PROTEIN BIOMARKERS SEEK VALIDATION

As proteomics researchers uncover potential disease markers by the hundreds, basic scientists and equipment manufacturers are still struggling with the problem of testing and validating this new trove of results. By Alan Dove

In 1896, administrators at Johns Hopkins Hospital in Baltimore approved an extravagance: they bought a suite of specialized equipment for culturing microorganisms, examining tissues, and analyzing body fluids. Stuffing this gear into a 12-foot-square room, the hospital created the world’s first dedicated clinical laboratory. The total bill was $50.

Fed by a rush of new discoveries in microbiology and pathology, clinical laboratories and their specialized tests soon became ubiquitous. Though the field required increasing quantities of expensive equipment and trained technicians, the enormous clinical benefits more than offset the mushrooming costs. Knowing exactly what was wrong with a patient—and knowing it as early as possible—became the benchmark of good medical care.

Like most revolutions, clinical testing soon reached a plateau. Even today, many deadly diseases remain extremely hard to diagnose. If you have strep throat or a thyroid condition, your doctor will know it very quickly, but cancer or Alzheimer’s disease might not be apparent until the disease is quite advanced.

Armed with new molecular tools, developed as the field of proteomics has grown, some researchers are now trying to tackle these tough cases with panels of protein biomarkers. Even in the early stages of a disease, the theory goes, cells in the body often change the expression levels of numerous proteins. Detecting those changes would allow clinicians to identify the disease quickly and more definitively. Reducing that theory to practice hasn’t been easy, but recent work suggests that it is at least possible.

Less Is More

The new strategy differs markedly from the old way of developing clinical tests. “The historical trend was you were more focused on a mechanism, and you developed tests for that,” says Martin Latterich, associate professor of pharmacy at the University of Montreal in Montreal, Quebec. With the advent of high throughput technologies, some researchers started comparing whole proteomes instead, looking at all of the proteins expressed in a sample from a sick patient, and comparing that panel to the proteome of a healthy control.

“Instead of looking at one thing at a time, we look at complex samples and don’t necessarily worry about if the protein itself is mechanistically involved,” says Latterich.

Casting such a broad net, the scientists inevitably find changes that are directly related to the disease mechanism, but they also find many surrogate biomarkers, which change in response to downstream events as the disease progresses. Critics of high throughput biomarker screening argue that this shotgun approach lacks the intellectual rigor of traditional mechanistic studies.

Latterich, who responded to that complaint in a 2005 editorial in *Proteome Science* (*Proteome Sci.* 3:8, October 28, 2005), prefers to see mechanistic and high throughput studies as complementary, with different biologists choosing to learn “less about more, or more about less.”

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“Validation really requires a joint effort between one lab that would be devoted to discovery and then another lab that has expertise in antibodies or ELISA.”

A more practical problem with modern biomarker screens is that they also uncover numerous false leads. “We’re really going into a lot of these studies blind; we don’t know exactly what the biomarker’s going to be. It’s not a case where we can look at all the differences and say ‘Aha, that’s the biomarker,’” says Timothy Veenstra, director of the laboratory of proteomics and analytical technologies, SAIC-Frederick at the National Cancer Institute (NCI) at Frederick, Maryland.

Indeed, many biomarker researchers cite validation—the process of winnowing a huge panel of potential biomarkers down to a much smaller set of usable ones—as the field’s biggest challenge. For Veenstra, whose lab focuses on biomarkers for prostate, breast, and ovarian cancer, the first problem is deciding which of the hundreds of protein expression changes in an initial screen are even worth pursuing. “Some you can throw out, maybe because they’re related to inflammation or acute phase response proteins that aren’t going to have any specificity for a particular cancer,” says Veenstra.

Even after throwing out obvious red herrings, researchers must still develop robust, reproducible tests for them. “Validation really requires a joint effort between one lab that would be devoted to discovery and then another lab that has expertise in antibodies or ELISA,” says Veenstra. ELISAs, or enzyme-linked immunosorbent assays, are the standard protein-screening tool in clinical labs, but developing a new ELISA test can take months. The more candidate biomarkers a team wants to take into validation, the more tests they need to develop.

To help address this bottleneck, manufacturers are starting to streamline the decades-old ELISA technique. One promising development is Perkin Elmer’s AlphaLISA system. Based on a proprietary antibody-binding bead system, the AlphaLISA eliminates the finicky washing steps of the standard ELISA procedure, and also makes the assay much easier to automate. The latter feature could be a particularly strong selling point as tests based on multiple biomarkers start to filter into already-overtaxed clinical laboratories.

Indeed, the first proteomics-based clinical assays may be just around the corner. Pharmaceutical and clinical testing companies have already shown they are willing to invest in the new generation of biomarkers, even though validation and testing can be costly. After NCI researchers discovered protein expression patterns that appear to correlate with ovarian cancer prognosis, the clinical laboratory firm Correlogic Systems of Rockville, Maryland, was quick to pick up the project. The resulting test, known as OvaCheck, remains in regulatory limbo, but experts seem confident that tests like it will soon reach the market. Meanwhile, the US Food and Drug Administration has started soliciting proteomic data from companies, in order to keep its staff up to speed on the technology as the new proteomics-based tests continue to move into the regulatory process.

Chip, Column, or Gel?

With numerous pharmaceutical and diagnostic companies working to validate biomarkers with traditional techniques such as ELISA, at least one company thinks it has found a better solution. At Protagen, in Dortmund, Germany, researchers are using protein chips to screen for autoantibody-based biomarkers. In a typical experiment, the scientists apply serum from a patient to a chip with a huge panel of recombinant human proteins, representing the proteome of the fetal brain. “We are comprehensively screening patient sera against this collection of 10,000 proteins, [and] are finding unique autoantibody recognition patterns that can be used as diagnostics,” says Christoph Huels, the company’s CEO.

One example of success using the protein biochip is the ability “to diagnose multiple sclerosis (MS) with more than 80 percent sensitivity and specificity,” according to Huels.

Using a chip-based system for both the discovery and validation phases could help get tests into the clinic faster, but the strategy has limitations. For example, it will reveal only biomarkers that involve autoantibody responses, and only when they target antigens that appear in the company’s panel of recombinant proteins.

Nonetheless, Huels argues that the approach may be more broadly useful than expected. “In the beginning we said it might be only applicable to autoimmune disease, [but] so far in each disease we have looked at it’s working,” he says. Besides MS, the company has found distinctive autoantibody patterns in Alzheimer’s disease and prostate cancer, and academic researchers have proven the principle in alopecia and dilated cardiomyopathy as well.

Though Protagen currently focuses on its own drug development programs, it is not the only one working with autoantibody-based screening chips. Scientists who want to buy the technology off the shelf can simply call Invitrogen, which offers several types of antibody discovery chips in its ProtoArray line. The chips can profile as many as 8,000 proteins in a single run, using as little as 1μL of serum or plasma as a starting sample.

For many gear makers and basic researchers, though, biomarker discovery focuses on more traditional technologies, especially liquid chromatography and mass spectrometry. Fortunately, these tools have been evolving rapidly in recent years (see “Mass Spectrometry for the Masses,” Science 319:1115, 2008), and companies have also introduced new equipment designed specifically for biomarker discovery.

One of the first problems biomarker screeners encounter is the chore of depleting the most common proteins from serum or other biological fluids, to allow them to detect the much scarcer but more informative protein signals that might make good biomarkers. While many groups turn to immunodepletion columns for this, Bio-Rad in Hercules, California, recently introduced an alternative, called ProteoMiner.
Instead of antibodies on the column, Bio-Rad uses “a hexapeptide library with diversity, so it’s a combinatorial chemistry–synthesized library,” says Aran Paulus, the company’s research and development manager for proteomics. Most serum proteins will bind to at least one hexapeptide in the column, but once that hexapeptide is saturated, the surplus protein washes through. After washing the column, the researcher can elute the bound proteins, which come out in a much smaller range of concentrations. According to the company, common proteins are depleted across the board, while rare proteins are retained without bias.

Whatever strategy a researcher uses for initial sample preparation, it often requires some additional cleanup before entering the mass spectrometer. For many modern biomarker screeners, that means a nanoscale high performance liquid chromatography system, which is often connected directly to the mass spectrometer. Unsurprisingly, equipment manufacturers are also trying to squeeze more performance out of this phase of the process.

One new strategy is to switch from the usual nanoscale system, which splits the fluid flow in order to achieve the correct flow rate, to a microfluidic pump that regulates its flow rate by feedback. “Our technology allows you to generate these flow rates in the ranges of 50–300 nanoliters per minute without flow splitting, and it provides good reproducibility,” says Remco van Soest, product manager for Eksigent Technologies in Dublin, California, which makes a feedback-regulated pump.

The mass spectrometer is the common endpoint for proteomic experiments, but some biomarker studies still perform their initial separations with a much older technique: 2-dimensional gel electrophoresis. Indeed, for many biochemists, 2-D gel technology is akin to the fictitious rock band Spinal Tap—though neither a critics’ nor a public favorite, it continues to fill a much-needed void.

“A lot of people find [the 2-D gel technique] very cumbersome to work with, because it’s long, it’s labor intensive, and it’s believed to be not very reproducible,” says Bio-Rad’s Paulus. “However, in our experience if you have the appropriate training and use the methods that are available, and use them religiously, you get very reproducible results,” he adds.

For those willing to invest the effort, 2-D gels offer distinct advantages over other chromatography techniques in proteomics. The gels preserve posttranslational modifications, which can be especially important in biomarker studies, as many diseases can perturb glycosylation or other modifications. Plus 2-D gels offer tremendous resolving power compared to conventional liquid chromatography, with a single gel run capable of separating thousands of proteins into distinct spots. “No other chromatography technology is coming even close. The resolution of 2-D gels is still unparalleled,” says Paulus.

The technique is also getting at least a little bit easier, thanks to equipment manufacturers who have continued to tweak it. Bio-Rad, for example, offers an extensive line of kits, reagents, and equipment to streamline every step of the 2-D gel process, from sample preparation to gel imaging and protein digestion. Once the proteins are removed from the gel and digested into peptides, biomarker researchers can feed them into a mass spectrometer just like any other sample.

### Hired Help
Considering the complexity of biomarker studies, it’s no surprise that many pharmaceutical companies—and even a few basic researchers—hire someone else to do at least some of the work. Indeed, as the field of biomarker discovery becomes more popular, more companies seem to be wading into the contract business, offering everything from occasional assistance with 2-D gels to complete biomarker discovery and validation services.

“We can carry studies all the way through from start to finish,” says Mike Pisano, CEO of NextGen Sciences of Cambridgeshire, UK. Like a handful of other firms, the company has been drawn into the biomarker business partly by the shifting needs of its pharmaceutical company clients.

NextGen started out in laboratory automation, but the rise of biomarker screening led it to acquire Pisano’s original company, Proteomic Research Services of Ann Arbor, Michigan, which specialized in protein purification. Now biomarker work has become a major part of the group’s business. “We developed assays for monitoring proteins quantitatively in clinical samples, and [what] we’ve found is that the pharmaceutical companies are very interested in looking at a set of proteins in clinical trials, in preclinical animal models, or in paraffin-embedded tissues,” says Pisano.

The products that emerge from these experiments may bear little resemblance to traditional clinical tests. Because they will rely on panels of proteins rather than simple assays, for example, interpretation could be more complicated. “From panels, in order to come to some meaningful conclusion, you have to classify the type of patient groups that have a certain type of pattern, and to link that to disease outcome or treatment option,” says Montreal’s Latterich. Equipping the next generation of clinical labs to do those types of analyses, of course, will probably cost more than $50.

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