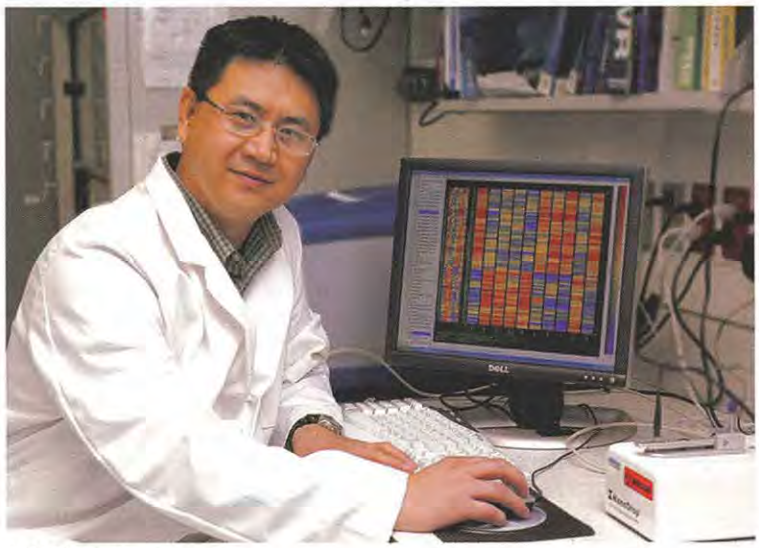
Small and Full of Promise

A number of microRNAs have been linked to the initiation and progression of various cancers. With their broad reach, miRNAs show promise both as tools to diagnose cancers and predict prognosis, and as targets for treatment or a therapy themselves.



JINGFANG JU

BY CIARA CURTIN

They are small, only about 22 nucleotides in size, but microRNAs hold a lot of potential as diagnostic or prognostic tools for cancer, or even as therapies.

MiRNAs are involved in development and regulation, two systems that tend to go awry in cancer. "MicroRNA plays quite a role in fundamental development and based on that, any changes in microRNA expression have a consequence," says Jingfang Ju, an assistant professor at the State University of New York at Stony Brook. One change, he adds, could have a broad impact on multiple genes and pathways. "From that point of view, I think microRNAs also are important, actually not only in cancer, but in many other diseases as well," Ju says.

MiRNAs have been implicated in both the initiation and progression of cancer. While working in Carlo Croce's lab, then at the Kimmel Cancer Center in Philadelphia, George Calin found that miR-15 and miR-16 are often deleted or down-regulated in people with familial chronic lymphocytic leukemia, the first finding that associated miRNAs with cancer. "I think [microRNAs are] a major component," says Calin, who is now an associate professor at the University of Texas MD Anderson Cancer Center.

Those and other mutations in miRNAs have been linked to various cancer types, and may be used as ways to diagnose the disease, determine patients' prognoses, and predict patients' responses to treatment. But before miRNA signatures of cancer can be used in the clinic, they must be validated, and best practices must be established for their collection and testing. And what they reveal should, ideally, inform patients and their physicians about a course of action, such as which therapy would be best in that particular case. Further, miRNAs could be the targets of that therapy or a treatment themselves.

The signature

A number of miRNAs have already been linked to cancer, its prognosis, or have been used to determine the best treatment. "As far as differentiating tumor subtypes, I think [microRNAs] are very good at that, or tissues where it came from," says Joanne Weidhaas, an assistant professor at the Yale School of Medicine. She adds: "I think microRNA changes are really going to predict that certain medicines are going to work or not work. There's definitely already evidence of that, some recent things coming out." A recent *Frontiers in Genetics* review by Stony Brook's Ju lists six miRNAs

that are diagnostic biomarkers for colorectal cancer, and eight that are prognostic. Another seven he lists are modulators of chemotherapy.

Ju and his lab have uncovered miRNAs associated with cancer patient response to chemotherapy and survival. "Not everyone responds to chemotherapy. ... They go through all the toxicity without any benefit," Ju says. He and his colleagues wanted to see if they could find a marker to distinguish responders from non-responders.

In colorectal cancer, they found a number of miRNAs associated with response to the chemotherapy drug 5-fluorouracil, including miR-181b and miR-21. Then Ju and his collaborators at Soochow University in China turned to gastric cancer — which is prevalent in China — to see if those or other miRNAs were associated with response to a new generation of 5-fluorouracil, S-1/oxaliplatin, that patients take as a daily pill. "And sure enough, we profiled a number of microRNAs and it turns out that we discovered two of them miR-181 and -21," Ju says. "[MiR]-21 is common for many tumor types, but these two in particular shows up and is related to the S1 patients' survival." He adds that these are likely not the only miRNAs involved.

As there are likely many miRNAs associated with diagnosing cancer as well as predicting its course or best therapy, assays for miRNAs could be multiplexed, says Dominik Duelli, an assistant professor at Rosalind Franklin University in Chicago. "What you can do if you are looking at microRNAs, you can look at many, many, many of them and if you know which microRNAs are involved in which process by basically one or a couple of reactions, you get a lot more information, in principle," he says.

One way to collect miRNA samples

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is from the blood or other body fluid, which is easier to collect than tumor tissue. About 67 percent of miRNAs, Duelli says, are faithfully released by cells, and their profile inside the cells matches their profile outside the cells. "But the problem is, some of our favorite microRNAs that we were looking at, at the time, in tissue had very good diagnostic and prognostic marker [potential], we couldn't find in the blood plasma or in ductal lavages or in other fluids," he says.

For that 67 percent, Duelli found that they are passively shed by the cells while the rest are released through an active process, likely in vesicles, as his lab reported in *PLoS One* in 2010. The vesicles contain cell-surface antigens that identify their cells of origin, which Duelli is putting to use. "That's how we can pull them out and then specifically profile the vesicles that come from an early breast cancer, a late breast cancer cell," he says. Eventually, such a test could be used to detect breast cancer in high-risk women before mammograms could, he adds.

The vesicles are stable in plasma, Duelli notes, and protect the miRNAs from the degradation activities of RNases.

Indeed, miRNAs appear to be stable in samples. The Fred Hutchison Cancer Research Center's Muneesh Tewari reported in a 2008 *PNAS* article that miRNAs are stable in plasma and serum, even after being stored at room temperature for 24 hours or several freeze-thaw cycles.

"You can take plasma, serum, and gastric juice — everything that people are ever thinking to use in research — and find microRNA," MD Anderson's Calin adds. "First of all, [it] is very cheap — plasma and serum. You can take it out of the blood that you are using for usual blood testing so you don't have to have a special collection of sample and so on. Second, you don't need the access to the tumor, which for solid cancer is a problem to go to a lung cancer, to go to a gastric cancer, it is a big problem. You profile the plasma or the serum from this patient and you can understand if they will respond to therapy, what is the prognosis, and so on."

But just what those miRNAs represent often requires validation. Some approaches — Duelli specifically points to total plasma profiling — to finding miRNAs associated with cancer might be picking up the body's reaction to cancer rather than a signal from the cancer itself, he says. Duelli's lab is searching for particles containing miRNAs that are specifically released from breast cancer cells by focusing on finding a signature in ductal lavage samples, then seeing if they can then pick that signature out in other body fluids like blood or urine.

Although, Tewari's lab at the Hutch recently reported in *Cancer Prevention Research* that levels of miRNAs in the blood closely track with blood cell counts. The researchers urge caution when interpreting results of circulating miRNA cancer biomark-

ers, as changes in blood cell counts may affect plasma miRNA levels.

Valid and useful

Before any miRNA can be used as a diagnostic or prognostic tool, it must be validated. In addition, methods for collecting and testing miRNAs need to be standardized.

Disease-associated miRNAs, like those Stony Brook's Ju found for gastric cancer, have often only been found in one population. The findings need to be confirmed in independent cohorts from other medical centers and even from other countries to be sure that the association is real and broadly applicable. As Ju points out, his study was done in Chinese patients, and there may be different associations in Caucasian cancer patients.

"All of the studies, I think, including ours, are basically pilot investigations," Ju says. "For example, the study we did is part of a phase III trial, so we only used samples from one center. Ideally, we need to collect all the patient samples from all the centers involved [and] do a large study. That'll be a nice validation." And that, he adds, is his team's next step.

Such multi-center validation will also help set best practices for how samples are collected, processed, and stored prior to being assayed for the miRNA of clinical interest.

Rosalind Franklin's Duelli found that how plasma is collected, and in which type of collection tube it is stored, makes a difference. Further, as his team reported in a January *Journal of Molecular Diagnostics* article, endogenous serum factors that co-purified with the miRNAs affect abundance measurements. These endogenous factors, Duelli says, inhibit the amplification by qPCR of miRNAs, a common way of measuring their presence and abundance.

In their article, Duelli's team proposed an approach to remove the

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inhibitors and restore miRNA levels through dilution and additional purification. He adds that researchers also need to consider whether the standard polymerase is the best for this application or if there is a better one that can be used instead. "It's a very technical point, but I hope this kind of paper will go towards people finding a more standardized approach and doing it," he says.

Then, the results from the assay have to give meaningful information to clinicians and patients. "Really, right now they could be used in the clinic, but it is hard to tell someone that, 'Hey you know what? You are three times more likely to die,'" Yale's Weidhaas says, adding that "what you want to say is: 'And this is the right treatment.'" Weidhaas is also a co-founder of MiraDx, which aims to translate miRNA findings into the clinic.

In addition, Weidhaas says that miRNA information could convince clinicians to treat patients when they otherwise would not. "It could be if it's a stage 2 patient and you are on the fence about treating them and you see they have this microRNA associated with bad outcome, it might push you in the clinic [to] say 'I'm on the fence, but I am going to lean towards recommending treatment. This is bad, but we have a solution,'" Weidhaas says. "That's most helpful, clinically."

As therapy

Down the road, miRNAs could be therapeutic targets. "Maybe someday it'll be microRNA replacement therapy or anti-microRNA, but before that, it could just be that those tumors need to get a different treatment," Weidhaas says. "It's really risk-stratifying people to direct personalized medicine."

As therapeutics themselves, miRNAs hold a lot of promise. "One particular microRNA can modulate a number of targets and pathways and in a way it's like we are realizing multi-targeted therapy by a single microRNA molecule," says Ju from Stony Brook.

MD Anderson's Calin adds that miRNAs have "a huge potential for therapy" because they can target a number of protein-coding genes at once. MiRNAs, he says, could act on dysregulated genes. For example, he points to the mutated miRNAs he found in chronic lymphocytic leukemia patients, miR-15 and miR-16, which normally target BCL2 and MCL1 to induce apoptosis. "You don't have to look only for specific targets, you have to look for targets distributed in one specific pathway," he says.

Despite the potential for miRNA-based therapeutics, however, there have been difficulties moving similar siRNA therapies into the clinic.

"The challenges of delivery are very big," Calin says. Indeed, Duelli says, "The question is: How do you do it?" Duelli notes number of approaches including using nanovesicles and lentiviruses are possible, but the issues of targeting and preventing off-target effects loom large.

"Obviously, it's difficult to deliver a nucleic-acid based drug to tumor cells at the moment," Ju adds, "but I think once the technical issues are overcome, it will be a reality soon hopefully." ■