MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis

James V. Tricoli and James W. Jacobson

Diagnostic Biomarkers and Technology Branch, Cancer Diagnosis Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Rockville, Maryland

Introduction

The Cancer Diagnosis Program of the National Cancer Institute sponsored a workshop entitled “MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis” in Rockville, Maryland on November 28 and 29, 2006. The purpose of this workshop was to bring together leaders in the microRNA (miRNA) field to discuss the potential for the application of miRNAs to translational research, with the ultimate goal of applying them as markers for cancer diagnosis. miRNAs are small (22–25 nucleotides in length) noncoding RNAs that can effectively reduce the translation of target mRNAs by binding to their 3′ untranslated region (UTR). This occurs through the assembly of an RNA-induced silencing complex composed of a variety of proteins including Argonaute. If the homology between the miRNA sequence and the target 3′-UTR is incomplete, then this complex reduces expression by blocking translation. If, however, the homology is complete, then degradation of the target miRNA can result. To date, more than 300 distinct human miRNAs capable of targeting thousand of genes have been identified.

Since their discovery, investigations into the function and role of miRNAs have revolutionized studies in molecular biology, particularly in the field of development. A variety of studies have shown the ability of individual miRNAs to regulate oncogene and tumor suppressor gene expression and others have shown that miRNA gene loss or mutation can contribute to tumorigenesis. miRNA expression patterns (or signatures) are now known to characterize the developmental origins of tumors more effectively than mRNA expression signatures and may provide a useful tool for the diagnosis and prognosis of human cancer. Several approaches have been developed to block the function of miRNAs resulting in the inhibition of their oncogenic effects. These accomplishments have revealed a potential for miRNA to be used as a clinical tool in cancer diagnosis and as a target for therapy. To address these issues, the workshop was divided into four sections featuring a total of 14 speakers. Dr. Paul Meltzer (National Cancer Institute, Bethesda, MD) served as chair for the workshop and began by discussing the recent effect of miRNA on translational research.

Dr. Meltzer highlighted several questions about miRNAs that need to be addressed, including how we will know when miRNA discovery is complete, how to verify miRNA tissue specificity, the effect of redundancy between closely related miRNAs, and the influence of polymorphisms in miRNAs and their targets. In addition, technical issues were brought up, including biospecimen requirements, technology platforms, data normalization, the effect of contaminating cells, and standardization of data reporting. [Cancer Res 2007;67(10):4553–5]

miRNA Gene Structure and Function

The ability to apply miRNAs to the diagnostic and therapeutic arenas stems from our basic scientific knowledge of miRNA gene regulation and function. The first two speakers, Dr. Michael T. McManus (University of California San Francisco, San Francisco, CA) and Dr. Bryant R. Cullen (Duke University, Durham, NC), provided an overview of the generation and functional role of miRNAs in the cell. Dr. McManus discussed the role of miRNAs in gene regulation and development. He described how Dicer is essential to proper miRNA processing and that Dicer mutations in mice are lethal at day 7.5 of development. He also pointed out that mutations in Dicer can result in effects other than the build-up of unprocessed miRNAs, including problems in chromosome segregation. Dr. Cullen discussed viral miRNAs and RNA interference. miRNAs have now been identified in several DNA viruses including every herpesvirus analyzed. Dr. Cullen explained that transforming herpesviruses express numerous viral miRNAs in latently infected transformed cells including EBV (23 miRNAs) and KSHV (12 miRNAs). He presented recent data showing that each of these miRNAs down-regulates a distinct and unique set of cellular mRNA when expressed in human B cells. These results suggest that viral miRNAs likely play a key role in the ability of some oncogenic viruses to transform human cells in vivo.

miRNA Expression and Cancer Signatures

The expression of individual miRNAs and miRNA signatures have now been linked to the diagnosis and prognosis of a number of human cancers. These include chronic lymphocytic leukemia, chronic myelogenous leukemia, and prostate, testicular, lung, breast, ovarian, pancreatic, and gastric cancers. Dr. Carlo M. Croce (The Ohio State Comprehensive Cancer Center, Columbus, OH) discussed miRNA signatures in B-cell lymphomas and provided data illustrating that many miRNA genes are located at fragile sites within the human genome. Two of these, mir-15a and mir-16-1, are located at the fragile site 13q14.3 and, along with 11 other miRNAs, show an association with disease progression in human CLL. Dr. Croce explained that homozygous deletion of mir-15a and mir-16-1 can lead to overexpression of the targeted miRNAs resulting in tumor proliferation and invasion. Dr. Scott Hammond (University of North Carolina at Chapel Hill, Chapel Hill, NC) discussed miRNAs as oncogenes and tumor suppressors and described his investigation of miRNA expression patterns in mammalian development and in cancer. He provided evidence that many miRNAs are down-regulated in some tumor cell lines due to reduced maturation during miRNA biogenesis. A number of miRNA genes are overexpressed in tumor cell lines and primary tumors. Seven of these cancer-associated miRNAs are clustered in a single primary transcript termed the miR-17 cluster (OncomiR-1) that is
located in a region amplified in lymphoma and several solid malignancies. Ectopic expression of these in a mouse model of lymphoma resulted in accelerated disease progression, reduced apoptosis, and more tumor dissemination. This work establishes miRNAs as oncogenes in human cancers and thus candidates for cancer marker development and targets of therapy. Dr. Lin Zhang (University of Pennsylvania School of Medicine, Philadelphia, PA) discussed miRNA gene copy number alterations in human epithelial cancers. Dr. Zhang cited work by Dr. Croce, which showed that 52% of miRNA genes reside in genomic regions that are altered in cancer. Dr. Zhang presented data confirming this result using an array-based comparative genomic hybridization study of 227 human tumors. Of the 283 known miRNA genes analyzed using high-resolution array-based comparative genomic hybridization, a large proportion exhibit DNA copy number alterations in ovarian (37%) and breast (73%) cancers and melanoma (86%). These findings support the notion that copy number alterations of miRNA genes are highly prevalent in cancer, may account partly for the frequent miRNA gene deregulation, and could be used as diagnostic cancer markers. Dr. Jun Lu (The Broad Institute of MIT and Harvard, Cambridge, MA) discussed miRNA profiles and the classification of human cancer. Dr. Lu described a bead-based miRNA profiling method that allows good detection fidelity, linearity, specificity, and reproducibility. He also discussed, using miRNA expression profiling to develop novel cancer diagnostics, understand cancer mechanisms, and assist in cancer therapeutic discovery. In a human cancer cohort, miRNA expression profiles distinguish the lineage of solid tumors, raising the prospect of using miRNAs to classify poorly differentiated cancer specimens. Dr. Lu presented data on changes in miRNA profiles of MCF-7 cells in response to histone deacetylase inhibitors and heat shock protein inhibitors and showed that the miRNA expression patterns are reflective of the mechanism of action of these drugs.

**Diagnostic and Treatment Potential of miRNAs in Cancer**

Recent evidence from translational studies suggests that miRNA signatures may be useful in categorizing, detecting, and predicting the course of an increasing number of human cancers. In addition, mechanistic studies on the role of miRNAs in oncogenesis and tumor progression have suggested therapeutic strategic targeting of miRNAs and miRNA regulated pathways. Dr. Joshua Mendell (Johns Hopkins University, Baltimore, MD) discussed c-Myc–regulated miRNAs in cellular transformation and tumorigenesis. Dr. Mendell presented data showing that c-Myc activates expression of a group of six miRNAs on human chromosome 13, known as the miR-17 cluster (OncomiR-1). Chromatin immunoprecipitation showed that c-Myc binds directly to this locus to activate transcription of these miRNAs. Dr. Mendell also showed that this group of miRNAs is widely overexpressed in human cancers and can promote tumorigenesis in animal models. He presented new data showing that miRNA stability and subcellular localization can be regulated in a sequence–specified manner. Every studied miRNA, including miR-29a, is predominantly cytoplasmic in cycling cells. Fractionation and *in situ* hybridization experiments show that miR-29b is partially localized to the nucleus and that a unique hexanucleotide terminal motif of miR-29b is a transferable element that specifies nuclear import and rapid decay. Dr. Mendell’s findings suggest that related miRNAs, generally believed to be redundant, may have distinct functions due to the presence of *cis*-acting regulatory motifs. Dr. Jun S. Wei (National Cancer Institute, Bethesda, MD) discussed the diagnostic and treatment potential of miRNAs. Dr. Wei explored the role of miRNAs in pediatric malignancies, profiling miRNA expression in a panel of 58 pediatric tumor xenografts and cell lines using in-house developed miRNA microarrays. The expression profiles of miRNAs showed distinct signatures for each tumor category. He presented data that showed consistency between microarray and real-time reverse transcription-PCR results that confirmed the expression of these cancer-specific miRNAs in corresponding clinical tumor samples for the neuroblastoma-specific and rhabdomyosarcoma-specific miRNAs. Dr. Wei described experiments to identify the targets of tumor-specific miRNAs by performing a parallel gene expression profiling study on the same set of xenograft samples correlating expression of miRNAs and mRNAs. The resulting data matrix will facilitate the identification of genes regulated by miRNA that may be potential targets for miRNA-based cancer therapies. Dr. Thomas Tuschl (Rockefeller University, New York, NY) discussed miRNA discovery and regulation using small RNA cloning approaches. Dr. Tuschl described the inhibition of miRNAs using antisense RNAs called antagonisms that target specific miRNAs. Antagonisms are stabilized by a 2′-O-methyl modification of the ribose sugar moiety that renders them highly resistant to nucleases and provide a high level of thermodynamic duplex stability. Dr. Tuschl showed that targeting miR-122 in the mouse liver using an anti-miR-122 antagonist resulted in complete degradation of miR-122 that is dose dependent. The antagonisms are highly specific and long lasting, with little or no short-term toxicity. Gene expression studies showed that the inhibition of miR-122 resulted in alterations in the level of numerous genes including those involved in cholesterol biosynthesis. These studies suggest that targeting specific miRNAs for the purpose of achieving a therapeutic benefit may be possible using antagonisms. Dr. Thomas Schmittgen (Ohio State University Comprehensive Cancer Center, Columbus, OH) discussed PCR-based miRNA expression profiling in pancreatic cancer. Dr. Schmittgen used a unique real-time PCR method to identify a large group of miRNAs that can distinguish pancreatic tumors from normal or benign pancreas. He provided evidence that real-time PCR is a superior method for miRNA expression analysis due to the sensitivity and specificity of the PCR. The expression signature correctly classified 28 of 28 pancreatic tumors, 11 of 15 adjacent benign tissues, and 6 of 6 normal pancreatic tissues. Interestingly, the majority of the differentially expressed miRNAs were increased in the pancreatic tumors, suggesting that they may be good therapeutic targets for antisense oligonucleotides or as markers to diagnose pancreatic and possibly other forms of cancer.

**Future Directions and Clinical Implications**

There are many issues to consider when validating a cancer marker or signature for use in the clinic. It is essential that we apply the lessons learned from gene expression and proteomic profiling to the emerging studies using miRNAs so as not to repeat previous errors in data analysis and signature validation. Dr. Cheryl Willman (University of New Mexico, Albuquerque, NM) discussed the lessons learned from gene expression profiling studies. Dr. Willman identified a number of key issues that need to be addressed to achieve meaningful and reproducible results in miRNA gene expression array studies. These include a well-defined clinical question, a statistically valid experimental design, selection
of highly characterized cases appropriate to the question and representative of the population, consideration of tumor heterogeneity, identification of normal controls, a robust platform, and robust statistical and computational analysis of diagnostics and predictors with independent validation. Dr. Willman pointed out that the results of any array study are dependent on case inclusion and statistical design, and may or may not be intrinsically stable or readily generalized. In addition, gene selection can markedly influence presentation of the data and has the potential to introduce bias. Finally, results must be fully validated on independent data sets. Dr. Kevin Dobbin (National Cancer Institute, Bethesda, MD) discussed the statistical similarities and differences between mRNA and miRNA array studies. Dr. Dobbin pointed out that there are typically far fewer features represented on miRNA arrays than on mRNA arrays. This makes array normalization more problematic. The normalization methods commonly used for mRNA arrays, based on median centering or lowess normalization, may not be adequate for miRNA arrays. There are also drawbacks to alternative exogenous or endogenous control-based methods used for normalization in miRNA studies. Methods for determining the number of arrays required for an mRNA experiment have been developed and, with minor modifications, can be applied validly to miRNA experiments as well. Dr. Dobbin provided an example of how preselecting "informative" genes to use in a cluster analysis produces bias that obscures the true structure in the data. The resulting cluster dendrogram can display significant structure even when none exists. Finally, more general issues related to designing reproducible and relevant studies (patient selection and potential clinical treatment decisions that may be affected) and what constitutes adequate study validation (internal versus external validation) were discussed, including the risk of inadequate cross-validation and how this risk can be reduced by using appropriate statistical software. Dr. Hui Wang (Agilent Technologies, Santa Clara, CA) discussed direct miRNA profiling from low-input total RNA. Dr. Wang stated that accurate miRNA measurements are challenging due to the large dynamic range of miRNA expression, high miRNA sequence homology, and the lack of consensus on normalization methods. She described studies of miRNA profiling directly from low-input total RNA (100 ng) without size-fractionation or amplification using a novel and highly efficient RNA labeling method and a unique probe design. To optimize the sequence specificity of the probe-target interactions, all probes on the microarray are empirically selected to have matched probe-target melting temperature (Tm). The Tm-balanced probes are generally capable of single-nucleotide discrimination. Dr. Wang provided an example from the human let-7 family of miRNAs where cross-hybridization of >3% was observed in only 4 of 56 potential cross-hybridization events. This probe design strategy has enabled the design of probes for all of the human miRNA sequences in the Sanger database. The larger dynamic range and the specificity of the assay offer a robust method for miRNA profiling. Dr. David Brown (Asuragen Corporation, Austin, TX) discussed methods and applications for miRNA expression analysis. Dr. Brown described a miRNA array platform that facilitates the quantitative analysis of more than 15,000 validated and predicted miRNAs. The platform is being used to identify miRNAs signatures that correlate with patient prognosis or response to therapy. This array platform can be used to evaluate formalin-fixed paraffin-embedded samples from cancer patients. He stated that the expression levels of as few as two miRNAs can be used to distinguish tumor, chronic pancreatitis, and normal pancreatic tissue samples. The presence of miRNAs in clinical samples such as formalin-fixed paraffin-embedded tissues, serum, plasma, urine, and saliva, as well as the strong correlation between miRNA expression and disease state and patient prognosis, suggests that miRNAs may ultimately prove to be valuable cancer diagnostic analytes.

The Future of miRNAs in Cancer Diagnosis and Treatment

The usefulness of miRNAs for the detection, diagnosis, prognosis, and possible treatment of human cancer will depend on carefully designed translational studies. In addition, it will require careful consideration of the best methods for sample collection, miRNA isolation, miRNA quantitation, and data analysis. To facilitate this, we need to gain a better understanding of specific miRNA characteristics, such as how targeting of multiple miRNAs by a single miRNA affects data interpretation in marker studies and the effect of miRNA isoforms on diagnostic utility. In the therapeutic arena, there is a requirement to target the correct miRNA sites without affecting miRNA targets of similar sequence. Whereas, as presented by Dr. Tuschl, early mouse studies using antagomirs show promise, actual treatment implementation in humans will likely present many unforeseen challenges. The miRNA workshop provided a timely opportunity to address these and other issues and to form a framework of understanding upon which future studies can be conducted.

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