MicroRNA Analysis: Is It Ready for Prime Time?

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Since their discovery, microRNAs (miRNAs)20 have shown great promise in a wide array of clinical applications. In some cases, the use of miRNAs as new diagnostic markers might help answer diagnostic dilemmas that gene expression analyses or other types of analyses have not been able to satisfactorily address. In other instances, it is easy to perceive certain miRNAs as targets for novel therapies that downregulate an entire pathway via the targeting of a single miRNA. When and if this concept will come to fruition remain unclear. Scientists agree that this field of biology is exciting, offers much promise, and has numerous advantages, compared with experiences with other biomolecules. In this Q&A, 5 individuals with extensive miRNA experience share their vision for where the miRNA field is heading and whether we, in the clinical laboratory, will experience its implications soon.

What are the most important characteristics of miRNAs that increase their potential as novel diagnostic or therapeutic targets?

George Calin: One important characteristic of miRNAs that makes them an exciting potential biomarker for diagnosis and/or a target for therapy is the fact that specific miRNAs target multiple components from the same pathway. For example, the miR-15a/16–1 cluster targets genes from the apoptotic pathway: BCL221 (B-cell CLL/lymphoma 2) and MCL1 [myeloid cell leukemia sequence 1 (BCL2-related)]. Any given miRNA can regulate numerous genes, and each gene can be regulated by different miRNAs.

Pierre Cordelier: In cancer research, miRNAs can differentiate normal from cancerous tissues and, more importantly, can discriminate different subtypes of cancer. The high stability of miRNAs in tissues and fluids is another key advantage that increases their potential as diagnostic markers over messenger RNA (mRNA). In addition, they can be quantified in very low amounts of material and in highly degraded samples. This is of prime importance to support their possible use as emerging biomarkers at the clinical level. It is also now well established that miRNAs target multiple miRNAs with high efficacy. Targeting (or restoring) a single miRNA would allow the correction of a complete pathway, in comparison with approaches targeting (or restoring) a single gene. This is particularly important to avoid tumor cell clonality, for instance, and makes miRNAs very appealing therapeutic agents.
Carlo Croce: Significant progress has been made in the genetic and epigenetic causes of cancer and therefore in the evaluation of targets for therapy, but the identification of additional alterations that cause or contribute to malignancy remains a high priority. In this regard, we have learned that miRNAs are highly modulated in tumors compared to normal tissues, and, more importantly, their expression is related to clinicobiological features of the neoplastic tissues. We have demonstrated that miRNA expression can be used to stratify patients for further therapies and that miRNA expression can also be useful in detecting the tissue of origin for cancers of unknown primary origin. The recent discovery that miRNAs can be present and measured in serum/plasma with concentrations reflecting pathological states, such as diabetes, lymphomas, and several solid cancers, represents a new approach for diagnostic screening. In this context, it is obvious to think that miRNAs can also be interesting therapeutic targets because of their target multiplicity: Each miRNA is able to repress at the same time the expression of hundreds of different genes. If a particular miRNA is linked to a tumor, this tumor will be completely dependent on its expression. Therefore, the inhibition of this particular miRNA can remove the block against the expression of many therapeutic proteins. Conversely, administration of an miRNA mimic can boost the endogenous miRNA population, thus repressing the detrimental genes.

Federico Monzon: There are 2 very important characteristics of miRNAs. First, miRNAs are small RNA molecules that are resistant to RNases and thus are well preserved in formalin-fixed, paraffin-embedded tissues. This inherent stability, therefore, makes them excellent candidates as biomarkers that can be used in the workup of routine diagnostic pathology specimens. Second, miRNA gene expression regulators are preserved in cell lineages and commonly deregulated in cancer. Thus, they have the potential to be used for diagnostics by detecting the up- or downregulation of miRNAs in tissues or body fluids.

Anna Szafranska-Schwarzbach: Perhaps the most noteworthy characteristic of miRNAs is the effect that miRNAs have on the regulation of cellular function, signaling networks, and biological processes. A single miRNA can regulate the expression levels of multiple mRNA targets; at the same time, many miRNAs can bind to one target mRNA. The targeted miRNAs in turn may cause major regulatory changes in the cell. This complex pattern of miRNA-driven regulation can lead to profound changes in cellular phenotypes even more pronounced than those triggered by modulation of an individual mRNA. As a result, in many cases the change in the expression of miRNAs seen in benign compared to diseased specimens may be a better indicator of a disease state than mRNAs by providing simpler profiles with more favorable technical characteristics.

The small size of miRNAs combined with their ability to target gene networks at multiple levels, via either promotion or suppression of tumorigenesis and metastasis, have resulted in extensive interrogation of miRNAs as potential drug targets. Approaches, such as synthetic antagonomers, have been designed to abolish or decrease miRNA activity, while miRNA mimetics have been designed to restore or increase the activity of miRNAs. These research strategies used to modulate the expression of deregulated miRNAs, in combination with the recent demonstration of effective and safe delivery systems in rodents, offer a glimpse at the promise of miRNA-based therapies for cancer treatment.

Which technology will most likely succeed in the clinical laboratory setting for analysis of miRNAs, and why?

George Calin: Quantitative real-time reverse-transcriptase PCR (qPCR) is easily performed in a clinical laboratory setting and produces data that are easier to analyze than those of gene expression arrays and deep sequencing; it is also more reproducible. This technology offers high throughput, since samples can be analyzed in the 384-well format, making it relatively inexpensive.

Pierre Cordelier: I agree qPCR surpasses microarray strategies for miRNA quantification. Indeed, qPCR is an extremely sensitive and accurate method, while being relatively inexpensive. On the other hand, in situ hybridization, which is technically challenging, may
provide additional information of miRNA expression at the cellular level.

**Carlo Croce:** miRNA qPCR assays and miRNA in situ hybridization have shown great versatility in miRNA research and have demonstrated their ability to detect miRNA expression in a very specific and sensitive way. I believe that they will be powerful tools for miRNA diagnostics and clinical research.

**Federico Monzon:** As indicated by others, most likely qPCR is better, due to the fact that it is already deployed in a large number of diagnostic laboratories. However, other technologies, such as NanoString (NanoString Technologies), could be more successful at developing diagnostic assays based on miRNA profiles, due to the ability to detect multiple miRNAs in a single assay.

**Anna Szafranska-Schwarzbach:** In the near term, qPCR-based assays have great potential to continue to advance the transition from the “bench to the bedside,” especially for the analysis of small panels of miRNAs. qPCR is a well-established, robust, and reproducible technology, with a number of key advantages, including its high sensitivity and specificity, potential for target multiplexing, and low RNA input requirement—all of which facilitate expression analyses, even in clinical specimens with a limited amount of material. A validation of a qPCR-based laboratory-developed test (LDT) is far less complex than for a test based on other platforms, such as microarrays or next-generation sequencing. This simplifies the effort needed to maintain compliance with CLIA and the College of American Pathologists regulations for the demonstration of accuracy, precision, sensitivity, and specificity. In the future, as miRNA panels become larger and more complex, it is probable that these panels will need to migrate to other platforms with high-throughput capabilities. It will be interesting to see the role that new technologies, such as next-generation sequencing and miRNA sequencing, will play in miRNA clinical testing.

**Is there currently enough evidence to support the use of miRNAs in clinical testing?**

**George Calin:** There is a plethora of data to support the clinical use of miRNAs. There are several mouse models (miR-155, miR-21, miR-15a/16), a huge amount of genomics data, and a very large number of functional studies. The number of translational studies has increased exponentially as well.

**Pierre Cordelier:** The discoveries in the field of miRNA research are very encouraging and promising. Recently, the first miRNA-targeted drug entered phase 2 clinical trials to treat patients infected with hepatitis C virus. It is tempting to speculate that miRNAs will be used routinely in clinical testing (as biomarkers and/or targets) in the near future.

**Carlo Croce:** In the past 8 years, 16 000 research publications on miRNAs were reported, and one point is perfectly clear: miRNAs are involved in every aspect of tumor biology. Both my and other laboratories have demonstrated that miRNAs can be used as a predictor of tumor progression, metastatic behavior, and therapeutic responsiveness to actual chemotherapy. Examples in this regard include the loss of miR-181 during the progression of chronic lymphoblastic leukemia, the upmodulation of miR-335 and miR-126 in metastatic breast cancer, the upmodulation of miR-221/222 in hormone-resistant breast cancer cells, and the dependence of lymphomas on miR-21. What we are still missing are more-comprehensive analyses of miRNA expression in very large cohorts of patient specimens to validate all the findings that we have accumulated in these years of bench and clinical research. This is the key area where we have to focus our efforts so that miRNAs can become a new tool in clinical and diagnostic medicine, since they can reduce both false-positive and false-negative results produced by the conventional diagnostic method.

**Federico Monzon:** No. The use of miRNAs as diagnostic or prognostic biomarkers has been extensively studied, but no application has been proven to have adequate clinical performance. They have been used for determination of tissue of origin in metastatic tumors, but the assay has not been validated in a large study. Another marketed application is the distinction between squamous and adenocarcinoma of the lung, but the assay has not been validated in a large study. Another marketed application is the distinction between squamous and adenocarcinoma of the lung, but the clinical value of using these assays compared to routine histopathology/immunohistochemistry has not been shown.

**Anna Szafranska-Schwarzbach:** Evidence is rapidly mounting to support the use of miRNAs in clinical testing. The miRNA diagnostics field continues to identify promising miRNA candidates, and these biomarkers are increasingly used to generate robust and reproducible tests for use in patient treatment decisions. At Asuragen, we developed and validated the miRInform® Pancreas LDT, which is based on the differential expression of miR-196a and miR-217. This test aids in discrimination of chronic pancreatitis from pancreatic ductal adenocarcinoma in formalin-fixed, paraffin-embedded specimens. We are currently finalizing the clinical validation of an assay based on the expression of 7 miRNAs for use in patients with benign, inconclusive, and nondiagnostic endoscopic ultrasound fine-
needle aspirate cytology results. In addition, we are in the process of validating a 9-miRNA signature in pancreatic cyst fluid specimens that can reliably differentiate patients with high-grade intraductal papillary mucinous neoplasms and other pancreatic cystic entities that require surgical intervention from those patients with low-grade intraductal papillary mucinous neoplasms, who can be treated more conservatively. A handful of other diagnostic assays have been developed or are being developed by us and others for a variety of diagnostic applications.

miRNAs are emerging as promising biomarkers for personalized medicine. One noteworthy example is a single-miRNA assay based on miR-210, which was recently reported to predict response to tamoxifen in breast cancer patients with an accuracy comparable to that of the Oncotype DX® assay (Genomic Health). The miRNA field will look very different in a couple of years after some of these promising findings are verified in large-scale clinical trials and implemented in routine clinical testing.

**Which area of miRNA research do you focus on, and how may it impact the use of miRNAs in the clinical lab?**

**George Calin:** My laboratory is involved with many different types of research using miRNAs and long noncoding RNAs. Our projects have focused on discovery efforts, genomics, and functional tests, with the goal of translating this information into something useful for the patient.

**Pierre Cordelier:** We are currently studying the expression and role of miRNAs in pancreatic adenocarcinoma. We have found that miRNAs can be used as prognostic and predictive biomarkers and as therapeutic targets to help alleviate the dismal prognosis of this disease.

**Carlo Croce:** My laboratory focuses on identifying the specific role of cancer-related miRNAs in the initiation and progression of malignancies. With the use of different high-throughput technologies, we have identified the specific miRNA signature, “miRNome,” of several different solid tumors and hematopoietic malignancies, including breast, lung, stomach, pancreas, and liver cancers: chronic lymphoblastic leukemia; acute lymphoblastic leukemia; and acute myeloid leukemia. In the second phase of our miRNA challenge, we are more involved in defining the molecular role of the differentially expressed miRNAs in tumors by trying to clarify whether the altered miRNA expression is a cause or consequence of malignant transformation. To this aim, we have established several different miRNA mouse models that have allowed us to show that miRNAs can be the cause of the malignant transformation and metastatic spread and that tumors strongly depend on the miRNAs’ expression. We are also trying to define the molecular pathways directly modulated and controlled by miRNAs so we can identify other potential therapeutic proteins that can be targeted through standard pharmacologic approaches. Given the apparently critical roles of miRNAs in every aspect of tumor biology, our experiments will be crucial for enabling a more thorough comprehension of miRNA biology in tumorigenesis that one day can lead to improved diagnosis and treatment of several human malignancies.

**Federico Monzon:** We are working on developing highly sensitive assays to detect lung cancer–associated miRNAs in sputum or bronchoalveolar lavage specimens. If successful, this would allow the development of a noninvasive or minimally invasive diagnostic assay for patients at high risk of developing lung cancer.

**Anna Szafranska-Schwarzbach:** Our group has been focused on using miRNAs as diagnostic analytes to address some of the unmet clinical needs, with particular attention on oncology applications such as pancreatic cancer. Our efforts have been directed at the identification of miRNAs that are indicative of different types of pancreatic cancer and disease-specific signatures. Our goal was to apply these miRNA signatures to help resolve clinical dilemmas in solid and cystic pancreatic lesions. In an effort to make the tests more accessible, we validated candidate biomarkers in compliance with CLIA and College of American Pathologists regulations for use as an LDT in our CLIA laboratory. These tests have the potential to improve the accuracy of tools that are currently used for preoperative diagnosis and provide the opportunity for earlier and more appropriate treatment intervention.

**Is it premature to think that we could have US Food and Drug Administration (FDA)-approved kit-based assays for miRNA analysis in the near future?**

**George Calin:** It is not premature. There are plenty of published data proving that miRNAs are better markers in some instances than proteins or mRNA (identifying cancer of unknown primary is only 1 important example). One of the detracting elements of miRNA analysis is the fact that qPCR is cheap and companies may not be willing to invest in taking such an assay to the FDA, because their return on investment may not be great. Instead, industry is focusing on more advanced technologies, such as deep sequencing, that may be more lucrative. It is interesting that although
90% of miRNA studies in cancer research were done in the US, the most advanced clinical trials on miRNAs are ongoing in Europe.

Pierre Cordelier: Aside from their potential as biomarkers, miRNAs will soon be used in “companion diagnostic kits” to predict response to targeted therapies. As a consequence, I believe that FDA-approved kit-based assays for miRNA analysis will emerge in the near future.

Carlo Croce: Based on what has been said, I think that it is not premature to have an miRNA-based kit as a diagnostic and prognostic tool. We need more experiments that will define clear miRNA signatures for all the different malignancies. We can imagine a set of miRNAs that can discriminate the normal tissues from tumors, give information on the nature of this tumor, its aggressiveness, and its responsiveness to actual therapeutic drugs. A “MicroPrint” that can provide individualized risk assessment for patients and allow doctors to tailor treatment protocols related to patients’ needs.

Federico Monzon: It is very likely that there will be FDA-approved assays. As mentioned above, miRNAs are very stable biomarkers. It is foreseeable that miRNA profiling will eventually become a reliable assay for specific tumor types or other diseases.

Anna Szafranska-Schwarzbach: miRNAs will continue to be introduced into the clinical market as LDTs but will eventually result in FDA-approved kit products. The CLIA-based approach has many advantages, which include raising physicians’ awareness and increasing their comfort level with miRNAs as diagnostic tools. From a laboratory standpoint, an LDT allows refinement of the testing procedures based on improved understanding of the technical and clinical practicalities of miRNA testing before embarking on the path of kit development. However, the principles and procedures for diagnostic-Kit manufacturing will likely become more widely adopted for the development of LDTs and the manufacture of test components and reagents. In the next 2 years, as the market matures and begins to adopt miRNAs as routine diagnostic analytes, miRNA-based LDTs should advance toward in vitro diagnostic-kit formats. This trend will likely be led by oncology-related products and, over time, extended to other clinical applications as well as companion diagnostics.

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