microRNAs entice as diagnostic key to multiple diseases

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June 2014—In research and development of diagnostics based on the small, non-coding RNAs known as microRNA, the potential clinical applications in cancer were the first to be explored and have hogged the spotlight. But the more light that is shed on microRNAs' mysteries, the more promise microRNA shows as a diagnostic and therapeutic tool in an array of diseases beyond cancer.

The numbers hint at the scope and magnitude of microRNAs' possible roles. More than 1,000 microRNAs are predicted to exist in the human genome, with each one potentially targeting hundreds of messenger RNAs. Therapies using microRNA to suppress hepatitis C viruses are already in human trials, while basic research in not only liver disease but also diabetes, cardiovascular disease, and neurodegenerative diseases, among others, is laying the groundwork for many more clinical trials within the next five to 10 years.

"The cancer field is ahead of us. But microRNAs are not only important in cancer," says Walter Lukiw, PhD, professor of neurology, neuroscience, and ophthalmology at the Louisiana State University Neuroscience Center. "MicroRNAs are ubiquitous and are proving to have huge effects in development, aging, and health and disease."

Demonstration of this fact has led to a tremendous acceleration of microRNA research, says Eric N. Olson, PhD, professor and chair of molecular biology at the University of Texas Southwestern Medical Center in Dallas. A key research interest in his laboratory is the molecular basis of heart development and disease—studies of how the heart forms, functions, and dysfunctions. The laboratory chose to zero in on microRNAs when it became apparent that they were regulated in many types of disease processes and play a crucial role in cardiovascular disease. (Small EM, et al. *Circulation.* 2010;121:1022–1032.)

He finds microRNAs particularly fascinating as biomarkers of disease. "They are secreted from cells, they circulate in all bodily fluids including blood, and you can diagnose various disorders in the cardiovascular system by the presence of microRNAs in the bloodstream," he says.

Dr. Olson describes microRNA as a kind of "dimmer switch" for cells. "They dial down gradually the expression of many different proteins in a cell, and it's the combined, gentle effect of the microRNA across many different proteins that ultimately influences how the cell behaves in response to a disease." The things that go awry in a cell that cause disease are controlled by microRNAs, he adds. "So if you can modulate the microRNA, you can modulate the disease."

Dr. Olson

At his medical center, research has indicated that microRNAs are not merely involved in the cardiovascular disease process but are actually front and center, Dr. Olson says. "We identified a collection of microRNAs in rodents and humans that changed in abundance during the progression of heart disease. We made genetic deletions in mice and also over-expressed microRNA genes, which caused interesting disease phenotypes." By manipulating the microRNAs, he explains, you can either diminish the symptoms of disease or worsen the disease. Based on that

research, “we began to explore in more detail how microRNAs might modulate disease progression in the cardiovascular system.”

Dr. Olson is optimistic about potential therapeutic applications, and has already co-founded a biotechnology company called miRagen Therapeutics, which is working on developing new drugs to control microRNAs’ biology over a variety of diseases. The company is now conducting studies of human disease in large animal models and carrying out preliminary toxicology studies on drug compounds involving microRNAs.
He thinks heart failure, fibrosis, and vascular disorders due to excessive vascular growth are the main likely candidates for microRNA therapies in cardiovascular disease.

What would give microRNAs an edge over other drugs? “MicroRNAs are based on an entirely different mechanism of action,” Dr. Olson points out. “The classical drugs we’re all familiar with generally act against single proteins in cells, most often inhibiting a cell surface receptor or protein kinase. But cells have ways of bypassing the activity of a drug by removing the protein to which the drug is targeted.”

“MicroRNAs, instead of targeting one protein to maximally inhibit it, target a large collection of proteins across a complicated biological pathway, so bypass mechanisms are less likely.” However, the same feature makes it difficult or even impossible to correlate the therapeutic effect of a microRNA-based drug with inhibition of a protein or cell. “There are many other therapeutic modalities that can be brought to bear on cardiovascular disorders,” Dr. Olson adds. “MicroRNAs are but one of the modalities in the armamentarium.”

It was a 2008 paper, the first reporting on the use of plasma-based microRNAs to detect pancreatic cancer, that drew Sok Kean Khoo, PhD, to study possible applications to neurodegenerative disease. By applying what has been learned in oncology, she felt that researchers could help the understanding of neurodegenerative disease catch up to microRNA applications in cancer. “I think microRNAs are definitely relevant to all diseases; it’s just that we haven’t done enough research yet,” says Dr. Khoo, distinguished associate professor of molecular genomics at Grand Valley State University in Michigan.

Dr. Khoo

Her interest is Parkinson’s disease. “We know that by the time you get diagnosed in the clinic, your neurons have already been degraded as much as 50 to 80 percent,” Dr. Khoo says. Molecular biomarkers that are objective and quantifiable would be useful in preempting this degradation. If microRNA could precisely measure the microexpression of Parkinson’s at the very early stages, before clinical signs appear, then it is possible that treatments could be developed and administered when they might be more effective, similar to the microRNA replacement therapy that is being developed in oncology.

Using a repository of plasma from Parkinson’s patients collected at Mercy Health Saint Mary’s and samples from Umeå University in Sweden, Dr. Khoo found a panel of circulating microRNAs that showed promise as early biomarkers. “We were very excited at the initial results.” While lower predictive values were shown in the validation set compared with the discovery set, Dr. Khoo was able to show that a minimum of four microRNAs could differentiate Parkinson’s patients from controls. She acknowledges that molecular biomarker research using samples from multiple clinical sites has challenges but was encouraged by the demonstration that using plasma-based circulating microRNAs as biomarkers is feasible.

Helped by a grant from the Michael J. Fox Foundation, Dr. Khoo is now testing those candidate biomarkers to see

whether they can use microRNAs to track the progression of Parkinson's disease. "Some patients have disease that progresses quickly and some still have moderate symptoms after 15 years, but we don't know why. We hope to identify microRNAs that can monitor Parkinson's progression and develop a different intervention to address those at risk of fast progression."

Supplementing a microRNA panel with bioimaging of the brain could increase the microRNA panel's usefulness in early detection. This combination of "wet" and "dry" biomarkers, Dr. Khoo says, could be a key to greatly improving diagnosis and staging of Parkinson's disease.

MicroRNA research in Parkinson's is still in its very early stages compared with some neurodegenerative diseases, she notes. "With Huntington's disease, we know the gene; it's very straightforward and detecting it is precise. Parkinson's is more like Alzheimer's in that more genes are involved." Dr. Khoo is hopeful that microRNA will shed light on cerebral palsy as well, which she is studying in collaboration with Michigan State University. "It's not neurodegenerative, but nobody really knows the etiology of the disease," she says.

While microRNA is promising, much work remains to be done. "We're able to detect it, but our sensitivity and specificity are not close to ideal, and we need to validate our findings and understand much more about the function of microRNA," Dr. Khoo says. "We have a lot of work to do to really understand how micro-RNA interacts with other genes and along what other pathways."

In a peer-reviewed paper published in 1992 about work done at the University of Toronto, Dr. Lukiw and his group were the first researchers to point out the possible involvement of small, non-coding RNAs in Alzheimer's disease. Later, at the Neuroscience Center of Louisiana State University in New Orleans, Dr. Lukiw began studying the abundance, speciation, and complexity of small non-coding RNAs, including microRNAs, and their mechanistic relationship with protein-coding messenger RNAs (mRNAs) in the Alzheimer's disease brain. However, there was a significant limitation. "We could only reliably look at upregulated microRNAs in Alzheimer's disease brains because our work was with postmortem tissue, and since microRNAs are known to rapidly degrade, a downregulated microRNA might just be a consequence of the postmortem interval. Since those early days," he says, "we have had great success with studying microRNA abundance, profiling, and activities in normal and stressed human brain cells in primary culture."

Nevertheless, Dr. Lukiw's group turned up interesting links between upregulated microRNA and downregulated messenger RNA in the Alzheimer's brain. For example, TREM2 (triggering receptor expressed on myeloid cells 2) is a receptor in the cells that senses toxic molecules such as extra amyloid that have built up in the brain, and gets rid of them by phagocytosis. In the Alzheimer's brain, there are defects in TREM2 and an increase in miR-34A, which seems to downregulate TREM2. "So microRNAs seem to be acting in specific areas that are important Alzheimer's disease pathways, and microRNAs also seem to be involved in Alzheimer's inflammatory signaling and innate immune signaling."

MicroRNAs are inside brain cells and in the fluid that surrounds brain cells, as well as the cerebrospinal fluid. "This suggests a way that the pathology can spread by microRNA-mediated signals, and it also suggests if you can see increases in specific microRNAs, they may be diagnostic for Alzheimer's disease." One of the more promising theories is that certain populations with different profiles of microRNAs, CSF, and serum may have a predisposition to Alzheimer's disease.

About five percent of Alzheimer's disease cases stem from a mutation in the genes, Dr. Lukiw notes, but the other 95 percent have what is called sporadic or idiopathic Alzheimer's, which has no known genetic component but may have an epigenetic component through microRNAs. When experimenting with human brain cells in primary culture in vitro, "if you stress these brain cells with the same kind of stressors you see in Alzheimer's disease, such as through TNX alpha or amyloid beta peptides, you see the same kinds of changes you see in Alzheimer's," he says.

As microRNA research advances, Dr. Lukiw expects that a panel of maybe 10 or 20 microRNAs could prove diagnostic for Alzheimer's within the next 10 or 20 years.

“I can see these panels not only for Alzheimer’s but also diabetes, metabolic syndrome, or other neurological diseases such as amyotrophic lateral sclerosis or schizophrenia. The more patients you look at, the more sure you are that these are actual markers for disease and not just variations in the genetics between different human populations.”

MicroRNA research in Alzheimer’s today is about five or six years behind microRNA research in cancer, Dr. Lukiw notes. “So cancer research got a jump-start on this type of diagnosis. But I would say there are now seven or eight large research groups looking at microRNA in Alzheimer’s or related progressive neurodegenerative disease, including prion, Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy, or mad cow disease.”

In liver disease, some non-cancer applications of microRNA research are already in clinical trials. Researchers have elucidated several key microRNAs that play a role in the hepatitis C virus life cycle. In fact, they have discovered a liver abundant miRNA that actually works to protect the virus from the body’s host defenses, says Ragunath Singaravelu, a doctoral candidate at the University of Ottawa in Ontario, Canada. For the last seven years, Singaravelu has been working in the laboratory of chemical biology and virology expert John Pezacki, PhD, adjunct professor at the University of Ottawa, on the role of microRNAs in hepatitis C.

Singaravelu

Research has shown that the HCV virus can essentially hijack miR-122, which protects the virus from innate antiviral pathways that want to degrade foreign RNA and stimulates the HCV life cycle, Singaravelu explains. “By having this microRNA hanging off the 5’ end of the molecule, they would be potentially protecting the virus from recognition by host sensors like RIG-I (*retinoic acid-inducible gene* 1) or degradation by exoribonuclease 2.” Based on this mechanism, miR-122 is being tested as a hepatitis C therapy that is now in phase two clinical trials, he says.

The microRNA research at the Pezacki laboratory is targeting a different aspect of the microRNA/virus interaction. It has focused more on microRNAs that contribute to the development of steatosis in hepatitis C patients. “Fifty percent of HCV-infected patients develop fatty liver, and generally the virus hijacks the lipid pathways to facilitate every step of its viral life cycle. The accumulation of fats helps the virus proliferate, and we’ve identified microRNAs whose modulated expression helps the virus accumulate fats in the liver. This steatosis is correlated with worsened disease outcomes and reduced response to therapy.”

“Using both in vitro cell culture systems and in vivo mouse models, we’ve correlated miR-27 expression levels in the liver to the level of steatosis induced by the virus. We’ve also shown that by modulating expression of miR-27, we can actually inhibit the virus.” So by identifying microRNA markers of steatosis such as miR-27, Singaravelu says, “we’re actually identifying microRNA biomarkers of disease progression, and potentially therapeutic response.”

Much further testing is needed, he adds, but these findings could soon have implications for therapy. “Right now, therapy for HCV is heading towards an interferon-free regimen, with a bunch of small molecules targeting separate viral proteins directly. The problem with these virus-targeted strategies is that the virus rapidly mutates. But one way to get around that is targeting a host pathway that the virus follows, because the virus can’t really mutate around the need for that host pathway. So that might be a nice complement to make the virus-targeted strategy more effective.”

Another study the Pezacki laboratory is working on involves a systematic approach to identify other microRNAs

that play a significant role in liver disease. “When you do a traditional microRNA profiling experiment, you compare HCV-infected cells with healthy cells. Generally we get over 100 differentially expressed microRNA. What’s not clearly understood is the biological relevance of these modulations. So we’ve essentially designed a systematic approach to figure out which pathway that microRNA is regulating.” Using that technique, Singaravelu and his coworkers have identified two other microRNAs that play a role in virus-induced steatosis and the virus’ hijacking of hepatic lipid metabolism to facilitate its life cycle.

Clinical trials of microRNAs in therapy are not slam dunks and could reveal unsuspected problems with promising therapeutic applications. “The main problem with microRNA as a therapy is potential toxicity,” Singaravelu says. “While a specific miRNA might regulate hepatic lipid metabolism, there’s also potential for it to regulate a whole entire other pathway that might be detrimental to the patient.”

Early detection is another clinical priority of microRNA research. “It is difficult to monitor progression of HCV, because people who progress to chronic infection are generally asymptomatic in the acute stage. So diagnosis is a huge problem because these patients seem fairly healthy until you get some of the later severe symptoms. Serum microRNA detection has the potential to be part of a noninvasive tool for early diagnosis.”

Gyongyi Szabo, MD, PhD, became interested in the potential usefulness of microRNAs because of the difficulties of treating liver disease. “In many liver diseases, we find there is a major imbalance of inflammatory processes going on, and sometimes blocking a pathway completely is dangerous,” says Dr. Szabo, who is associate dean for clinical and translational sciences and professor and vice chair for research in the Department of Medicine, University of Massachusetts Medical School. “MicroRNAs, which can regulate pretty much every biological process, can offer a kind of intermediate regulation, fine-tuning without totally blocking the expression of messenger RNA. That’s the concept that particularly attracted me to microRNAs.”

Clinicians often want to know what is happening in the liver of a particular patient. “But we have very limited markers, whether in the blood or the urine, to evaluate liver function. Many of these diseases have components of inflammation that may cause damage, but we have no way of knowing how much inflammation is there and for how long. It contributes to scarring and that eventually leads to cirrhosis, but we don’t have any good serum markers to tell how much scarring has occurred in the liver.”

In research she led two years ago, Dr. Szabo’s laboratory induced various types of liver damage in mouse models and showed that certain microRNAs, particularly miR-122, are highly abundant in hepatocytes or liver cells. An increase of these microRNAs in circulation correlates closely with traditional markers of liver injury, such as levels of alanine aminotransferase or aspartate aminotransferase.

In addition to miR-122, another group of microRNAs is associated with inflammation. Her laboratory found that in miR-155, for example, one of the major master regulators of inflammation was highly increased and may be useful for further evaluation for biomarker discovery. While these are preliminary findings, Dr. Szabo believes they are encouraging and provide good leads for further research. “We need to go back to look at human samples and potentially evaluate these questions in real patient populations.”

The University of Massachusetts has been the home institution for some of the leading basic research in microRNAs, and Dr. Szabo’s focus is largely on the translation of these discoveries to diagnostics and future therapeutics. Clinical applications are at least a few years away, she estimates. But further study of the microRNAs linked to liver disease is essential, she believes.

“They are giving us insight into better understanding of the disease mechanism that will allow us to have targeted choices for therapeutic interventions.”

Perhaps the most promising applications of microRNA research involve their use in the diagnosis and treatment of kidney disease linked to diabetes. Current research around kidney disease and its progression falls far short of what is needed, says Christos Argyropoulos, MD, PhD, assistant professor of nephrology at the University

of New Mexico. “The current therapies we have for diabetes, mainly blockers of the renin-angiotensin-aldosterone axis, are only effective 30 percent of the time. The other 70 percent of the cases we have no impact on. And all the trials we’ve run in the last four or five years to develop new therapies have ended up being failures.”

Dr. Argyropoulos started research in this field in 2009. He found himself drawn to microRNAs as a means for understanding differences between patient samples at different stages of kidney disease. “We were interested in what happens in the transition between people with no evidence of disease and diabetes in stages two and three. And we found that microRNA was differentially expressed two years before the detection of protein in urine, which is how we monitor for diabetic nephropathy in the clinic.”

Delaying the progression between stages of diabetes is an important clinical goal, and it’s possible that microRNAs could be used to select people who need to be started on therapy. “Maybe patients who have these abnormal signatures in their urine may benefit from earlier treatment,” Dr. Argyropoulos says.

With his new position in New Mexico, where the prevalence of diabetes and diabetic kidney disease is high, Dr. Argyropoulos is exploring design of a clinical trial. “We have to validate the biomarkers a little better, and we expect that to take another year or so. Then we plan to put patients on an interventional protocol.”

One purpose of the trial would be to find people who are likely to sustain kidney damage and treat them early. “The other angle we would apply to later stages,” Dr. Argyropoulos says. “We know that about 25 percent of patients with diabetes will develop kidney disease, and of those about 70 to 80 percent may progress to more advanced kidney damage. You want to know who are the people who will do badly. If you examine patients and find microRNAs that correlate with more severe stages of diabetic kidney disease, you can use that prognostically, and tell other people that their kidney disease is likely to stay at the same level.”

There would potentially be additional bioinformatics benefits to such data, he says. “Some of these microRNAs are involved in other disease processes like vascular biology or heart function, and it’s possible we could use what we measure in the urine to predict damage to other organs, such as the heart or eyes or vasculature. Nowadays, we cannot do that because the correlation between albumin in the urine and risk of cardiovascular complications of diabetes is not perfect. So maybe by using microRNA, we can get more precise early detection of disease in other organs.”

He expects that the research protocol they are planning will combine several inquiries. “Diabetes is so common a disease, it absolutely makes no sense to separate the complications, because most of them tend to go together. So I would put everything in the same protocol for development of microRNAs in diagnostics.”

Some biotechnology companies are already developing microRNAs as therapeutic agents, which he sees as an essential pursuit even though so much about microRNAs remains unknown. At the 10th Annual Conference on MicroRNA as Biomarkers and Diagnostics, which took place in March, Dr. Argyropoulos was struck by a common theme. Among the diverse range of participants from industry, drug safety, and academia there was unanimity on the need for better research to make sure the biology of microRNAs is understood.

Despite the array of research now underway, the secrets of microRNAs’ structure and meaning have yet to be unlocked, Dr. Olson agrees. With respect to microRNAs circulating outside cells, he highlights key questions for the future: “What are they doing there, where do they come from, where are they going? There is a lot of interest in those questions right now.”

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