MicroRNA markers show staying power

Anne Paxton

March 2014—Not many components of human cell biology have been discovered and immediately dubbed "junk." But micro-RNAs, small noncoding RNA molecules first identified in 1993, fall into that category. Like Hans Christian Andersen's Ugly Duckling, microRNAs began their life after discovery with people scoffing at them. People even laughed at researchers who thought microRNAs held promise in diagnosing cancer.

No one is laughing now. Molecular biologists now understand that microRNAs inhibit the translation and stability of messenger RNAs, the genes that control such cellular processes as inflammation, cell-cycle regulation, stress response, differentiation, apoptosis, and migration. Research on multiple fronts is producing ever more provocative findings about the dysregulation of microRNA and how that process plays an essential role in the development and progression of cancer.

With three to four papers on the subject published per day, new clinical applications are under active development across the gamut of cancer diagnostics: in chronic lymphocytic leukemia; in colon, breast, and prostate cancer; and in cancers like lung, liver, ovarian, and pancreatic that are notoriously difficult to detect early and treat effectively.

Companies such as Asuragen, which launched the first microRNA diagnostic test in 2008, and Rosetta Genomics are integrating microRNA biomarkers into their molecular diagnostics offerings. And some leading researchers view microRNAs as the most exciting cancer diagnosis and treatment development in decades.

"I don't think we've ever had a biomarker that controlled or regulated cell processes to the extent that microRNA does," says Gregory J. Tsongalis, PhD, director of molecular pathology at the Geisel School of Medicine at Dartmouth College in Hanover, NH.

Back in the era of the Human Genome Project, "When we initially isolated our DNA and RNA, there were all of these small fragmented things we would discard as trash, as degraded nucleic acids. But it turns out in those nucleic acids were these small RNA sequences, microRNAs, that have changed the way we look at things biologically," Dr. Tsongalis says.

It was Dartmouth researcher Victor Ambros, PhD, who discovered microRNAs in 1993, and the molecular pathology laboratory there has continued to study multiple aspects of microRNAs. People are cautiously optimistic about prospects for microRNAs in cancer diagnostics, Dr. Tsongalis says. "We have seen biomarkers come and go—some that showed great promise, then petered out because they didn't end up being as specific or sensitive as expected. But I'm not sure that will be the case with microRNA."

MicroRNAs' relevance has been discovered in a number of different diseases, though the majority of the work has been in human cancer. "There, microRNA markers, either alone or in panels, have been shown to be really, really specific for different tumor types, to distinguish between malignant and benign tissues," he says. "Or to distinguish a tumor versus an inflammatory process."

The field is continuing to push these types of biomarkers into more clinical settings, Dr. Tsongalis says. Researchers at Dartmouth are using small panels of microRNAs to resolve clinically important questions of whether the lesions or cells in cytology samples are malignant or not, and the hospital has a pancreatic cancer panel in occasional clinical use. "We don't run it on every patient, but ones where there is a question of whether we have an autoimmune pancreatitis or pancreatic cancer, we would use those panels to make that distinction. We had a recent case at our GI tumor board of potential pancreatic cancer, and the cytology result was suspicious for malignancy but we couldn't make the diagnosis. We have the data now to show that our microRNA panels can do that pretty readily. They've shown fantastic accuracy."

One application that has been of particular interest at Dartmouth is chronic lymphocytic leukemia. “Carlo Croce’s group has done a lot of work in that area. They’ve shown, and we’ve confirmed, there are certain microRNAs you can use to distinguish people with indolent CLL, that may never be troublesome to them, from those with very aggressive CLL.” That difference changes the patients’ potential responsiveness to therapy, he notes, as well as their overall prognosis. Micro-RNA is also becoming the standard diagnostic in liver cancer to distinguish high and low risk.

Another research focus has been methods for testing less invasive sample types. One of micro-RNAs’ advantages is their inherent stability. Because microRNAs are resistant to RNases, they are well preserved in formalin-fixed paraffin-embedded tissue, making them excellent candidates as biomarkers in workups of routine pathology specimens. “We’ve been really successful in using profiling techniques with traditional pathology specimens from resected tissue,” Dr. Tsongalis says. “But we’ve also been very successful in using the microRNA profiling technique off cytology specimens and small biopsies, which are becoming more and more the diagnostic specimen of choice for certain tumors. And now we and others are showing we can even do microRNA detection in the circulation.”

The stumbling block is making sure the biomarkers are accurately validated, Dr. Tsongalis says. “It becomes a little bit different than just validating an assay. We know the quantitative assay works well, but we have to make sure the expression levels really link back to the patient and the clinical phenotype. So we’re moving away from the analytical validation to spending more time doing the clinical validation, which in the molecular laboratory we usually don’t do.” That process involves making sure that many different patients with different types of lesions and different disease stages are tested.

Carlo Croce, MD, for one, is convinced that in the near future microRNA will be used not only for cancer diagnosis and prognosis but also as cancer drugs that can be used to treat and prevent tumors. It was in the cancer genetics program at Ohio State University, which Dr. Croce directs, that George Calin, MD, PhD, first showed in 2002 how microRNAs played a central role in cancer.

For his work on the role of microRNA in various cancers over the past 20 years, Dr. Croce, professor and chair of Ohio State’s Department of Molecular Virology, Immunology, and Clinical Genetics, has become the seventh most cited author in biology, according to Thomson Scientific’s Essential Science Indicators. In Dr. Croce’s view, microRNAs will have a huge clinical impact as noninvasive blood tests become used for early cancer detection. “I believe that very soon some of these tests will be commercially available, and that will change the face of diagnostics,” he says.

With his research group at Ohio State, Dr. Croce started studying leukemia in 2004 and moved rapidly to solid tumors, having now studied about 16 different solid malignancies, he says. That includes several studies of lung cancer in which his group is looking at the expression of micro-RNA in the blood to develop noninvasive tests for those at risk of developing lung cancer. “We are also working extensively on breast cancer where we can distinguish different types by using microRNA profiling. We can treat specific groups with specific drugs directed to kinases that have been dysregulated because of the dysregulation of microRNAs.”

The field is evolving rapidly, he points out. “In the beginning, there was not much standardization, and a lot of people carried out studies using technologies like gene chips that were not very good. Now the technology is very sensitive and, in general, the findings have become pretty reliable.”

Dr. Croce is especially optimistic about the potential impact of micro-RNAs in noninvasive screening. “Let us say you are looking for tumor of the prostate. There is a not-very-sensitive blood test now, the prostate- specific antigen test. But one day there will be a test with microRNA that will detect development of a malignant tumor and you will be able to intervene very early.” Most men over 70 have prostate cancer but not an invasive form of prostate cancer, he notes. “You should not operate on people without invasive prostate cancer, but unfortunately we don’t have a test yet that can distinguish between invasive and noninvasive prostate cancer. One day we will

have a microRNA test to do that, and it will have enormous implications in how we deal with prostate cancer."

The same should be true of ovarian cancer. "If you can detect it at stage one, the tumor can be removed and the patient can be cured." With microRNAs as biomarkers, he predicts such a diagnostic will be available soon.

Now that more than 1,000 microRNAs have been identified, it's likely the majority have been found, and one of the main goals in profiling is understanding the significance of those sequences and how their dysregulation is linked to the pathogenesis of cancer, says oncologist Todd A. Fehniger, MD, PhD, assistant professor of medicine in the Division of Oncology at Washington University School of Medicine. Most recently, he has researched microRNA mutations in acute myeloid leukemia, in collaboration with the group of Daniel Link, MD, at Washington University. "Recurrent mutations in miR-142-3p were discovered by The Cancer Genome Atlas, and then confirmed and followed up by Dr. Link's lab. This had not been described before, and the mutations were in the 'business end' of the microRNA, the seed sequence" (Trissal M, et al. Dysregulation and recurrent mutations of miRNA-142 in de novo AML. Poster presented at: 55th ASH Annual Meeting and Exposition; Dec. 7–10, 2013; New Orleans). These mutations also resulted in dysregulation in the second miR-142 sequence, miR-142-5p, suggesting an alteration in microRNA processing, revealed by using small RNA sequencing.

"There's a lot of interest in using either micro-RNA expression or recurrent mutations in micro-RNAs to inform how we treat patients," Dr. Fehniger says. "But as far as a useful screening test in a large population of individuals where the disease is a rare event, the test parameters that have been described don't seem like they're quite there yet." For example, a study reported in JAMA in January described a panel of 38 micro-RNAs in whole blood that had 85 percent sensitivity and 64 percent specificity (Schultz NA, et al. Micro-RNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA.* 2014;311(4):392–404). "But you can't really use that level of sensitivity and specificity in a screening population to detect extremely rare early-stage pancreatic cancers. So I think there is still work to be done if we're going to use that approach in rare solid tumors."

Using microRNAs for risk stratification, on the other hand, is likely to be more feasible in the short term, he says. "A very interesting study used a set of six microRNAs in stage two colon cancer to try to define people who were going to do very well with the surgery alone and not relapse versus those who probably would relapse [Zhang JX, et al. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. *Lancet Oncol.* 2013;14(13):1295–1306]. This is an important clinical application for a medical oncologist where you need to decide who is going to benefit the most from additional chemotherapy treatment."

But to be generally medically useful, Dr. Fehniger notes, "just like everything else in diagnostics, these tests need to be validated, confirmed by several groups and again in prospective trials before we're confident enough to use a microRNA signature, for example, to change treatment for a patient." That milestone is five to 10 years down the line for most cancers, he estimates.

Remarkable new discoveries remain to be made, and he thinks micro-RNAs are going to be an active area of study for decades to come. "I approach microRNAs like any other scientific question. If microRNA is recurrently dysregulated in cancer, it's probably dysregulated for a reason, and through understanding the biology of that microRNA in the setting of a cancer cell, we're going to learn a lot about the disease. It will be through understanding what the microRNAs do inside the cancer cells that's really going to inform new therapeutics, and it's that end of the spectrum of microRNAs that many medical oncologists such as myself are most interested in coming to fruition."

Early detection has been the focus for Feng Jiang, MD, PhD, associate professor of pathology, University of Maryland School of Medicine, who has been exploring microRNAs as potential sensitive and specific noninvasive biomarkers for lung cancer.

Using next-generation sequencing on bronchial washing specimens of 26 lung cancer patients and 26 healthy controls, Dr. Jiang and his colleagues identified 12 microRNAs that displayed different expressions in the healthy

subjects and those with cancer. A separate study of two cohorts of 36 cancer patients and 36 healthy controls allowed the researchers to develop an optimized marker panel, which they then validated in independent sets of cases and controls.

Why was NGS necessary? “Earlier sequencing technology does not have the ability to identify normal microRNA which show changes in certain types of tumors,” Dr. Jiang explains. “We used more advanced NGS because through deep sequencing we were able to systematically and comprehensively define or identify normal micro-RNA, which had not been identified by earlier technology.”

To his knowledge, no other paper published has directly used NGS analysis on tumor tissue to identify microRNA. His results have been submitted for publication and are now under review.

In the second phase of this study, he is continuing to research development of biomarkers that can be used for early detection of lung cancer through microRNAs in plasma, serum, or sputum samples. But clinical applications will depend on tests in several larger populations. “If it works, we will apply for a large-scale prospective trial. Right now, we want to combine the biomarkers together with CT scans to increase the sensitivity and specificity for lung cancer early detection. But a CT scan is much too expensive to be used for a screen. In the future, we’re hoping to develop a biomarker that would be cost-effective to screen the general population.” The goal, he says, is to reduce mortality associated with lung cancer.

For diagnostic purposes, the key advantage of microRNA is that the gene is small, he notes. “Small genes can be more easily detected in the sample and they are less affected by the quality of the specimen. Because the storage of tumor tissue degrades it, if you are measuring protein changes, the assay heavily depends on the quality of the specimen. You can use microRNA without concern about the degradation of the specimen.” In addition, microRNA is easier to detect compared with methylation, for which an assay must employ PCR. “This gives microRNA a big advantage compared to other candidates like proteins.”

Dr. Jiang expects that microRNAs will be combined with different categories of genes or proteins to produce useful profiles. “I don’t think we can use microRNAs to solve everything. In the future we will probably use different classes of biomarkers together.”

MicroRNA research has progressed substantially since 2004 when Muller Fabbri, MD, PhD, became involved. Dr. Fabbri is assistant professor of pediatrics and molecular microbiology and immunology at the University of Southern California Keck School of Medicine and Children’s Hospital Los Angeles.

Dr. Fabbri’s current research is exploring how microRNAs affect the tumor microenvironment, especially as molecules that are shuttled between cancer cells and surrounding cells. “In the early years, when people were simplifying things, they might just look for target genes of these microRNAs and see that some tended to target tumor suppressors, others to target oncogenes. However, with more and more literature, it has become clear that it’s a little risky to label microRNAs just as tumor suppressors or oncogenes, because it’s turning out it really depends on the type of tumor and in some cases on the species.”

For example, he points out, in lung cancer a microRNA might act as an oncogene in humans but not in mice. He reported this shifting nature of micro-RNAs in a paper that compared the phenomenon to Robert Louis Stevenson’s Dr. Jekyll and Mr. Hyde (Fabbri M, et al. Regulatory mechanisms of micro-RNAs involvement in cancer: the strange case of Dr. Jekyll and Mr. Hyde. *Expert Opin Biol Ther.* 2007;7(7): 1009–1019).

In 2007, Dr. Fabbri published a paper in which it was observed that one cluster of two micro-RNAs is able to affect expression of 14 percent of the genome. “So of course you cannot think that in this 14 percent of the genome there are only oncogenes or only tumor suppressor genes. There’s a mixture.” He compares the phenomenon to that of forces in physics. “If you have different forces applying in different directions, then you take the direction which is the result of all the forces applied. I like to see microRNAs in this way.”

Dr. Fabbri believes researchers are very close to understanding the activity of microRNAs. “The signatures of dysregulated microRNAs have been basically described since 2004. And every day there are more and more papers showing targets and validating the targets of specific microRNAs.” Now, for example, if you give a pathologist a tumor and he or she doesn’t know which tumor it is, “just by looking at which micro-RNAs are down- or upregulated, it is possible to guess with a good amount of success the tissue of origin.”

This is a great breakthrough, he says. “As we speak, there are still eight or 10 percent of cancers of unknown primary origin that are metastasized, and the clinician is not able to see the primary tumor. So this is very important if we can guess the tumor of origin from the microRNA expression profile, because you can give your patient a better treatment with fewer side effects.”

The specific signatures of microRNAs also have prognostic implications for risk stratification, he adds. “If you take patients who already have a clear diagnosis—for example, chronic lymphocytic leukemia patients—then one of the biggest challenges for clinicians is that you don’t really know what the outcome of that disease is. It can be indolent or aggressive, but clinicians don’t have good tools to guess.” In a paper he coauthored, Dr. Fabbri showed that microRNA expression can help with this identification, which allows a clinician to treat more aggressive disease types more quickly (Calin GA, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med.* 2005;353:1793–1801). He envisions the use of microRNAs in conjunction with current chemotherapy and radiotherapy within five or six years.

Dysregulation of some micro-RNAs seems to occur early in a disease’s natural history. “You don’t have to wait for stage four lung cancer to see certain microRNAs popping up, so they are really good diagnostic biomarkers,” Dr. Fabbri says. “They’re also really good, after a patient has been operated on, at predicting recurrence of the disease when the disease is not even detectable yet. And they can be identified not only in the blood but in all human biological specimens you can possibly imagine, depending on the cancer—for example, saliva, in the case of head and neck cancer.”

Dr. Fabbri says it’s important to understand that microRNAs do more than just silence genes. “So far, people have focused on micro-RNAs as an obstacle to expression. They bind to the target gene and prevent it from becoming a protein, so they are excellent regulators of gene expression, but they don’t just do that. There is much more to their mechanism of action.”

He blames traditional dogma of molecular biology for the initial belief that microRNAs were “junk DNA.”

“A gene is a piece of DNA transcribed into messenger RNA which is translated into a protein. People just focused on this DNA/RNA/protein link, and if a piece of DNA was not doing this, it was considered not helpful. But cells do not spend a single molecule of their energy to do something that is not helpful or not doing anything. Fortunately, people realized at some point that 98 percent of our DNA does not encode for protein. And this whole group of what used to be called junk—microRNA is only a small fraction of it—is now called ‘noncoding DNA.’”

However, not all researchers are as sanguine about microRNAs’ prospects in the short term. Federico Monzon, MD, a molecular pathologist at Invitae Genetics Laboratory in San Francisco, studied expression of microRNAs in different types of renal tumors when he was an assistant professor of pathology at Houston Methodist Hospital. “We explored micro-RNAs as tools for renal tumor subtype classification because they are amenable to developing diagnostic assays that are easily implemented in the laboratory.”

Now in industry, where he focuses on genetic testing based on next-generation sequencing, Dr. Monzon does believe microRNA shows promise. “There’s a good amount of literature describing specific microRNA profiles associated with tumors. But these profiles have not been developed as robust clinical tools yet.”

“My skepticism is mostly that we don’t have strong links with outcomes. We know a lot about how specific microRNAs might be increased in one tumor or another, but we don’t yet have a validated way to use that information to make clinical decisions, and that’s where the gap is.”

Of therapeutic applications, he says: “We have associations, but we don’t know how to modulate microRNAs, so if we discover that an aggressive tumor has a specific microRNA profile, it doesn’t necessarily mean there is something we can do for the patient. If you have a specific profile that could identify who responds to traditional therapy, that would be useful, and it’s still possible. We just need to find out with the appropriate research.”

Dr. Tsongalis, of Dartmouth, would agree that microRNA may not pan out with every application now being researched. But, he says, “I’m not sure people have really picked up on the impact microRNA could have, diagnostically. There will be certain areas where it will end up as the biomarker of choice. It’s just a matter of people getting used to the concept and being able to relate microRNAs back to what the clinical question is. And once that happens, I think there are a number of applications that this will be routine for.”

MicroRNAs are still evolving, he emphasizes. “But we’re moving toward clinical use very quickly. The data is supporting a lot of different applications on the clinical side, so the naysayers, I think, will be paying a little bit more attention. Because this is not your average biomarker.”

[hr]

Anne Paxton is a writer in Seattle.