNAs represent LNA-modified ASOs.
The antagomirs are single-stranded 23-nucleotide RNA molecules complementary to the targeted miRNA that have been modified to increase the stability of the RNA and protect it from degradation.

TABLE 4. *Continued*

Mechanism of action: AMOs are ASOs against miRNAs, and therefore produce ASO-miRNA duplex through sequence complementarity, leading to RNAse-H mediated cleavage of the target miRNA gene.
The LNA anti-miRNA have the same mechanism as the ASO/AMO.
The miRNA/antagomir-duplexes induce degradation of the miRNA and recycling of the antagomir in a way still not completely known.
Example: NCT01200420 [192]
Oligonucleotide: Miravirsen (LNA) phase 2
Condition: Hepatitis C virus
Molecular target: miR-122
Rationale: Miravirsen is a novel LNA therapeutic agent that inhibits miR-122. Since the replication of hepatitis C virus is dependent on this miRNA, by blocking it researchers aim to prevent the development of hepatocellular carcinoma.
Purpose: The main purpose of this study is to determine the safety and tolerability of multiple dosing of miravirsen in subjects infected with chronic hepatitis C, who are at high risk of developing hepatocellular carcinoma. Another purpose includes assessing the pharmacokinetics of miravirsen and the assessment of miravirsen's effect on hepatitis C viral titer.
Other examples:
NCT00466583: A Phase 1, Open-Label, Dose Escalation Study Evaluating the Safety and Tolerability of EZN-2968, a Locked Nucleic Acid Antisense Oligonucleotide Against Hypoxia-Inducible Factor-1α, Administered as a Weekly 2-Hour Intravenous Infusion in Adult Patients With Advanced Solid Tumors or Lymphoma. [193]
NCT00285103: An Open-labeled, International, Multicenter, Dose Escalating, Phase I/II Study of SPC2996,an LNA Antisense Molecule Against Bcl-2, in Patients With Relapsed or Refractory Chronic Lymphocytic Leukaemia. [194]
NCT01186328: T2009-007: A Phase I Study Evaluating the Safety, Tolerability and Biological Activity of EZN-3042, a Survivin mRNA Antagonist, Administered With Re-induction Chemotherapy in Children With Relapsed Acute Lymphoblastic Leukemia (ALL) [IND 108753]. [195]

AML indicates acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; ASO, antisense oligonucleotide; AMO, Anti-microRNA oligonucleotide; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; LNA, locked nucleic acids.

Novel approaches can be developed in the near future. One is a combination approach that consists of boosting the therapeutic synergy between miRNA and small interfering RNA (siRNA). The dual inhibition of a specific oncogene by its single-targeting siRNA and a multitargeting miRNA can be used to obtain improved therapeutic efficiency. Although siRNA-mediated silencing of EphA2, an ovarian cancer oncogene, results in a reduction in tumor growth, the additional inhibition of EphA2 by mir-520d-3p further "boosts" its antitumor effects.[205] Finally, a therapeutic strategy in which cells are stimulated to secrete oncogenic miRNA-loaded vesicles and the patient with cancer is subsequently treated with dialysis can be envisioned as a way to "wash out" noncoding oncogenes and induce the tumor suppressor proteins inside the cancer cells.

Instead of a Conclusion: The New Wave – Long ncRNAs in the Clinic

Even though miRNAs have a leading role in published literature, new categories of ncRNAs have recently acquired significance in the field. The size range of ncRNAs is vast (from 200 nt to tens of thousands of nts), and at least 10 times longer than miRNAs (and in some instances, thousands of times larger). They include large intergenic ncRNAs (lncRNAs) and ultraconserved genes, which were found to be abnormally expressed in cancer and involved in tumorigenic mechanisms.[3,206] Because the spectrum of ncRNAs is much greater than that for miRNAs (the estimates are of tens of thousands of ncRNA transcripts vs thousands of microRNAs in the human genome), the impact on any aspect of basic and translational cancer research will be huge.

LncRNAs promise to be as biologically important as (or most likely more important than) miRNAs. They can regulate the transcription of target genes by protein binding, interference, hybridization, and epigenetic mechanism as chromatin remodeling, methylation, and histone modification.[207] They have also been linked to every hallmark of cancer: sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis, and resisting cell death. LncRNAs have strong potential to become biomarkers due to their specific localization in cells and tissues. The tissue specificity of lncRNAs can also enable them to be valuable therapeutic agents. Hence, they could serve for diagnostic, prognostic, and monitoring purposes.[208]

Despite this, the truth is that we are only experiencing the beginning of our understanding of the complexity of the lncRNA world. It is certain that their structures and repertoire of functions exhibit robustness in cancer, and that these functions are adversarial to those of the proteomic field. Nevertheless, what is known about cancer is just the tip of the iceberg; lncRNAs have been linked to a wide array of known human pathologies/diseases, especially within the last 5 years. Some of the pathologies include cardiovascular disease,[209,210] inflammatory disease,[211,212] diabetes,[213,214] Alzheimer disease,[215] infectious diseases,[216] schizophrenia,[217] ischemic disease/stroke,[218] neuromuscular disease,[219] immune response,[220,221] and so on. Certainly, this is an exciting time for the RNA therapeutic arena, and we are looking forward to more results from clinical studies investigating novel strategic approaches. ■

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