No miR Hype: MicroRNA's Cancer Role Expands

Long considered a mere slave to DNA, carrying the genetic message from chromosomes to the protein-making machinery of the cell, RNA has come into its own.

RNA interference, discovered in 1998, is now a standard laboratory tool for knocking down gene expression. Drug therapies using small interfering RNA are now in clinical testing for treating a respiratory virus and age-related macular degeneration. And a rush of discoveries in the last 4 years have linked another class of small RNAs, known as microRNAs, to cancer. Our current knowledge of microRNAs "might be the tip of the iceberg," said Nobel Prize winner Phillip Sharp, Ph.D., of the Massachusetts Institute of Technology in Cambridge, at this year's meeting of the American Association of Cancer Research.

Research into microRNAs in cancer is exploding. MicroRNAs—miRs for short—are noncoding RNAs, about 22 nucleotides long, that bind to specific messenger RNA (mRNA) targets and either block their translation into proteins or trigger their degradation. They're well conserved through evolution, suggesting an important biological role. About 350 microRNAs have been identified in humans, with the total predicted to eventually reach 1,000 or more. Because each microRNA has dozens, perhaps hundreds, of targets, "most human genes will probably be influenced in some way by a microRNA," said Frank Slack, Ph.D., of Yale University in New Haven, Conn.

Emerging From Obscurity

"The whole class of genes has been a surprise to many people," Slack said. "They're so small, and they were just missed for many years." Because mutations that inactivate microRNAs are rare, functional knockouts are uncommon so microRNAs went unnoticed for decades. Scientists assumed the bands at the bottom of their electrophoresis gels represented degraded RNA or other artifacts and ignored them. Lin-4, the first known microRNA (cloned by Victor Ambros, Ph.D., at Harvard in 1993), was considered a weird quirk of worm larval development. Only after Harvard's Gary Ruvkun, Ph.D., cloned the second microRNA, let-7, in 2000 did the search for more begin in earnest. In 2001 research groups in the United States and Germany reported finding dozens of new microRNAs. "That really opened the floodgates," said Slack.

Frank Slack

The first link between microRNAs and cancer came the following year. In the early 1990s three groups had identified a region of chromosome 13 that was deleted in more than half of all cases of chronic lymphocytic leukemia (CLL). They assumed the region contained tumor suppressor genes. Ohio State University in Columbus researcher Carlo Croce, M.D., searched fruitlessly for these genes for the better part of a decade until 2002, when he finally found the genes for two microRNAs in the deleted region. Croce and postdoc George Calin, M.D., showed that both genes were absent or had reduced activity in two-thirds of CLL patients, strongly suggesting that the microRNAs—dubbed miR-15 and miR-16—were tumor suppressors. Croce's lab has since confirmed this supposition, showing that miR-15 and miR-16 induce apoptosis by targeting the key survival protein Bcl-2, which is overexpressed in CLL.

Croce's lab has linked microRNAs to solid tumors as well. "MicroRNAs are only part of the story," said Calin. "But noncoding RNAs are involved in a lot of human cancers."

In lung cancer, for example, the let-7 microRNA also acts as a tumor suppressor, with similar therapeutic implications. After helping clone the let-7 gene 5 years ago, Slack searched for let-7 targets in the roundworm, finding the worm version of human ras, a critical oncogene in lung cancer. Meanwhile, groups in Japan and at the biotech company Ambion showed that let-7 was poorly expressed in lung cancer, which suggested its tumor suppressor function. Slack confirmed this in 2004 by showing that let-7 regulates ras levels in cell culture.

New Class of Oncogenes

But microRNAs are not always tumor suppressors. They can act as cancer-causing oncogenes as well. In 2004

Masao Seto, M.D., at the Aichi Cancer Center Research Institute in Nagoya, Japan, identified a new gene on chromosome 13 that is often amplified in cancer, and he showed that it encoded a cluster of seven microRNAs. Scott Hammond, Ph.D., at the University of North Carolina in Chapel Hill had already noticed that the cluster was overexpressed in many cancer cell lines. Hammond teamed up with Greg Hannon, Ph.D., of the Cold Spring Harbor Laboratory in New York to see if the cluster, called miR-17-92, could promote cancer. It did: In a mouse model of lymphoma, expression of the microRNA cluster accelerated tumor growth. (*See* News, August 3, 2005, Vol. 97, No. 15, p. 1114.)

These microRNAs may act as oncogenes in other cancers besides lymphoma. At April's AACR meeting, Hannon reported similar results in a mouse model of breast cancer. "The number of animals is so far still relatively small," Hannon said, "but we are seeing acceleration of the onset of tumorigenesis."

No one yet knows how these microRNAs are promoting cancer. "It's been a tough one to figure out," Hammond said. Because each microRNA has hundreds of potential targets, demonstrating its role in biology and in cancer is enormously time-consuming. And each of the seven microRNAs in the miR17-92 cluster appears to act independently, adding to the complexity. But Hannon, Hammond, and others are making progress.

"We're trying to address whether miR17-92 is important in tumor initiation, tumor progression, tumor maintenance, or all of the above," Hannon said.

Besides miR17-92, three other microRNAs are confirmed oncogenes. There will probably be more, since many microRNAs are overexpressed in various human cancers. "It's … hard to imagine that at least some of these don't have a functional role in cancer," Hammond said.

In 2004, Croce's group reported that more than half of known microRNA genes were located in cancer-associated genomic regions or in fragile sites—areas of chromosomes prone to breakage, amplification, and fusion with other chromosomes.

"Their paper was kind of mind-blowing," said Slack. "That really suggests [that] those microRNAs are all playing a role in cancer."

Arrested Development

What exactly are these tiny RNAs doing in cancer? There are two schools of thought: Either they are activating or inhibiting specific cancer gene targets, with a direct impact on tumor growth, or they're mopping up many genes overexpressed in cancer to reduce the stress on genetically unstable cancer cells. "I suspect we'll find microRNAs that function at each end of the spectrum, and everywhere in between," said Hannon at the AACR meeting.

One theory of microRNAs and cancer focuses on the important role of microRNAs in development. For example, lin-4 and let-7, the first identified microRNAs, regulate the timing of development in roundworms. Since cancer cells have many characteristics of undifferentiated cells, it's possible that microRNA expression in cancer causes cells to recreate the development process but without moving past the undifferentiated, proliferating stage.

MicroRNAs "weren't put on the earth, or designed, to function in cancer," Slack observed. "They were designed to function during development to maybe shut off cell division or shut off the cell cycle so cells could differentiate."

In cancer, this developmental process may somehow start and then stop, due to mutations or misregulation of key microRNAs. The result: uncontrolled cell proliferation.

The recent microRNA discoveries have obvious clinical implications. CLL, a disease of white blood cells that won't die, is the most common leukemia. It is poorly understood at the molecular level. MicroRNAs provide a new window on the disease and could prove useful for prognosis and treatment. Last year Croce's group reported in the *New England Journal of Medicine* that a 13-microRNA signature could distinguish aggressive from slow-growing CLL. In theory, delivering miR-15 and miR-16 to tumors would trigger apoptosis.

The same theory could prove true for let-7 in lung cancer. "Our hope is that we can use let-7 as a potential diagnostic tool to diagnose lung cancers early in patients," Slack said. "And, secondly, potentially use let-7 as a way to knock out activated ras in those lung cancers."

Detection and Treatment

Although no microRNA diagnostics or therapies yet exist, companies are working on them. In March, Ambion spun off a new molecular diagnostics company, Asuragen, based in Austin, Texas. Ambion spent 4 years developing techniques for manipulating and expressing microRNAs and analyzing their function, techniques that Asuragen is now using for potential diagnostic tests in cancer and other diseases.

"There are some clear opportunities to apply microRNAs to detect cancer in individual patients," said David Brown, Ph.D., Asuragen's director of discovery. Prognostic tests could also be in the works. "MicroRNA expression could tell you a lot about [patients], whether they're going to respond to therapy."

Brown pointed out that microRNAs are much more stable than mRNAs, which makes detecting them relatively simple. And microRNAs, unlike mRNAs, can be easily recovered from the formalin-fixed tumor samples typically stored in U.S. hospitals. Although there are far fewer individual microRNAs than

MicroRNAs currently implicated in cancer

MicroRNA	Cancer Role	Cancer Type	Mechanism
miR-15	tumor suppressor	CLL	Bcl-2 inhibition
miR-16	tumor suppressor	CLL	Bcl-2 inhibition
miR-155	oncogene	lymphomas	unknown
let-7	tumor suppressor	lung cancer	ras inhibition
miR-17-92 cluster	oncogene	B cell lymphoma	unknown
miR-372	oncogene	testicular	inhibit p53 pathway
miR-373	oncogene	testicular	inhibit p53 pathway

mRNAs, "their importance in biology is probably as great," Brown said. "They're the functional counterpart of transcription factors … and in fact they might be more important." Asuragen is currently evaluating different diagnostic test approaches using microRNAs.

MicroRNA therapy is probably farther off, but the early signs are hopeful. A group led by Rockefeller University researcher Markus Stoffel, M.D., reported last December in *Nature* that chemically modified antisense oligonucleotides—short strings of DNA bases complementary in sequence to their targets—injected into mice potently silenced a target microRNA in the liver. The Stoffel group dubbed these oligonucleotides "antagomirs." Hammond thinks antagomirs should be more effective against cancer-causing microRNAs than classic antisense therapy has been against protein-coding mRNAs, because antagomirs compete with microRNA targets for binding. That's an easier task than interfering with the protein translation machinery, which is the classic antisense mechanism. And antagomirs should benefit from the antisense field's long struggle to overcome problems of delivery, stability, and cellular uptake.

"You should be able to accelerate the development of these [microRNA] inhibitors just by … borrowing the techniques that antisense has already developed," Hammond said.

But the microRNA field is still too new to expect microRNA cancer therapies anytime soon. "The roles of these [microRNA] molecules are still far from clear," said Nagesh Mahanthappa, Ph.D., director of business development and strategy for Alnylam Pharmaceuticals, a Cambridge, Mass., biotech company. "Until we have greater clarity on that I don't think you'll see antagomirs as the focus of today's therapeutic efforts per se."

Alnylam specializes in RNA therapy. The company has an small interfering RNA treatment in early clinical trials, and it made the antagomirs used in the Stoffel experiments. Mahanthappa says he views the potential of microRNA cancer therapies with "measured optimism." Their real therapeutic potential, he noted, will depend on future revelations about how microRNAs function in the biology of cancer and other diseases.

"The antagomir technology is definitely going to be part of the long-term therapeutic vision of [Alnylam]," he said. "And undoubtedly there are going to be even more discoveries in the coming months and years relating to the roles of small RNAs."

—Ken Garber

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BIG JOB FOR LITTLE RNAs

MicroRNAs Found Cavorting With p53

By Mary Beckman

The tumor suppressor protein p53 has long held the spotlight as master of ceremonies in the meteoric rise of malignancies. Now, a class of small RNAs that has been waiting in the wings appears poised to take center stage—or at least dance backup.

Over the last several years, more and more of the small RNAs called microRNAs have been uncovered performing a variety of duties in cancer (*see* J Natl Cancer Inst 2006;98:885–7). Now, several research groups have unearthed microRNAs working with one of the most infamous players in cancer biology: tumor suppressor p53.

The finding comes as no surprise, given the number of microRNAs being found in cells. "We expected that some of the targets of such a prominent transcription factor as p53 would be microRNAs," says molecular biologist Guido Bommer, M.D., at the University of Michigan School of Public Health in Ann Arbor.

The past year has seen a burst of studies that link p53 to a family of microRNAs called miR-34. Work from many different laboratories revealed that this set of three microRNAs are involved in the cell cycle and apoptosis, two cellular systems that cancer perturbs. p53 activates miR-34, and miR-34 slows or stops the production of other proteins. "There are about five to 10 experimentally validated targets of miR-34," Bommer says.

The three small RNAs that make up the miR-34 family reside in different places in the genome: miR-34a resides on one chromosome, and miR-34b and miR-34c on a different one. MiR-34b and -34c are produced as one copy and then trimmed down (the combined form is sometimes called miR-34bc). The three RNAs are almost identical, but different kinds of cells produce different amounts of them.

MicroRNAs can prevent proteins from being produced by virtue of their nucleotide sequence. A short sequence complements a sequence found on the target's messenger RNA, and those two RNAs stick together. That link either blocks the message from being copied into protein by the protein-making machinery of the cell or it degrades the message altogether.

To find microRNAs that were controlled by p53 in the first place, cancer biologist Joshua Mendell, M.D., Ph.D., of Johns Hopkins University School of Medicine and his colleagues used colon cancer cells that had been engineered to lack p53. The team damaged the cells' DNA and monitored which microRNAs increased production. By comparing results from the cells with and without p53, the researchers found eight microRNAs ramped up by p53. "MiR-34a was the most robustly induced," Mendell says.

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Molecular biologist Gregory Hannon, Ph.D., of Cold Spring Harbor Laboratory in New York and colleagues performed similar experiments in embryonic mouse cells. In addition to comparing microRNAs in cells that contain p53 versus those that do not, the team curbed the production of p53 and then let the cells produce p53 again. The production of miR-34 increased in parallel with that of p53 in the cells that resumed making the tumor suppressor protein.

But just because both molecules arise together does not necessarily mean that they work together. To determine whether p53 directly works with miR-34, Mendell's team mutated the nucleotide sequence in miR-34 that was suspected to interact with p53 and found that the cells could no longer increase their production of the microRNA. Hannon's group found that when they used antibodies to fish out p53 from mashed-up cells, miR-34a and miR-34bc came along for the ride. Together, these experiments showed that the protein and the RNA molecule work side by side.

Mendell's group then tested what happens when miR-34 ramps up in the absence of any DNA damage. They infected colon cancer cells with copies of the microRNA, and about a quarter of the cells underwent apoptosis, or programmed cell death. However, when they repeated the experiment in the cells without p53, only 10% of the cells did so. "That tells us that there are multiple mechanisms by which apoptosis happens," Mendell says.

A second group, led by Moshe Oren, Ph.D., of the Weizmann Institute of Science in Israel, also demonstrated miR-34a's role in apoptosis: Inactivating miR-34a prevented p53 from causing cultured cells to self-terminate. And increasing the amount of the microRNA increased p53-induced apoptosis, mirroring Mendell's result.

Bommer found one way in which miR-34a could induce apoptosis. The protein Bcl2 normally protects a cell from undergoing programmed cell death. When he mixed molecules carrying the regulatory region of Bcl2 RNA and miR-34a RNA, the micro molecule prevented the Bcl2 RNA from being converted into a protein. When miR-34a levels go up in a cell,

Bommer suggests, they could turn off Bcl2, allowing cells to kill themselves.

In addition to causing apoptosis, the microRNA can simply stop a cell from growing. Cultured colon cancer cells normally produce small amounts of miR-34a. Bommer's group overproduced miR-34a in those cells and found that the cells quit growing. This result suggested that miR-34a controls some molecular players in the cell cycle.

Support for this result came from Hannon's laboratory. His team produced the miR-34 molecules in cultured cells of four tumors and looked for proteins whose production fell over the next 24 hours. He further investigated three proteins as possible targets of miR-34, two of which are involved in the cell cycle: cyclin E2; cyclin-dependent kinase 4; and a protein involved in liver cell growth, hepatocyte growth factor receptor.

Hannon then asked whether miR-34 carries p53's cease-and-desist signal to the targets. MiR-34 sticks to a particular six-nucleotide sequence in messenger RNA molecules, and Hannon's group mutated that sequence in their three proteins of interest. The mutation allowed the kinase and the growth factor receptor to continue to be produced even in the presence of miR-34a, and cyclin E2 recovered to about 80%. These results suggest that p53 uses miR-34 to turn off some genes.

Moving from culture to real life, Oren's team showed that p53 activates miR-34 in animals: Irradiating mice increases their production of miR-34a. Furthermore, enhanced production of miR-34a in animals can actually protect against cancer. Hannon injected mice with a type of liver cancer cell whose p53 is temporarily suppressed, allowing the cells to grow malignantly. Once the suppression of p53 was lifted, the cells pumped out high levels of the miR-34s. And the tumors stopped growing, strongly suggesting that miR-34a can function as a tumor suppressor similar to p53.

While miR-34a seems to be stealing the spotlight, miR-34b and -34c also have roles, depending on the tissue type. Bommer examined levels of miR-34 in a variety of tissues and found that the lung had the highest level of miR-34bc. Delving into lung cancer, he found that six of 14 lung tumor types had lost much of their miR-34bc. In these samples, miR-34a was not consistently up or down, he says.

Many questions remain to be answered about miR-34. For example, Mendell says that how miR-34 works its magic is not clear. "The microRNA could be regulating one target or modulating expression of many transcripts to a small degree," he says. And with some pancreatic cancer cells, the cells retain p53 instead of losing it and get rid of miR-34 instead. "It's possible that miR-34 is a tumor suppressor," he says.

Bommer's group is currently trying to knock out the miR-34 genes in mice to see what happens in those animals. Along with all the tissue culture results, such work will help researchers understand how big of a role the tiny molecule plays in the huge scourge that is cancer.

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