The American Journal of Pathology, Vol. 171, No. 3, September 2007

DOI: 10.2353/ajpath.2007.070070

Review

MicroRNAs in Tumorigenesis

A Primer

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MicroRNAs (miRNAs) are a family of 21- to 25-nucleotide, noncoding small RNAs that primarily function as gene regulators. It is surprising that these tiny molecules, so diverse and consequential in their biological functions, have been hidden for so many years. Thanks to their discovery, cancer research has found a new arena. Aided by innovative molecular techniques, the research of miRNAs in oncology has progressed rapidly in recent years. miRNA abnormalities are becoming an emerging theme in cancer research. Specific functions of miRNAs, many of which are relevant to cancer development, are becoming apparent. The value of miRNAs in cancer classification and prognostication is being explored, and new therapeutic strategies targeting miRNAs are being developed. Because there is great promise that miRNA research will provide breakthroughs in the understanding of cancer pathogenesis and development of new valuable prognostic markers, pathologists should be adequately informed of this rapidly progressing field. Here, we offer a review on the basics of miRNA biology and the emerging role of miRNA in cancer pathogenesis, classification, and prognostication, including highlights of the involvement of specific miRNAs in different tumor types. ***(Am J Pathol 2007, 171:728–738; DOI: 10.2353/ajpath.2007.070070)***

Basic Biology of MicroRNAs

A Brief History of MicroRNA Discovery

The first microRNA (miRNA), *lin-4*, was discovered in 1993 as a small noncoding RNA that regulates *Caenorhabditis elegans* development.[1] Although there was evidence suggesting that *lin-4* negatively regulates *lin-14* protein expression by a novel antisense mechanism, the exact nature of this RNA was a mystery at that time. In 2000, the second miRNA *let-7* was characterized as a 21-nucleotide small RNA that regulates *C. elegans* development. The discovery that *let-7* was evolutionarily conserved among animal species including vertebrate, ascidian, hemichordate, and mollusk indicated that these small RNAs are fundamentally important to many biological processes, such as development,[2] and to many pathological processes. Since the discovery of *lin-4* and *let-7*, many more miRNAs have been identified using various experimental and computational methods. Estimates of the total number of human miRNAs range up to more than 800, at least three times more than the original estimate.[3]

miRNAs are encoded in animal and plant genomes and are believed to have originated as a host defense against foreign genetic material such as RNA viruses and transposable elements. Interestingly, viruses such as Epstein-Barr virus, cytomegalovirus, Kaposi sarcoma herpes virus, Simian virus 40, and human immunodeficiency virus-1 encode miRNAs that may play a role in viral pathogenesis.

Biogenesis of miRNA

The biogenesis of metazoan miRNAs begins with the transcription of miRNA genes to generate primary pri-miRNA transcripts in the nucleus (Figure 1). Regions of pri-miRNAs have short, imperfectly base-paired stem-

Supported by the Department of Pathology and Laboratory Medicine, Weill Cornell Medical College (to W.T.).

Accepted for publication May 23, 2007.

J.E.D. is a founder and owns stock in Third Wave Technologies, the company that developed and markets Invader tests for analysis of nucleic acids.

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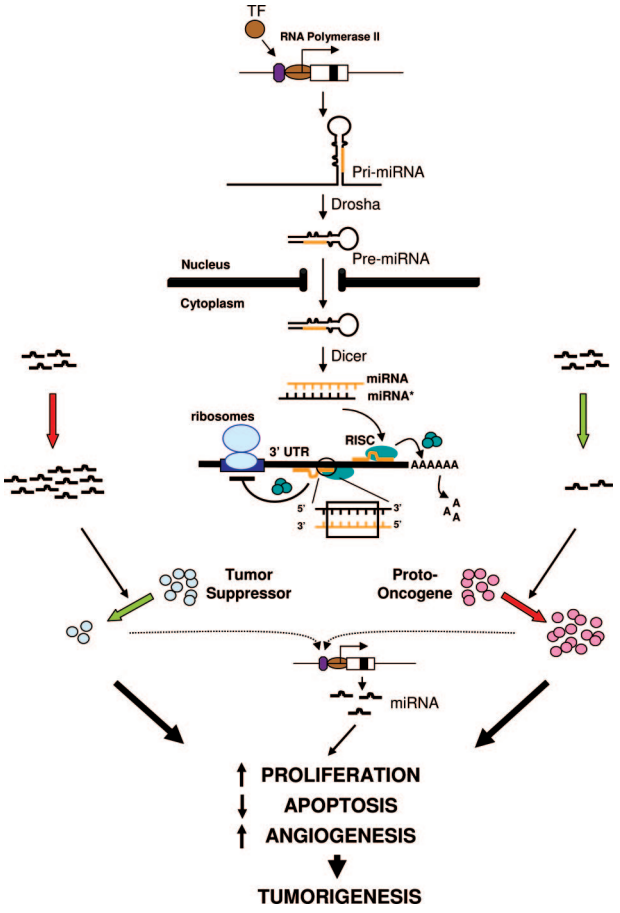

Figure 1. Oncogenesis by deregulation in miRNA expression. Primary miRNAs (pri-miRNAs) are transcribed from miRNA genes by RNA polymerase II. These miRNA precursors may be spliced and polyadenylated. Transcription from these miRNA genes is probably under the regulation of transcription factors (TF) that respond to multiple signals and can also be epigenetically controlled. The pri-miRNAs are processed by RNAase III enzyme Drosha to pre-miRNAs, which are ~70-nucleotide RNAs with an imperfect RNA duplex structure. The pre-miRNAs are transported from the nucleus to the cytoplasm by Exportin-5. Within the cytoplasm, the pre-miRNAs are further processed by Dicer, another RNAase III enzyme, generating miRNA:miRNA* hybrids. On binding of the short RNA duplexes to specific proteins such as Argonaut 2, the miRNA* is released, and the remaining complex containing the mature miRNA is called RISC (RNA interference silencing complex). The miRNA directs the association of the RISC to specific sites in the 3′-untranslated region of a targeted mRNA. If base-pairing between the miRNA and its target is perfect, an endonucleolytic function of Argonaut 2 cleaves the mRNA, leading to its rapid destruction. Imperfect complementarity can elicit translational repression or cause instability of the mRNA. The latter effect is possibly mediated by a deadenylation mechanism. These functions all require Watson-Crick pairing between the seed sequence at the 5′ end of the miRNA and a complementary site in the 3′ portion of the targeted mRNA. Deregulation of miRNAs can occur in the transcription of miRNA genes due to an alteration in miRNA gene copy number, methylation status of the promoter, or changes in activity of the TFs that control their transcription. Some of these TFs may themselves be protein products of proto-oncogenes or tumor suppressor genes. TFs whose amounts are controlled by one miRNA may, in turn, control production of other miRNAs. miRNA can also be deregulated as a result of global or miRNA-specific abnormalities in miRNA processing. Alterations in the amounts of miRNAs and their precursors that can result in cancer are indicated on the left and right panels. Red and green arrows emphasize increases and decreases from normal, respectively. In tumorigenesis, excessive accumulation of a miRNA that normally regulates synthesis of a tumor suppressor may promote cancer by causing underexpression of the tumor suppressor (left). Conversely, underexpression of a miRNA that normally controls the expression of a proto-oncogene can lead to excess accumulation of that gene product (right). Imbalances in the amounts of proto-oncogene products or tumor suppressors may promote cell proliferation, decrease apoptosis, and stimulate angiogenesis, all of which contribute to tumor formation.

loop structures containing sequences that will become the miRNA. These hairpin structures are released from the pri-miRNAs by Drosha, an RNaseIII enzyme, as ~70-nucleotide precursors called pre-miRNAs. Pre-miRNAs are transported into the cytoplasm, where they are further processed by Dicer, another RNaseIII enzyme, that cuts off the terminal loop and generates an imperfect double-stranded RNA duplex called miRNA/miRNA*. The double-stranded RNA duplex binds to a protein (Argonaut 2) as part of the RNA-induced silencing complex (RISC). The strand of the duplex that is complementary to the miRNA/miRNA*, is released. The RISC, containing its miRNA, binds to the target mRNA and direct mRNA cleavage or translational repression based on sequence complementarity. RISC is also involved in the RNA interference pathway of small interfering RNA,[4,5] which is structurally and functionally related to miRNA.

Functions of miRNA

miRNAs act as gene regulators, the functions of which are mediated through translational repression or mRNA degradation via binding of miRNAs to target sites in the 3′-untranslated regions of protein-coding transcripts. Early studies of miRNA and the related RNA interference showed that the degree of sequence complementarity determines whether the target mRNA is degraded or its translation into protein is repressed.[6,7] According to this model, perfect sequence complementarity leads to endonucleolytic cleavage and degradation of the mRNA, whereas less strict complementarity results in translational repression. This model is supported by the observation that most plant miRNAs have near perfect sequence complementarity with their target mRNAs and mediate mRNA cleavage, whereas animal miRNAs lack such perfect or near-perfect sequence complementarity and mediate translational repression as exemplified by *lin-4*. miRNAs may inhibit translation at a postinitiation step by interfering with translation elongation.[8] Although translation repression seems to be the major mechanism of miRNA-mediated gene regulation in animals, it may not be the only possible consequence of the miRNA:mRNA interaction that forms imperfect duplexes. Recently, studies have show that several animal mRNAs, such as *lin-4*, *let-7*, miR-1, and miR-124, also mediate mRNA degradation, despite binding sites that are only partially complementary to the miRNAs.[9,10] miRNA degradation in these cases seems to be secondary to deadenylation of the mRNA rather than direct cleavage of mRNA mediated by perfect miRNA:mRNA pairing.[11]

Translational repression by the miRNA:mRNA interaction depends on perfect Watson-Crick base-pairing between the seed region (nucleotides 2 to 7 or 8) at the 5′ end of the miRNA and the target site.[12,13] The seed region of the miRNA is also critical in the down-regulation of mRNA levels by miRNA.[10] Although the miRNA-mediated mRNA cleavage generally requires only one mRNA binding site, the number of miRNA binding sites in a target mRNA can affect the efficiency of translational repression.[14] Several computer algorithms have been

created for prediction of mammalian miRNA targets.[15] These algorithms are based largely on the critical "seed pairing" in conjunction with several other parameters. Often many targets are predicted, many of which are possibly false positive. Because partial base-pairing between a miRNA and potential target can affect protein but not mRNA levels, experimental validation of the vast majority of animal miRNA target predictions has been difficult. However, in those cases in which the level of a targeted mRNA is affected by miRNAs, direct analyses of mRNA levels may support miRNA target predictions.[9,10] Identification of the downstream targets for miRNAs is crucial to the understanding of their functions.

Many, and perhaps all, miRNAs have multiple targets.[16] Thus, miRNAs potentially regulate thousands of human genes, of which many are involved in transcriptional regulation or other basic cellular functions, such as control of the cell cycle. Given the seemingly unlimited potential of miRNAs to regulate expression of multiple cellular genes, it comes with no surprise that miRNAs play important roles in diverse biological processes.

Studies in animal models have revealed essential functions of miRNAs in developmental control. Many miRNAs exhibit a temporal or tissue-specific pattern of expression, suggesting that they play a critical role in tissue and organ development, function, and maintenance. In *C. elegans*, lin-4 and *let-7* control the timing of larval development, and *lsy-6* is important in neuronal patterning.[17] In *Drosophila*, the miRNA *bantam* regulates development by promoting cell proliferation and inhibiting apoptosis through modulating the expression of the proapoptotic gene *hid*.[18] In mammals, miRNAs have been shown to regulate cell fate, including lineage differentiation in a number of tissue types. For example, miR-181 has been shown to modulate hematopoietic lineage differentiation.[19] miR-223 regulates human granulopoiesis by a regulatory pathway involving transcription factors NFI-A and CCAAT/enhancer-binding protein-α.[20] miR-1 and miR-133 promote myogenesis and myoblast proliferation.[21] Brain-specific miR-124a and miR-9 molecules affect neural lineage differentiation in embryonic stem cell-derived cultures.[22] miR-143 is involved in human adipocyte differentiation.[23] In addition, miRNAs regulate homeostasis in cell growth. For example, miR-15a, miR-16, and miR-21 are involved in apoptosis.[24,25] A polycistronic miRNA cluster on human chromosomal band 13q31, miR-17-92, which consists of seven miRNAs, participates in control of cell proliferation and angiogenesis.[26,27] Because of the essential role of miRNAs in such a wide array of biological functions, abnormalities in miRNA expression are likely to contribute significantly to many pathological processes such as the development of cancer.

Detection of miRNAs

Northern blots were the first detection method used in the quantification of miRNAs. However, Northern blotting requires a relatively large amount of RNA and is relatively insensitive in measuring miRNA expression levels. Quantitation of individual miRNAs can also be performed using real-time reverse transcriptase-polymerase chain reaction (RT-PCR),[28,29] modified Invader assay,[30] or confocal laser-induced fluorescence detection.[31] All these methods require only nanograms of total RNA, have high sensitivity, and are able to differentiate between mature miRNAs and their precursors as well as to discriminate between highly homologous mature miRNAs, for example, different members of the same miRNA family. In addition, they have the ability to finely discriminate expression levels.

The real-time RT-PCR method makes use of a stem-loop reverse transcription primer to generate cDNA templates, which are then amplified using specific primers and detected with specific fluorescence probes, similar to the standard real-time RT-PCR for the detection of mRNA. This method has a very broad dynamic range, but it requires relatively costly equipment for the PCR process and detection of the amplicons. The Invader assay directly detects specific RNA molecules using an isothermal signal amplification process with a fluorescent read-out. It does not require expensive instrumentation and can be easily adopted for high-throughout read-out of multiple samples. The confocal laser-induced fluorescence detection method for miRNA eliminates the need for reverse transcription, PCR amplification, or other signal amplification steps. It involves the use of two short locked nucleic acid-modified probes that are labeled with different fluorophores and hybridized to the two halves of the miRNA. The expression of that miRNA can be quantitated by counting the miRNA molecules that hybridize to both of these probes using a two-color confocal scanner.

None of the above methods is optimal for the simultaneous analysis of hundreds of miRNAs present in a single sample. For this purpose, oligo-array-based technologies have been developed.[32,33] Although miRNA microarrays are well suited for large-scale screening and are very sensitive, they are semiquantitative and cannot finely discriminate expression levels that differ by a smaller margin. In addition, they cannot discriminate between highly similar miRNAs because of the inability to optimize hybridization conditions for all miRNAs. However, the use of locked nucleic acid-modified probes[34] has improved the ability of miRNA microarrays to measure more accurately levels of highly homologous miRNAs.

Recently, methods have been developed to profile miRNAs from formalin-fixed, paraffin-embedded tissues and to localize miRNA expression at the cellular and subcellular levels by *in situ* hybridization. RNA-primed, array-based Klenow enzyme assay was successfully used to profile miRNA expression from formalin-fixed, paraffin-embedded tissues of human brain and oligodendrogliomas, and the results were confirmed with Northern blots and refined by *in situ* hybridization.[35] *In situ* hybridization for miRNA allows determination of expression in single cells within a heterogenous cell population and facilitates comparison of miRNA expression in different cell populations in their natural contexts without the need of cell sorting or separation. This method is well suited for evaluating clinical archived samples and thus is particularly useful for advancing miRNA research in pathology.

miRNA in Cancer Development

The importance of miRNAs in tumorigenesis is underscored by the association of cancers with genomic alterations that have the potential to deregulate their repression. Perturbations in miRNA expression have been observed or are predicted to affect the activities of targeted mRNA encoding proteins that have oncogenic or anti-oncogenic functions. In addition, animal studies have directly demonstrated a role of certain miRNAs in oncogenesis.

miRNAs Are Associated with Genomic Alterations

Several studies have demonstrated an association of altered miRNA expression and cancer. A disproportionate fraction of genes encoding miRNAs are in regions where regulation of miRNA expression can potentially be disrupted by chromosomal abnormalities.[36] Slightly more than one-half of the 186 miRNA genes studied are located in cancer-associated genomic regions, which include minimal regions of loss of heterozygosity, minimal regions of amplification, and less commonly, chromosomal breakpoint regions. For instance, genes encoding miR-15a/miR-16a are located on 13q14, a region that is often deleted in chronic lymphocytic leukemia (CLL). The miR-17-92 polycistron is associated with 13q31, a region that is amplified in some B-cell lymphomas. In a case of precursor B-lymphoblastic leukemia/lymphoma, a gene encoding miRNA-125b-1 is translocated to the immunoglobulin heavy chain gene locus.[37] In a study with higher genomic resolution, 283 miRNA genes in 227 human ovarian, breast, and melanoma cancer specimens were analyzed for copy number changes using an array-based comparative genomic hybridization method.[38] Of the 283 miRNAs analyzed, 37.1% in ovarian cancer, 72.8% in breast cancer, and 85.9% in melanoma were located in genomic regions exhibiting gene copy number alterations. In all three types of cancer, the copy numbers of 26 miRNA genes increased, whereas the copy numbers of 15 other miRNA genes decreased. The fact that the same miRNAs are affected in several different cancers may indicate that some miRNAs participate in the generation of a "common" tumor phenotype; conversely, alterations of certain miRNAs are specific to a particular type of cancer.

A comprehensive correlative expression analysis of miRNAs located in abnormal chromosomal regions has demonstrated a high correlation between alterations in DNA copy number and expression of miRNAs,[38] suggesting that genomic alterations, particularly genomic amplification or deletion, can be a major mechanism of activation of the oncogenic function of miRNA or inactivation of its tumor suppressor function.

miRNA Expression Profiles Are Broadly Altered in Cancers

Conflicting reports have been published on whether the global amounts of miRNAs increase or decrease in cancers. Expression profiles of 217 mammalian miRNAs in cancers (colon, kidney, prostate, urinary bladder, lung, and breast) compared with normal tissues, using a bead-based flow cytometric miRNA expression profiling method,[39] reported a global decrease of miRNA expression in cancers. It was suggested that miRNAs play a role in driving cell differentiation, and the decrease of miRNA expression found in cancer cells might contribute to the generation and maintenance of cancer cells, including less differentiated cancer stem cells.

In contrast, Volinia et al,[40] using a miRNA microarray platform, observed that overall amounts of miRNAs were not reduced among 228 miRNAs in six different solid tumor types (breast, colon, lung, pancreas, prostate, and stomach cancers). Rather, 26 miRNAs were found to increase in amount, whereas only 17 miRNAs were downregulated in these cancers. miR-21 was overexpressed in all six cancers, miR-17-5 and miR-191 were overexpressed in five, and 21 miRNAs were overexpressed in at least three cancers. The different conclusions of these two studies may be due to differences in RNA isolation, the choice of internal standards used to normalize for losses during sample isolation, and the methods used to analyze the miRNAs.

Both of these studies demonstrate that miRNA expression is broadly altered in cancers. Although some of the changes may just be bystander effects as a result of the tumor phenotype, perhaps as a result of a decrease in the effectiveness of the Drosha processing machinery,[41] other changes in miRNA expression may directly contribute to oncogenesis.

miRNAs as Oncogenes and Tumor Suppressor Genes

The location of miRNA genes in fragile sites and the broad alteration of miRNA expression in tumors provide circumstantial evidence of an etiological role of miRNAs in tumor formation. Stronger, more direct evidence of the involvement of miRNAs in oncogenesis should come from the demonstration of the oncogenecity or tumor suppression function of miRNAs in cultured cells and, preferably, in animal models, although the latter approach is not always available or feasible. Therefore, the cancer-related functions of miRNAs are often inferred from the biological properties of a few experimentally validated target genes.

Because miRNAs are negative regulators of gene expression, changes in the amounts of these RNAs can be tumorigenic if they target mRNAs for either a tumor suppressor or an oncogene (Figures 1 and 2). For example, overaccumulation of a miRNA that targets the mRNA of a tumor suppressor would result in less of that protective factor. In contrast, reduced accumulation of a miRNA that targets the mRNA of a proto-oncogene could lead to excessive amounts of the oncogenic protein. The net outcome of both pathways is an imbalance of the activities of tumor suppressor genes and oncogenes.

Not only can miRNAs control expression of canonical protein-coding oncogenes and tumor suppressor genes,

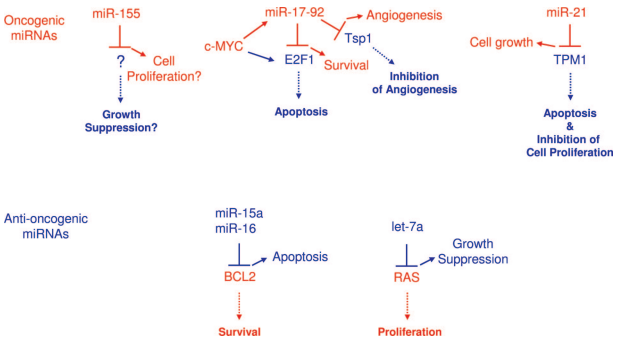

Figure 2. Target interactions and biological effects of main cancer-associated miRNAs. Top: MiR-17-92 polycistron cluster, miR-155, and miR-21 are well-known oncogenic miRNAs. MiR-17-92 is within a regulatory loop between c-MYC and E2F1. Both miR-17-92 and E2F1 are transcriptionally activated by c-MYC. MiR-17p and miR-20a within the miR-17-92 cluster target E2F1 for translation inhibition, thus preventing its abnormal accumulation in the setting of an activated c-MYC and modulating its proapototic capacity. As a result, the oncogenic effect of c-MYC is enhanced through reduction of its apoptotic program. MiR-17-92 also modulates translation of anti-angiogenic thrombospondin-1 (Tsp1), thereby promoting angiogenesis. The exact biological effects of miR-155 and its targets are not known. However, it is postulated to play a role in cell proliferation. MiR-21 mediates tumor growth by inhibiting apoptosis and suppressing cell proliferation, which is possibly mediated, at least partly, through inhibition of the tumor suppressor gene tropomyosin I (TPM1). Bottom: MiR-15a, miR-16, and let-7 are examples of tumor suppresssor miRNAs. MiR-15a and miR-16 suppress BCL2 translation and lead to increased apoptosis. Let-7 inhibits RAS translation and reduces stimulation of growth. Decreased levels of these miRNAs result in reduced cell apoptosis and increased cell proliferation. Gene products and their biological effects that promote tumor formation are indicated in red; those that suppress tumor formation are marked in blue.

they can themselves be the transcriptional targets of oncogenes and tumor suppressor genes. Thus, protein-coding proto-oncogenes (or tumor suppressor genes) can fine-tune their oncogenic (or anti-oncogenic) functions by modulating expression of downstream miRNAs, which in turn regulate their oncogenic or anti-oncogenic targets. This is best illustrated by the miR-17-92 cluster, which is transcriptionally activated by the proto-oncogene c-*Myc*.[42] Two of the miRNAs within this cluster, miR-17-5p and miR-20a, repress translation of E2F1, a protein that enhances both cell cycle progression and apoptosis. Transcription of the *E2F1* gene is increased by c-MYC. However, because c-MYC also activates transcription of the miR-17-92 cluster, causing the levels of miR-17-5p and miR-20a to increase, the increase in E2F1 levels is modulated. This feedback mechanism may help prevent accumulation of excessive amounts of E2F1, which may otherwise sensitize cells to apoptosis, thereby shifting its function toward cell division. Moreover, c-MYC promotes angiogenesis because increased transcription of the miR-17-92 cluster results in down-regulation of the anti-angiogenic thrombospondin-1 and related proteins, such as connective tissue growth factor.[27]

As might be expected, changes in the copies of genes encoding miRNAs are often responsible for the activation of oncogenes and inactivation of tumor suppressor genes,[38] but other mutations and even chromosomal modifications have been shown to play roles in these processes. For example, 11 of the 28 overexpressed miRNAs detected in breast cancer exhibit gains in gene copy number.[38] Epigenetic deregulation of the primary transcripts encoding miRNAs, such as abnormal DNA methylation or histone modification, have also been implicated in changing the levels of some miRNAs.[43] Treatment of human cancer cells with DNA methylation inhibitors results in up-regulation of 17 of 313 miRNAs analyzed, suggesting that expression of some miRNAs in cancer is down-regulated by epigenetic silencing.[43] Because miRNAs can be regulated at the Drosha processing step,[41] it is also conceivable that altered expression may be caused by miRNA-specific abnormalities in Drosha processing. Although sequence variants of miRNA precursors are rather common in cancer cells, mutations that affect the production of normal mature miRNAs are uncommon.[44]

miRNAs as Oncogenes

An animal model developed by Tam et al[45] showed that *BIC*, the gene encoding miR-155, cooperated with c-*Myc* to promote lymphomagenesis in chickens. At that time, it was noted that *BIC* was unlikely to encode a protein, but the identity of the biological product was unknown. We now know that *BIC* encodes miR-155, so this was the first demonstration that a miRNA could have oncogenic potential.[45] More recently, Costinean et al[46] generated transgenic mice carrying a miR-155 transgene with B-cell-targeted expression and showed that the transgenic mice developed preleukemic pre-B-cell proliferation at as early as 3 weeks of age and eventually developed frank high-grade B-cell malignancies at 6 months of age. That study supports a major etiological role of miR-155 in lymphoma development.

Overexpression of miR-17-19b, a truncated version of miR-17-92, in transgenic mice expressing c-*Myc* could accelerate lymphomagenesis, suggesting miR-17-92 acts cooperatively with c-MYC in lymphomagenesis.[26] In contrast to c-*Myc*-associated B-cell lymphomas that show extensive tumor cell apoptosis, lymphomas that developed in the setting of miR-17-19b overexpression in *c-Myc* transgenic mice did not show extensive tumor cell apoptosis, suggesting possible anti-apoptotic functions of miR-17-92.

We expect more *in vivo* animal models for direct demonstration of the oncogenic functions of miRNAs in the future. In the meantime, evidence that miRNAs can function as oncogenes comes from identification of tumor suppressor genes as putative miRNA targets for miRNAs that are overexpressed. For example, miR-21, which is overexpressed in breast carcinomas, promotes cell growth by inhibiting the putative tumor suppressor gene tropomyosin 1.[47] Two tumor suppressor genes, *RB1* and *TGFBR2*, were validated as targets for miR-20a and miR-106 by luciferase reporter assays.[40] However, it has yet to be demonstrated whether these miRNAs can directly regulate the endogenous protein levels of their putative target genes.

As illustrated by the aforementioned regulatory network between c-MYC, miR-17-92, and E2F1, miRNAs may promote tumor formation not only by down-regulating bona fide tumor suppressors but also by modulating functions of proto-oncogenes to enhance their oncogenic

activities while keeping their anti-oncogenic effects, such as apoptosis induction, to a minimum.

To complicate matters further, a recent study suggested a tumor suppressor role for miR-17-5p, a miRNA encoded in the miR-17-92 polycistron. Overexpression of miR-17-5p in breast cancer cell lines harboring low levels of this miRNA inhibits proliferation.[48] These results raise the possibility that a miRNA may function as an oncogene or a tumor suppressor depending on the cell-type-specific microenvironment, which may provide a different repertoire of available target genes.

miRNAs as Tumor Suppressor Genes

miRNAs have been implicated as tumor suppressor genes. The most well-characterized examples are miR-15a and miR-16, which are down-regulated by hemizygous or homozygous deletion or other unknown mechanisms in 68% of CLLs.[49] In one case, a point mutation in the precursor molecule for miR-16 has been shown to compromise the generation of mature miR-16.[50] This mutation is associated with a family history of CLL,[50] whereas the other, normal allele is deleted. Although this type of classic mechanism of inactivation for a tumor suppressor gene seems to be rare, it does provide further evidence for tumor suppressor roles for miR-15a and miR-16.

The tumor suppressor activities of miR-15a and miR-16 in CLL are further supported by the observation that these two miRNAs negatively regulate the expression of the anti-apoptotic factor *BCL2.* Thus down-regulation of miR-15a and miR-16 could result in higher BCL2 protein level and higher anti-apoptotic activity.[24]

However, questions about the tumor suppressor role for miR-15a and miR-16 in the majority of CLL are raised by a study that employs direct cloning and real-time RT-PCR to quantitate miRNAs.[51] This study did observe reduction in miR-15a and miR-16 levels, although the percentage of cases with such alterations is only ~10%, one-half of which harbor homozygous deletions in 13q14. Cases with heterozygous deletions were not found to have significant changes in miR-15a and miR-16 levels compared with cases without deletions. Furthermore, this study did not identify an inverse correlation of BCL2 expression and miR-15a/miR-16 levels among the CLL cases. However, this data cannot completely exclude *BCL2* as a target for miR-15a/miR-16, because it is likely that *BCL2* is simultaneously regulated by other factors. Further experiments are necessary to resolve the issue of the frequency of alterations in miR-15a/miR-16 expression and the involvement of BCL2 up-regulation in the pathogenesis of CLL.

Down-regulation of miRNAs that target well-known proto-oncogenes is also observed in other tumors. *Let-7* expression is lower in lung carcinomas than in normal lung tissue. Its role as a tumor suppressor is supported by experiments showing that *let-7* negatively regulates expression of the *RAS* proto-oncogene.[52]

In general, it seems that miRNAs do not function as "classic" tumor suppressors, which are typically inactivated through a "two-hit" mechanism of inactivation. Unlike many protein-coding tumor suppressors, the functions of which are completely abrogated in cancers, the tumor suppressor functions of these miRNAs are manifest when their levels are reduced below critical levels, either by a reduction in gene dosage or some other mechanisms, which in turn lead to an increase in levels of oncogenic gene products to an extent that will cause a tumor phenotype. Thus, miRNA tumor suppressors tend not to be "all-or-none" effectors.

miRNAs in Stem Cell Activation

Several studies have shown miRNAs are involved in the self-renewal of stem cells. In mice and zebrafish, the absence of Dicer-1, an RNaseIII enzyme essential for miRNA biogenesis, results in lethality early in development and loss of stem cell populations.[53,54] Mutation in Argonaute genes, encoding key components of the miRNA processing complex RISC, causes stem cells to lose their ability of self-renewal. Hatfield and colleagues[55] reported that in *Drosophila melanogaster*, miRNA pathways play important roles in the division of stem cells. Germline stem cell mutant for *dicer-1* showed delay in the G_1 to S transition, suggesting that miRNAs may be involved in cell cycle control in these cells. An attractive hypothesis is that miRNAs help to regulate the timing of stem cell division by modulating the amounts of the cell cycle regulator p21. An important question to be answered is which set of miRNAs are crucial for normal stem cell maintenance and whether such miRNAs are involved in cancer stem cell function.

miRNA Abnormalities in Different Types of Cancer

Abnormalities in some miRNAs are associated with a single type of tumor, whereas changes in others occur in more than one tumor type (Tables 1 and 2). Several miRNAs, such as miR-155, miR-21, miR-17-92, and *let-7*, seem to function in tumorigenesis across several tissues. These miRNAs probably modulate pathways that are essential to initiation and maintenance of tumor phenotype regardless of cell origin. Interestingly, in those studies that profile miRNA expression in a particular type of solid tumor, the miRNAs are mostly overexpressed, and no widespread down-regulation of miRNAs is observed.

Hematopoietic Tumors

CLL. As discussed above, alterations in copy number and/or expression of miR-15a and miR-16 have been identified in CLL.[49,51] NZB mice, which are predisposed to a lymphoproliferative disorder similar to CLL, harbor a mutation associated with miR-16 down-regulation at the miR-16 locus syntenous to human 13q14, further supporting its pathogenetic role in CLL.[56] Abnormalities in miR-15a and miR-16 may contribute to pathogenesis of CLL by up-regulating the anti-apoptotic factor BCL2.[24]

Table 1. Tumor-Associated MicroRNAs and Their Potential Prognostic Utility

Tumor type	miRs*	Exp.	Method of exp. analysis	Potential prognostic utility	Reference
Brain, GBM	miR-21	↑	N		25, 65
	miR-221, miR10-b	↑	M, N		
	miR-128, miR-181a, miR-181b	↓	M, N		
Breast ca	miR-21	↑	M, N	miR-21 and miR-145 expression levels correlate with proliferation index	40, 61
	miR-125b, miR-145	↓	M, N		
Colorectal ca	miR-143, miR-145, miR133b	↓	N	miR-31 expression level correlates with cancer stage	62–64
	miR-31, miR-135b, miR-96, miR-183	↑	R		
Hepatocellular ca	miR-18, miR-224	↑	M, N	Higher miR-18 expression level is inversely correlated with tumor differentiation	66, 67
	miR-199a, miR-195, miR-200a, miR-125a, miR-122	↓	M, N		
Lung, NSCLC	*let-7*	↓	M, N, RT	Low *let-7* and high miR-155 expression levels correlate with poor survival in adenocarcinoma	52, 68, 69
	miR-126*	↓	M, RT		
	miR-21, miR-205, miR-155	↑	M, RT		
Lung, SCLC	miR-17-92 cluster	↑	N, S		70
Lymphoma/leukemia, DLBCL	miR-155	↑	N, I	Higher levels in ABC type than in GC type	57
Lymphoma/leukemia, CLL	miR-15a, miR-16-1	↓	N		49–51
	miR-21, miR-150, miR-155	↑	Cloning, RT		
	miR-92, miR-222	↓	Cloning, RT		
Lymphoma/leukemia, others	miR-155	↑	N		26, 58
	miR-17-92 cluster	↑	RT		
Pancreatic endocrine and acinar tumors	miR-103, miR-107, miR-21, miR-204	↑	M, N	Overexpression of miR-21 correlates with high proliferation index and liver metastasis	71
	miR-155	↓	M, N		
Testis, germ cell tumors	miR-372, miR-373	↑	RPA, *In situ*		72
Thyroid, papillary ca	miR-221, miR-222, miR-146b	↑	M, N, RT		73
Thyroid, follicular ca	miR-197, miR-346	↑	M, RT		75

*MicroRNAs of which alterations in expression are only identified by microarray method are excluded.
ABC, activated B cell; ca, carcinoma; DLBCL, diffuse large B-cell lymphoma; Exp., expression relative to normal tissue; GBM, glioblastoma multiforme; GC, germinal center; I, Invader assay; *In situ*, *in situ* hybridization; M, miRNA microarray; N, Northern blot; NSCLC, non-small cell lung cancer; RT, real-time quantitative PCR; RPA, RNase protection assay; S, Southern blot; SCLC, small cell lung cancer.

CLL is also the first human tumor for which a global miRNA profile with potential clinical utility was proposed. Using microRNA microarray, a unique miRNA expression profile consisting of 13 miRNAs that can differentiate the poor prognostic group (high ZAP-70 expression and unmutated IgV_H) from the good prognostic group (low ZAP-70 expression and mutated IgV_H) was identified.[50]

Other B-Cell Lymphomas. In line with its oncogenecity in animal models, miR-155 levels were elevated 12- to 60-fold relative to the control B cells in diffuse large B-cell lymphoma cells.[57] Interestingly, miR-155 levels in the activated B-cell subtype of diffuse large B-cell lymphoma were two- to threefold higher than that in the germinal center subtype. High expression of miR-155 was also observed in Hodgkin's lymphoma and primary mediastinal B-cell lymphoma.[58] There are conflicting results on whether miR-155 is highly expressed in Burkitt lymphoma cells.[59,60] miR-155 is likely to have a broad role in lym-

Table 2. Tumor-Associated MicroRNAs and Their Validated Target Genes

miRs	Tumor type	Exp.	Target genes	Reference
let-7	NSCLC	↓	*RAS*	52
miR-15a, miR-16	CLL	↓	*BCL2*	24, 49
miR-17-92 polycistron	Breast ca, B-cell lymphomas, SCLC	↑	*AIB1* (miR-17-5p), *E2F1* (miR-17-5p, miR-20a), *TGFBR2* (miR-20a), *Tsp1* and *CTGF*	26, 27, 40, 42, 48, 70
miR-21	Breast ca, GBM	↑	*TPM1*	25, 47, 61
miR-106a*	Colon ca, pancreas ca, prostate ca	↑	*RB1*	40
miR-221, miR-222, miR-146b	Thyroid, papillary ca	↑	*KIT*	73, 74
miR-372, miR-373	Testis, germ cell tumors	↑	*LATS2*	72

*MicroRNA expression levels in tumors were demonstrated by microarray only and have not been validated by other methods.
ca, carcinoma; Exp., expression relative to normal tissue; GBM: glioblastoma multiforme; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

phomagenesis. However, the elucidation of the exact mechanisms of how miR-155 contributes to lymphomagenesis requires identification of its biological targets. Interestingly, alterations in miR-155 expression are not absolutely specific for lymphoid tumors. Its deregulation has also been documented in solid tumors (see below).

He et al[26] showed that overexpression of the miR-17-92 polycistron is associated with gene amplication in these B-cell lymphomas. It is conceivable that miRNAs in this polycistron cooperate in lymphoma development by affecting multiple pathways related to tumorigenesis.

Solid Tumors

Breast Carcinoma. Using miRNA microarray, Iorio et al[61] identified 29 miRNAs that were differentially expressed in breast cancer compared with normal breast tissue. Among these 29 miRNAs, the most significant difference was seen in miR-125b, miR-145, miR-21, and miR-155. Furthermore, expression of specific miRNAs could be correlated with a number of pathological features such as ductal versus lobular histological type, lymph node metastasis status, estrogen receptor and progesterone receptor status, ERBB2 expression, proliferation index, and p53 expression by immunohistochemical staining. Similarly, the miRNA profiling study of solid tumors by Volinia et al[40] identified 15 miRNAs that are up-regulated in breast cancer.

Colorectal Carcinoma. miRNA serial analysis of gene expression generated a colorectal "microRNAome" for the human colorectal cancer cell lines, which included 133 novel miRNA candidates in addition to 200 known mature miRNAs.[62] In the miRNA profiling study of solid tumors by Volinia et al,[40] 21 miRNAs were found to be up-regulated in colon cancer compared with normal colon mucosa, and one miRNA is down-regulated. In another study, real-time RT-PCR analysis of 156 miRNAs in colon cancers and normal colonic mucosa allowed the identification of 13 miRNA that exhibit significantly altered expression in colon cancers and demonstrated that the expression level of miR-31 was correlated with the stage of tumor.[63]

Direct cloning of miRNA sequences showed that miR-143 and miR-145 levels were present at lower levels in colorectal adenomas and cancers than in normal colorectal mucosa.[64] Real-time RT-PCR analysis confirmed the down-regulation of miR-145 in colorectal cancers.[63]

Glioblastoma Multiforme. Analysis of the expression of 245 miRNAs in glioblastoma multiforme cell lines with the miRNA microarray method identified nine overexpressed and four underexpressed miRNAs that are associated with glioblastoma multiforme.[65] Chan et al[25] reported miR-21 overexpression in glioblastoma multiforme and suggested that overexpressed miR-21 may be anti-apoptotic.

Hepatocellular Carcinoma. miRNA microarray analysis of 25 paired hepatocellular carcinomas and surrounding nontumor tissue identified three overexpressed miRNA and five underexpressed miRNA.[66] This miRNA expression signature could predict tumor status with approximately 100% accuracy. In addition, the levels of four miRNAs are inversely correlated with the degree of hepatic differentiation of the tumor. A different pattern of miRNA expression was also observed between chronic hepatitis and liver cirrhosis. In another study, miR-122 was found to be down-regulated in 10 of 20 hepatocellular carcinomas compared with paired normal liver tissue.[67]

Lung Carcinoma. Volinia et al[40] analyzed the expression of 228 miRNAs in lung cancer and identified 35 miRNAs that are up-regulated and three that are down-regulated compared with normal lung tissues. Unique miRNA signatures were also found for different histological types of lung cancer.[68] Two miRNAs have been implicated as prognostic markers in lung cancer. In a study of 143 non-small cell lung carcinoma specimens, 105 adenocarcinomas, and 25 squamous cell carcinomas, Takamizawa et al[69] found association of low *let-7* expression level with shorter postoperative survival. In addition, high levels of miR-155 is associated with poor survival by multivariate analysis.[68] In small cell lung cancer cell lines, Hayashita et al[70] showed that the miR-17-92 cluster was overexpressed. *In vitro* study demonstrated overexpression of miR-17-92 led to enhanced cancer cell proliferation.[70]

Pancreatic Endocrine and Acinar Tumors. Twelve nontumor pancreas and 44 pancreatic tumors, including 12 insulinomas, 28 nonfunctional endocrine tumors, and four acinar tumors, were subjected to miRNA array analysis.[71] Pancreatic tumors, regardless of histological types, could be distinguished from normal pancreas by the overexpression of miR-103 and miR-107 and the lack of miR-155. Acinar pancreatic carcinoma can be separated from endocrine tumors by a set of 10 miRNAs. miR-204 is preferentially expressed in insulinomas. miR-21 overexpression is associated with high Ki-67 proliferation index and the presence of hepatic metastasis.

Because overexpression of miR-155 is frequently seen in other tumor types, miR-155 down-regulation in pancreatic tumors seems rather unusual. Further experiments are necessary to confirm this observation and address its pathogenetic significance.

Testicular Germ Cell Tumor. Voorhoeve et al[72] reported that miR-372 and miR-373 participate in testicular germ cell tumor development. They showed that miR-372 and miR-373 collaborate with RAS^{V12} in tumorigenesis. One of the potential oncogenic mechanisms of miR-372 and miR-373 is by inactivating a tumor suppressor called "large tumor suppressor homolog 2."

Thyroid Cancer. By comparing the miRNA expression patterns in the papillary thyroid cancer and the nonneoplastic thyroid tissue with a miRNA microarray containing 460 known miRNAs, He et al[73] identified a miRNA expression signature consisting of 23 miRNAs, of which 17 showed overexpression between 1.5- to 19.3-fold, and six showed less than twofold reduction in expression. Five overexpressed miRNAs were sufficient to predict cancer status, and the three most overexpressed miRNAs, miR-146, miR-221, and miR-222, were associated with the down-regulation of *KIT*,[73] which was validated in a separate study as a target for miR-221 and miR-222.[74] miRNA profiling of follicular thyroid carcinoma, on the other hand, identified only two overex-

pressed miRNAs, miR-197 and miR-346.[75] Thus, a unique set of miRNAs is deregulated in papillary thyroid cancer and follicular thyroid carcinoma, suggesting that different pathways are involved in their pathogenesis.

miRNAs in Cancer Classification and Prognosis

The existence of unique miRNA expression profiles for different tumor histological types and subtypes, due largely to the tissue specificity of miRNAs, should be a useful adjunct in classification of tumors that otherwise cannot be accurately diagnosed by morphology and immunophenotype. Using miRNA expression profile methods, Lu et al[39] were able to correctly classify 12 of 17 poorly differentiated carcinomas. In contrast, an mRNA-based method only correctly classified one of those carcinomas.

There is emerging evidence that miRNAs can be used for cancer prognosis. Because reduced expression of *let-7* and high expression of miR-155 in human lung cancers was associated with poor survival, these miRNAs may be used to identify those patients for closer monitoring and adjunct therapy.[69] A unique miRNA expression profile consisting of 13 miRNAs correlates with the two prognostic subgroups of CLL as defined by somatic hypermutation status and ZAP70 expression.[50] Two of these 13 miRNAs were confirmed to be differentially expressed in an independent study.[51] These miRNAs or a subset of them may be used as an adjunct to the present molecular markers for the stratification of prognostic subgroups.

Conclusions

Although miRNAs are undoubtedly key players in oncogenesis, much more work needs to be done before we can dissect the contribution of each of the miRNA in tumorigenesis. This requires sensitive quantitation of miRNA expression and identification of miRNA target genes. The latter should go beyond computer prediction and will require careful examination of the ability of the miRNA in question for specific interaction and to regulate the endogenous target genes. Characterizing the many potential targets of a miRNA is not a trivial undertaking, and it will be a while before all of the potential targets of a miRNA are identified and studied with respect to their role in oncogenesis.

How is the discovery of miRNAs going to improve the practice of pathology? We have seen the potential use of miRNAs in diagnosis, classification, and prognosis of tumors. Because miRNAs are frequently differentially expressed between normal and cancerous tissues, a precise panel of miRNAs can be used to differentiate between malignant and reactive lesions when such a distinction cannot be ascertained by conventional means. A similar panel of miRNAs may also be useful for discriminating between tumors of different histiogenic origins or between histological subtypes of a certain tumor type. Because the number of miRNAs is much lower compared with the messenger RNAs, miRNA profiling may be superior to conventional cDNA microarray for use as an adjunct in this type of classification. In addition, miRNAs may serve as useful prognostic biomarkers. A short panel of miRNAs can potentially be used to predict clinical behavior of the tumor, for example, predilection for lymph node or distant metastasis, or risk of relapse.

Here, we have stressed the impact that miRNA profiling might have on cancer diagnostics. However, it is very likely that similar applications will be discovered in diagnosis of other diseases, both infectious and metabolic. This is an exciting and emerging field that will have great impact on both diagnosis and treatment. Currently, a wide variety of methods are used to analyze these RNAs, and it may be wise to exhibit caution in extrapolating results obtained in different manners. Hopefully, standardized methods will become generally accepted and practiced, which would facilitate direct comparisons between studies and tests.

Finally, because miRNAs are small in size and are likely to modulate multiple oncogenic pathways, they serve as potential effective targets for cancer therapy. Antagomirs, chemically engineered antisense nucleotides for miRNAs, can be used *in vivo* to silence specific endogenous miRNA.[76] This may represent a therapeutic strategy to silence overexpressed miRNAs in human cancer. Other strategies aiming to correct the abnormal expression of miRNAs associated with cancer are likely to be developed in the future.

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