Putting extracellular RNA to the diagnostic test

Tracking RNA in body fluids could reveal early signs of myriad diseases. **By Elie Dolgin**

The body's tissues routinely communicate with each other through RNA messages sent back and forth between cells. So, it seemed obvious to scientists that, by eavesdropping on these extracellular communiqués carried in blood, saliva, urine and other fluids, they should be able to intercept dispatches indicative of health and disease.

If only it were that easy. "When people got into this, we were all a bit naive," says Louise Laurent, a perinatologist at the University of California, San Diego (UCSD).

Laurent is one of a growing number of scientists trying to develop minimally invasive RNA tests for the early detection and clinical management of cancer, heart disease, neurodegeneration and other ailments. But the inherent diversity of extracellular RNA (exRNA) molecules, and the packages that transport them, poses a considerable challenge. "I don't think anybody expected the complications of the biology," says Laurent, whose own research focuses on using exRNA to predict complications in pregnancy.

Heterogeneity of the RNA repertoire can make it difficult to discern clinically useful biomarkers amid the background molecular noise. And then, to confound matters further, all sorts of technical challenges are associated with the collection, processing and analysis of exRNAs from biological samples. These can make it hard to compare results from different laboratories – a necessary step in the discovery and validation of exRNA biomarkers.

Take, for example, methods for isolating extracellular vesicles (EVs) – envelopes of fatty molecules, typically about one-thousandth the size of a human cell, that protect their cargo from the RNA-degrading enzymes found in most biological fluids. In a study of some 1,700 experiments involving the vesicles, researchers found more than 1,000 unique protocols for extracting them from biofluids[1]. The procedural distinctions were often seemingly minor – involving, say, a different rotor type for spinning samples to separate their molecular components. But as study author An Hendrix, a cancer biologist who studies EVs at Ghent University in Belgium, points out, "changing a few parameters can really influence the vesicles that you obtain from a sample."

DAVID PARKINS

Research on exRNA for diagnostics has been intensifying over the past five years, and universities and companies are diving into the field in the hope of coming up with medically useful techniques. Making progress on both the biological and technical fronts, scientists have begun to tease apart how RNA molecules find themselves bound up inside EVs and other carriers, and they are discovering what role these molecular missives have under various physiological or pathological conditions.

Thanks to initiatives such as the Extracellular RNA Communication Consortium (ERCC), new methods are also being developed to improve the standardization and reproducibility of exRNA detection. The consortium, a US$160-million, 10-year programme, was launched in 2013 by the US National Institutes of Health (NIH) to jump-start the clinical development of exRNA-based diagnostics and therapeutics.

Although obstacles to widespread clinical adoption remain — not least, the ability to obtain pure populations of vesicles — some 'liquid biopsy' tests that rely on exRNA signatures in biofluids have already hit the market, providing actionable information for patients facing an uncertain cancer diagnosis. Similar diagnostic probes could follow for diseases of all kinds.

"There's tremendous growth in the field," says Danilo Tagle, a molecular geneticist who is associate director for special initiatives at the NIH's National Center for Advancing Translational Sciences in Bethesda, Maryland, and is helping to coordinate the ERCC. "It's driving companies now to commercialize a number of these approaches."

Fluid assets

At a laboratory in Waltham, Massachusetts, technicians routinely process thousands of vials of urine each month. They pull out all the EVs from each sample, and then isolate the many RNAs they contain.

This is the home of Exosome Diagnostics, a subsidiary of Bio-Techne and the first company in the world to offer an EV-based diagnostic assay for clinical use. The test, known as ExoDx, is designed for older men whose blood levels of prostate-specific antigen (PSA) are slightly elevated, to help them decide whether to get a biopsy of their prostate.

A prostate biopsy involves inserting a needle roughly the width of a pinhead through the rectum and extracting a small nib of tissue. The procedure often leaves men in pain, with bleeding, infections and bladder trouble. But without a biopsy, it can be difficult to know which individuals with PSA scores in the 'grey zone' of 2–10 nanograms per millilitre have aggressive high-grade tumours that need to be cut out, and whom can safely be left alone. Current estimates are that less than one-quarter of men with middling PSA results turn out to have aggressive cancer.

ExoDx aims to spare more men from invasive biopsies, and the overtreatment that often follows, by quantifying the levels of three particular RNA molecules found in EVs from urine samples. Two relate to genes that encode regulatory proteins — one cancer-promoting and one tumour-suppressing — while the third is associated with a gene that carries the instructions for making a non-protein-coding RNA implicated in prostate cancer development. By assessing these genes' relative activity, the test is able to estimate an individual's risk of aggressive prostate cancer.

In two clinical trials involving a total of more than 1,000 men with intermediate PSA levels, the test proved highly predictive of who had a worrying cancer, and so should consider a biopsy, and who had more benign disease and could reasonably opt for a watch-and-wait approach[2,3].

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The company is expanding into other clinical areas. A second EV-based urine test, now in the works, would predict early which kidney-transplant recipients are at risk of rejecting their donor organs. A blood test to detect gene mutations on the basis of both exRNA and circulating tumour DNA in people with lung cancer is also under development. "This is not just a diagnostic. This is a platform," says Exosome's co-founder and chief scientific officer, Johan Skog.

Many other molecular diagnostics firms and academic research teams are also looking at exRNA as a way to spot warning signs of cancer or aid in risk stratification. For example, Cepheid, in Sunnyvale, California, and Pacific Edge, in Dunedin, New Zealand, offer urine tests that measure levels of five protein-encoding RNAs, to identify bladder cancer in its earliest stages or to monitor for signs of post-treatment recurrence. That strategy follows the logic of Exosome's urine test — collect a body fluid close to the source of the malignancy and probe it for RNA shed by cancer. The same approach has been taken to test spinal fluid for RNAs associated with brain cancer and saliva for RNAs linked to mouth cancer. But tumours also emit RNAs that spread throughout the body.

Most teams have gone looking for these systemic exRNA footprints in blood. However, David Wong, an oral biologist at the University of California, Los Angeles, has shown that they are detectable in saliva, too. Working with clinical collaborators at the Samsung Medical Center in Seoul, Wong has examined the spittle of some 2,500 individuals and identified a saliva signature comprising 9 RNAs — some human, some bacterial — that is highly predictive of who will develop stomach cancer, the most common malignancy in South Korea.

Spit test

Irrespective of the body fluid under consideration, exRNAs might form only part of the diagnostic equation. That's why Freenome is trying to capture the totality of a tumour's biology by taking a multiomics approach to the problem of colorectal cancer screening. It is inspecting proteins, DNA, epigenetic biomarkers and other circulating indicators of disease alongside RNA in blood samples.

"We need the breadth of different diagnostics," says Jimmy Lin, chief scientific officer of Freenome, which is based in South San Francisco, California. "One single analyte alone isn't able to detect all the information" necessary to unmask cancers lurking in the molecular shadows, he explains.

Freenome scientists reported at a conference this year (see go.nature.com/3ge6wjc) that the company's platform, which relies on machine-learning algorithms to sift through its reams of biological data, picked up more than 90% of cancer cases — and outperformed an approved stool-based test that is the only current alternative to a colonoscopy in colon-cancer screening. Owing to the unpleasant and inconvenient nature of colonoscopy, many people skip routine testing.

A handful of exRNA-focused start-ups are branching out beyond cancer diagnostics and directing their efforts to diseases of the heart and brain. For instance, Dyrnamix in Lexington, Massachusetts, is developing exRNA diagnostics to personalize treatment for people with cardiovascular disease. Meanwhile, Neurodex, a few kilometres away in Natick, is capturing neuron-derived exosomes from blood, and then scrutinizing the RNAs and proteins inside them in the hope of spotting early indications of Alzheimer's disease.

"In general," says Neurodex chief scientific officer Erez Eitan, a neuroscientist by training, "neuro people don't like RNA." Unlike cancer, he explains, the pathology of neurological disease is at the protein level — not in the DNA or RNA — and so the research community

outlook

JOHAN SKOG/ EXOSOME DIAGNOSTICS

Johan Skog processes samples in Exosome's laboratory in Waltham, Massachusetts.

doesn't know much about the role of RNA in neurodegeneration.

That partly explains why most experimental blood tests for presymptomatic detection of Alzheimer's measure for levels of β-amyloid fragments, activated tau or some other protein whose build-up in the brain has come to define the disease. This strategy, however, is akin to rummaging through someone's rubbish bin to determine how they live, says Victoria Risbrough, a neuroscientist at the Veterans Affairs Center of Excellence for Stress and Mental Health in San Diego, California. A better way of understanding their quirks and idiosyncrasies, Risbrough suggests, would be to read their communications – which is exactly what exRNA represents.

"The RNA gives you a sense of what might be driving some pathology," says Risbrough, who is collaborating with UCSD neuropathologist Robert Rissman to develop diagnostics for traumatic brain injury. "Instead of sifting through the trash, you're looking at their text messages."

Value add

A broad screening test for brain disease remains the ultimate goal. But according to Kira Sheinerman, co-founder and chief executive of DiamiR in Monmouth Junction, New Jersey, the initial customers for most exRNA diagnostics will probably be drug companies running clinical trials of investigational therapeutics.

With DiamiR's panel of brain-enriched and inflammation-associated microRNAs found in blood, for example, Sheinerman and her colleagues have shown that they can predict with 84% accuracy whether an older person with no signs of cognitive impairment will go on to develop Alzheimer's disease. That kind of information, Sheinerman says, could help drug developers and clinical researchers to better identify people with presymptomatic Alzheimer's who might be good candidates for an experimental therapy that's undergoing clinical evaluation.

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And even if these exRNA tests never become widely used, "there's value in generating new hypotheses of the disease", says neurologist Joseph Quinn at the Oregon Health and Science University in Portland. Working with molecular neurobiologist Julie Saugstad, Quinn has discovered a series of microRNAs with diagnostic potential for Alzheimer's that could also point to possible biological pathways for future drug development. However, Quinn cautions, the basic science of this process is still not well understood. As a result, his efforts in the field of exRNA diagnostics – like those of so many other researchers – remain in an exploratory phase.

Part of that exploration involves developing tools to refine the methods for isolating EVs. Although some researchers have had success profiling exRNAs without first plucking out EVs from their body fluids of origin, this bulk approach to RNA analysis can often miss subtle signals of biological relevance. So, the field is steadily moving away from total RNA sequencing of human biofluids and towards strategies that zero in on particular vesicles secreted by organs of interest. "It's homing in on where the signal is," says Saumya Das, a cardiac electrophysiologist at Massachusetts General Hospital in Boston and a co-founder of Dyrnamix.

Conventionally, scientists have attempted to extract EVs by spinning samples in an ultracentrifuge and then relying on differences in size and density between molecular components to obtain the vesicles of interest. But this approach is not perfect. "There's still some contamination," says Esther Nolte-'t Hoen, an immune cell biologist at Utrecht University in the Netherlands. "You will not get 100% purity based on size and density."

Techniques in development include filtering EVs through tiny pores of various diameters, or using binding agents to pull target EVs out of a sample. Whatever the method, a validated reference material is necessary for accurate calibration – something that has only recently started to become available.

Last year, for example, Hendrix and her colleagues at Ghent University developed a bioengineered EV that can be spiked into biofluids and then experimentally tracked to help check the accuracy of sample preparation and analytical protocols[4]. "The field has long been searching for such a reference material," says Hendrix, who has shared her traceable EVs with dozens of labs around the world. "After every meeting where I present the technology, I get multiple requests," she says.

Hendrix and others, including Nolte-'t Hoen, have also been actively involved in community building and data-reporting initiatives to ensure that studies of RNA-containing EVs, including those focused on disease diagnostics, can be properly interpreted and replicated. "It is maybe not scientifically the most exciting thing to do," Nolte-'t Hoen says, "but it's very necessary."

For its part, the NIH, through its massive multimillion-dollar consortium, is now focused on finding better ways to isolate individual EVs and analyse their cargoes. Few exRNA-based diagnostic tests in development today take this level of precision – many don't enrich for EVs at all – and that could be fine for many clinical applications.

But, says Tagle, "to demonstrate rigour, you need to be able to know where your source of information is coming from – and that will, in the end, lead to more robust and reproducible results."

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