A gene of rare effect

A mutation that gives people rock-bottom cholesterol levels has led geneticists to what could be the next blockbuster heart drug.

BY
STEPHEN S. HALL

When Sharlayne Tracy showed up at the clinical suite in the University of Texas (UT) Southwestern Medical Center in Dallas last January, the bandage wrapped around her left wrist was the only sign of anything medically amiss. The bandage covered a minor injury from a cheerleading practice led by Tracy, a 40-year-old African American who is an aerobics instructor, a mother of two and a college student pursuing a degree in business. "I feel like I'm healthy as a horse," she said.

Indeed, Tracy's well-being has been inspiring to doctors, geneticists and now pharmaceutical companies precisely because she is so normal. Using every tool in the modern diagnostic arsenal — from brain scans and kidney sonograms to 24-hour blood-pressure monitors and cognitive tests — researchers at the Texas medical centre have diagnostically sliced and diced Tracy to make sure that the two highly unusual genetic mutations she has carried for her entire life have produced nothing more startling than an incredibly low level of cholesterol in her blood. At a time when the target for low-density lipoprotein (LDL) cholesterol, more commonly called 'bad cholesterol', in Americans' blood is less than 100 milligrams per decilitre (a level many people fail to achieve), Tracy's level is just 14.

A compact woman with wide-eyed energy, Tracy (not her real name) is one of a handful of African Americans whose genetics have enabled scientists to uncover one of the most promising compounds for controlling cholesterol since the first statin drug was approved by the US Food and Drug Administration in 1987. Seven years ago, researchers Helen Hobbs and Jonathan Cohen at UT-Southwestern reported[1] that Tracy had inherited two mutations, one from her father and the other from her mother, in a gene called *PCSK9*, effectively eliminating a protein in the blood that has a fundamental role in controlling the levels of LDL cholesterol. African Americans with similar mutations have a nearly 90% reduced risk of heart disease. "She's our girl, our main girl," says Barbara Gilbert, a nurse who has drawn some 8,000 blood samples as part of Cohen and Hobbs' project to find genes important to cholesterol metabolism.

Of all the intriguing DNA sequences spat out by the Human Genome Project and its ancillary studies, perhaps none is a more promising candidate to have a rapid, large-scale impact on human health than *PCSK9*. Elias Zerhouni, former director of the US National Institutes of Health (NIH) in Bethesda, Maryland, calls *PCSK9* an "iconic example" of translational medicine in the genomics era. Preliminary clinical trials have already shown that drugs that inhibit the PCSK9 protein — used with or without statins — produce dramatic reductions in LDL cholesterol (more than 70% in some patients). Half-a-dozen pharmaceutical companies — all aiming for a share of the global market for cholesterol-reducing drugs that could reach US$25 billion in the next five years according to some estimates — are racing to the market with drugs that mimic the effect of Tracy's paired mutations.

Zerhouni, now an in-house champion of this class of drug as an executive at drug firm Sanofi, headquartered in Paris, calls the discovery and development of *PCSK9* a "beautiful story" in which researchers combined detailed physical information about patients with shrewd genetics to identify a medically important gene that has made "super-fast" progress to the clinic. "Once you have it, boy, everything just lines up," he says. And although the end of the *PCSK9* story has yet to be written — the advanced clinical trials now under way could still be derailed by unexpected side effects — it holds a valuable lesson for genomic research. The key discovery about *PCSK9*'s medical potential was made by researchers working not only apart from the prevailing scientific strategy of genome research over the past decade, but with an almost entirely different approach.

As for Tracy, who lives in the southern part of Dallas County, the implications of her special genetic status have become clear. "I really didn't understand at first," she admits. "But now I'm watching ads on TV [for cholesterol-lowering drugs], and it's like, 'Wow, I don't have *that* problem.'"

A HEART PROBLEM

Cardiovascular disease is — and will be for the foreseeable future, according to the World Health Organization — the leading cause of death in the world, and its development is intimately linked to elevated levels of cholesterol in the blood. Since their introduction, statin drugs have been widely used to lower cholesterol levels. But Jan Breslow, a physician and geneticist at Rockefeller University in New York, points out that up to 20% of patients cannot tolerate statins' side effects, which include muscle pain and even forgetfulness. And in many others, the drugs simply don't control cholesterol levels well enough.

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"The *PCSK9* story is a terrific example of an up-and-coming pattern of translational research."

The search for better treatments for heart disease gained fresh impetus after scientists published the draft sequence of the human genome in 2001. In an effort to identify the genetic basis of common ailments such as heart disease and diabetes, geneticists settled on a strategy based on the 'common variant hypothesis'. The idea was that a handful of disease-related versions (or variants) of genes for each disease would be common enough — at a frequency of roughly 5% or so — to be detected by powerful analyses of the whole genome. Massive surveys known as genome-wide association studies compared the genomes of thousands of people with heart disease, for example, with those of healthy controls. By 2009, however, many scientists were lamenting the fact that although the strategy had identified many common variants, each made only a small contribution to the disease. The results for cardiovascular disease have been "pretty disappointing", says Daniel Steinberg, a lipoprotein expert at the University of California, San Diego.

More than a decade earlier, in Texas, Hobbs and Cohen had taken the opposite tack. They had backgrounds in Mendelian, or single-gene, disorders, in which an extremely rare variant can have a big — often fatal — effect. They also knew that people with a particular Mendelian disorder didn't share a single common mutation in the affected gene, but rather had a lot of different, rare mutations. They hypothesized that in complex disorders, many different rare variants were also likely to have a big effect, whereas common variants would have relatively minor effects (otherwise natural selection would have weeded them out). "Jonathan and I did not see any reason why it couldn't be that rare variants cumulatively contribute to disease," Hobbs says. To find these rare variants, the pair needed to compile detailed physiological profiles, or phenotypes, of a large general population. Cohen spoke of the need to "Mendelize" people — to compartmentalize them by physiological traits, such as extremely high or low cholesterol levels, and then look in the extreme groups for variations in candidate genes known to be related to the trait.

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The pair make a scientific odd couple. Hobbs, who trained as an MD, is gregarious, voluble and driven. Cohen, a soft-spoken geneticist from South Africa, has a laid-back, droll manner and a knack for quantitative thinking. In 1999, they set out to design a population-based study that focused on physical measurements related to heart disease. Organized with Ronald Victor, an expert on high blood pressure also at UT Southwestern, and funded by the Donald W. Reynolds Foundation in Las Vegas, Nevada, the Dallas Heart Study assembled exquisitely detailed physiological profiles on a population of roughly 3,500 Dallas residents[2]. Crucially, around half of the participants in the study were African Americans, because the researchers wanted to probe racial differences in heart disease and high blood pressure. The team measured blood pressure, body mass index, heart physiology and body-fat distribution, along with a battery of blood factors related to cholesterol metabolism — triglycerides, high-density lipoprotein (HDL) cholesterol and LDL cholesterol. In the samples of blood, of course, they also had DNA from each and every participant.

As soon as the database was completed in 2002, Hobbs and Cohen tested their rare-variant theory by looking at levels of HDL cholesterol. They identified the people with the highest (95th percentile) and lowest (5th percentile) levels, and then sequenced the DNA of three genes known to be key to metabolism of HDL cholesterol. What they found, both in Dallas and in an independent population of Canadians, was that the number of mutations was five times higher in the low HDL group than in the high group[3]. This made sense, Cohen says, because most human mutations interfere with the function of genes, which would lead to the low HDL numbers. Published in 2004, the results confirmed that rare, medically important mutations could be found in a population subdivided into extreme phenotypes.

Armed with their extensive database of cardiovascular traits, Hobbs and Cohen could now dive back into the Dallas Heart Study whenever they had a new hypothesis about heart disease and, as Cohen put it, "interrogate the DNA". It wasn't long before they had an especially intriguing piece of DNA at which to look.

THE MISSING LINK

In February 2003, Nabil Seidah, a biochemist at the Clinical Research Institute of Montreal in Canada, and his colleagues reported the discovery of an enigmatic protein[4]. Seidah had been working on a class of enzymes known collectively as proprotein convertases, and the researchers had identified what looked like a new member of the family, called NARC-1: neural apoptosis-regulated convertase 1.

"We didn't know what it was doing, of course," Seidah says. But the group established that the gene coding the enzyme showed activity in the liver, kidney and intestines as well as in the developing brain. The team also knew that in humans the gene mapped to a precise genetic neighbourhood on the short arm of chromosome 1.

That last bit of geographical information pointed Seidah to a group led by Catherine Boileau at the Necker Hospital in Paris. Her team had been following families with a genetic form of extremely high levels of LDL cholesterol known as familial hypercholesterolaemia, which leads to severe coronary artery disease and, often, premature death. Group member Marianne Abifadel had spent five

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Single-minded: Helen Hobbs and Jonathan Cohen's approach to heart-disease genetics yielded a target for drugs that could compete with statins.

fruitless years searching a region on the short arm of chromosome 1 for a gene linked to the condition. When Seidah contacted Boileau and told her that he thought *NARC-1* might be the gene she was looking for, she told him, "You're crazy", Seidah recalls. Seidah bet her a bottle of champagne that he was correct; within two weeks, Boileau called back, saying: "I owe you three bottles."

In 2003, the Paris and Montreal groups reported that the French families with hypercholesterolaemia had one of two mutations in this newly discovered gene, and speculated that this might cause increased production of the enzyme[5]. Despite Seidah's protests, the journal editors gave both the gene and its protein product a new name that fit with standard nomenclature: proprotein convertase subtilisin/kexin type 9, or *PCSK9*. At around the same time, Kara Maxwell in Breslow's group at Rockefeller University[6] and Jay Horton, a gastroenterologist at UT-Southwestern[7] also independently identified the *PCSK9* gene in mice and revealed its role in a previously unknown pathway regulating cholesterol[8].

The dramatic phenotype of the French families told Hobbs that "this is an important gene". She also realized that in genetics, mutations that knock out a function are much more common than ones that amplify function, as seemed to be the case with the French families. "So immediately I'm thinking, a loss-of-function mutation should manifest as a low LDL level," she says. "Let's go and see if that's true."

GOING TO EXTREMES

Hobbs and Cohen had no further to look than in the extreme margins of people in the Dallas Heart Study. In quick order, they identified the highest and lowest LDL readings in four groups: black women, black men, white women and white men. They then resequenced the *PCSK9* gene in the low-cholesterol groups, looking for mutations that changed the make-up of the protein.

They found seven African Americans with one of two distinct 'nonsense' mutations in *PCSK9* — mutations that essentially aborted production of the protein. Then they went back and looked for the same mutations in the entire population. Just 2% of all black people in the Dallas study had either of the two *PCSK9* mutations — and those mutations were each associated with a 40% reduction of LDL cholesterol in the blood[9]. (The team later detected a 'missense mutation' in 3% of white people,which impaired but did not entirely block production of the protein.) The frequency of the mutations was so low, Hobbs says, that they would never have shown up in a search for common variants.

When Hobbs and Cohen published their findings in 2005, they suggested that *PCSK9* played a crucial part in regulating bad cholesterol, but said nothing about whether the mutations had any effect on heart disease. That evidence came later that year, when they teamed up with Eric Boerwinkle, a geneticist at the University of Texas Health Science Center in Houston, to look for *PCSK9* mutations in the Atherosclerosis Risk in Communities (ARIC) study, a large prospective study of heart disease that had been running since 1987. To experts such as Steinberg, the results[10] — published in early 2006 — were "mind-blowing". African Americans in ARIC who had mutations in *PCSK9* had 28% less LDL cholesterol and an 88% lower risk of developing heart disease than people without the mutations. White people with the less severe mutation in the gene had a 15% reduction in LDL and a 47% reduced risk of heart disease.

How did the gene exert such profound effects on LDL cholesterol levels? As researchers went on to determine[11], the PCSK9 protein normally circulates in the bloodstream and binds to the LDL receptor, a protein on the surface of liver cells that captures LDL cholesterol and removes it from the blood. After binding with the receptor, PCSK9 escorts it into the interior of the cell, where it is eventually degraded. When there is a lot of PCSK9 (as in the French families), there are fewer LDL receptors remaining to trap and remove bad cholesterol from the blood. When there is little or no PCSK9 (as in the black people with mutations), there

are more free LDL receptors, which in turn remove more LDL cholesterol.

The UT-Southwestern group, meanwhile, went back into the community looking for family members who might carry additional *PCSK9* mutations. In September 2004, Gilbert, the nurse known as 'the cholesterol lady' in south Dallas because of her frequent visits, knocked on the door of Sharlayne Tracy's mother, an original member of the Dallas Heart Study. Gilbert tested Tracy, as well as her sister, brother and father. "They tested all of us, and I was the lowest," Tracy says. Zahid Ahmad, a doctor working with Hobbs at UT-Southwestern, was one of the first to look at Tracy's lab results. "Dr Zahid was in awe," Tracy recalled. "He said, 'You're not supposed to be so healthy!'."

It wasn't just that her LDL cholesterol measured 14. As a person with two dysfunctional copies of the gene — including a new type of mutation — Tracy was effectively a human version of a knockout mouse. The gene had been functionally erased from her genome, and PCSK9 was undetectable in her blood without any obvious untoward effects. The genomics community might have been a little slow to understand the significance, Hobbs says, "but the pharmaceutical companies got it right away".

> "We didn't understand why everybody wasn't doing what we were doing."

THE NEXT STATIN?

This being biology, however, the road to the clinic was not completely smooth. The particular biology of PCSK9 has so far thwarted efforts to find a small molecule that would interrupt its interaction with the LDL receptor and that could be packaged in a pill. But the fact that the molecule operates outside cells means that it is vulnerable to attack by monoclonal antibodies — one of the most successful (albeit most expensive) forms of biological medicine.

The results of early clinical trials have caused a stir. Regeneron Pharmaceuticals of Tarrytown, New York, collaborating with Sanofi, published phase II clinical-trial results[12] last October showing that patients with high LDL cholesterol levels who had injections every two weeks of an anti-PCSK9 monoclonal antibody paired with a high-dose statin saw their LDL cholesterol levels fall by 73%; by comparison, patients taking high-dose statins alone had a decrease of just 17%. Last November, Regeneron and Sanofi began to recruit 18,000 patients for phase III trials that will test the ability of their therapy to cut cardiovascular events, including heart attacks and stroke. Amgen of Thousand Oaks, California, has also launched several phase III trials of its own monoclonal antibody after it reported similarly promising results[13]. Among other companies working on PCSK9-based therapies are Pfizer headquartered in New York, Roche based in Basel, Switzerland, and Alnylam Pharmaceuticals of Cambridge, Massachusetts. (Hobbs previously consulted for Regeneron and Pfizer, and now sits on the corporate board of Pfizer.)

Not everyone is convinced that a huge market awaits this class of cholesterol-lowering drugs. Tony Butler, a financial analyst at Barclays Capital in New York, acknowledges the "beautiful biology" of the *PCSK9* story, but wonders if the expense of monoclonal drugs — and a natural reluctance of both patients and doctors to use injectable medicines — will constrain potential sales. "I have no idea what the size of the market may be," he says.

"Everything hinges on the phase III side effects," says Steinberg. So far, the main side effects reported have been minor, such as reactions at the injection site, diarrhoea and headaches. But animal experiments have raised potential red flags: the Montreal lab reported in 2006 that knocking out the gene in zebrafish is lethal to embryos[14]. That is why the case of Tracy was "very, very helpful" to drug companies, says Hobbs. Although her twin mutations have essentially deprived her of PCSK9 throughout her life, doctors have found nothing abnormal about her.

That last point may revive a debate in the cardiology community: should drug therapy to lower cholesterol levels, including statins and the anti-PCSK9 medicines, if they pan out, be started much earlier in patients than their 40s or 50s? That was the message Steinberg took from the people with *PCSK9* mutations in the ARIC study — once he got over his shock at the remarkable health effects. "My first reaction was, 'This must be wrong. How could that be?'And then it hit me — these people had low LDL from the day they were born, and that makes all the difference." Steinberg argues that cardiologists "should get off our bums" and reach a consensus about beginning people on cholesterol-lowering therapy in their early thirties. But Breslow, a former president of the American Heart Association, cautions against being too aggressive too soon. "Let's start out with the high-risk individuals and see how they do," he says.

Not long after Hobbs and Cohen published their paper in 2006, they began to get invited to give keynote talks at major cardiology meetings. Soon after, the genetics community began to acknowledge the strength of their approach. In autumn 2007, then-NIH director Zerhouni organized a discussion at the annual meeting of the institutes' directors to raise the profile of the rare-variant approach and contrast it with genome-wide studies. "Obviously, the two approaches are opposed to each other, and the question was, what was the relative value of each?" says Zerhouni. "I thought the *PCSK9* story was a terrific example of an up-and-coming pattern of translational research" — indeed, he adds, "a harbinger of things to come".

Hobbs and Cohen might not have found their gene if they had not had a hunch about where to look, but improved sequencing technology and decreasing costs now allow genomicists to incorporate the rare variant approach and to mount large-scale sweeps in search of such variants. "Gene sequencing is getting cheap enough that if there's another gene like *PCSK9* out there, you could probably find it genome-wide," says Jonathan Pritchard, a population biologist at the University of Chicago, Illinois.

"What was amazing to us," says Hobbs, "was that the genome project was spending all this time, energy, effort sequencing people, and they weren't phenotyped, so there was no potential for discovery. We didn't understand, and couldn't understand, why everybody wasn't doing what we were doing. Particularly when we started making discoveries." ■

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