

Beyond Sequencing: New Diagnostic Tests Turn to Pathways

By Ken Garber

In cancer, as in life, DNA doesn't have the last word. Although some gene mutations can predict response to certain targeted therapies, single-gene testing has limits.

"Most of biology and probably the large fraction of cancer biology doesn't occur at a chromosomal level," said NCI geneticist Ken Buetow, Ph.D., at the 2010 American Association for Cancer Research conference on molecular diagnostics. "It occurs at a network level." Many alterations in many genes interact to create the cancer phenotype, Buetow said, with the frequency of any single alteration "very rarely above single-digit percentages."

What's needed, according to Buetow and others, are diagnostic tests that can examine multiple points in these networks simultaneously and use the information to predict how likely a given treatment is to work. "Clearly, we have to move from looking at single molecules and even single pathways to looking at networks and how they interact if we're going to move forward," said Gordon Mills, M.D., Ph.D., chair of the department of systems biology at the M. D. Anderson Cancer Center in Houston.

Such pathway and network diagnostic tests may be on the way. Some of the most advanced are designed to detect alterations in proteins, since proteins—especially phosphorylated signaling proteins—more directly reveal the activation state of pathways and networks than do DNA sequence or gene expression. "We give you contextual understanding of the net result of all the physiological, of all the genetic change, and all the epigenetic change, and probably other control system changes,"

said David Parkinson, M.D., CEO of South San Francisco, Calif., biotech company Nodality, which is developing some of the new proteomic diagnostic tests. "That's the level at which drugs work or don't work."

The success of these methods will depend on the ability of companies to validate the tests' effectiveness and reproducibility in clinical trials. For some, such trials are already under way.

Going With the Flow

By focusing on protein activation, Nodality identifies alterations in intracellular signaling pathways, apoptosis, and DNA repair to monitor drug treatment effects and creates tests to predict which drugs may be effective in specific patients. Called single-cell network profiling (SCNP), the method uses flow cytometry to detect changes in cells. In flow cytometry, a stream of single cells in fluid suspension, stained with fluorescent-labeled antibodies, are illuminated by a laser, which excites the fluorescence. Some of the fluorescent light, focused on sensors, generates electrical signals proportional to the number of fluorescent molecules in each cell. These signals, processed by computer, are plotted onto graphs that depict numbers of cells with the desired properties.

Flow cytometry in the past relied mainly on cell surface markers to select cells, but Stanford University scientists reported in 2002 that multiple phosphorylated intracellular proteins could be simulta-

neously distinguished by using antibodies. In 2004, Stanford's Garry Nolan, Ph.D., reported in *Cell* that such information, in human acute myelogenous leukemia (AML), could be used to identify patterns

of response and resistance to chemotherapy, as well as new signaling pathways. In 2006, Stanford spun off Nodality to commercialize the technology.

In SCNP, tumor cells are fixed, made permeable, and stained with fluorescent antibodies for flow cytometry detection of target proteins. But first a special "perturbation" step occurs. The cells are stimulated with some kind of input, such as growth factors, cytokines, or a drug. "The additional trick here is to come at the cell and invoke it with various pokes and proddings to actually stimulate a signaling network to form," explained Nolan at a 2007 lecture, "because it's the signaling network that is informative."

In research studies using SCNP, said Parkinson, as many as 150 different stimuli have been applied to cells before flow cytometry. For clinical tests, that number has been reduced to single digits, and antibodies will be used against about a dozen different proteins to profile activated pro-tumor pathways.

Several drug clinical trials, most for AML, are testing SCNP. (Because flow cytometry requires cells in suspension—not attached to a surface—SCNP is harder to use for solid tumors.) At the 2010 annual meeting of the American Society of

Hematology, Nodality reported results in two AML trials: in pediatric patients and in patients older than 60 years. Using SCNP, investigators found that distinct combinations of signaling "nodes," such as phosphorylated proteins in the JAK/STAT and PI3 kinase families, predicted response to induction chemotherapy and likelihood of relapse in the pediatric group, as well as treatment response in the older patients.



Gordon Mills, M.D., Ph.D.

"Clearly, we have to move from looking at single molecules and even single pathways to looking at networks. . ."

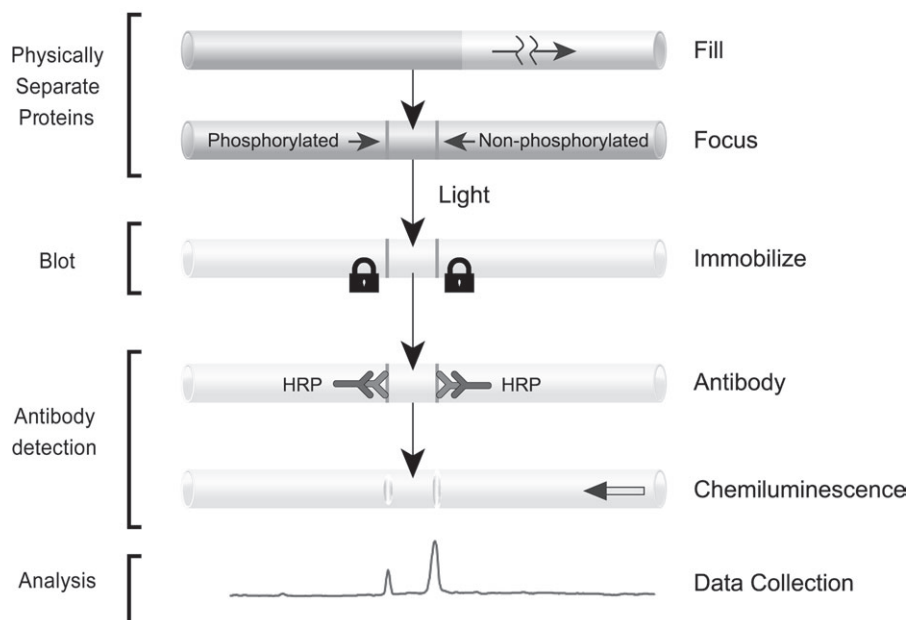
This information could, if validated, direct likely non-responders to alternative therapies, and patients likely to relapse could receive timely bone marrow transplants.

These trials have helped Nodality refine protein signatures, or “classifiers,” for such tests. Parkinson said that, to validate these tests, investigators will analyze tumor samples in a blinded fashion from past AML trials with known patient outcomes. The validation studies for these two AML populations could be completed next year, with commercial test introduction to follow. Other SCNP tests are in the works. “Our goal is to actually provide a suite of clinical tests that allow hematologists and oncologists to make clinical decisions based on that individual patient’s biology,” Parkinson said.

Western Blotting With a Charge

Another proteomic testing technology with the same goal is the nanofluidic proteomic immunoassay (NIA). Developed in the laboratory of Dean Felsher, M.D., Ph.D., at Stanford, using instrumentation from Santa Clara, Calif., biotech company Cell Biosciences, NIA has the potential to identify active cellular networks with more sensitivity, and with less tumor material, than SCNP. The test is probably not as close to commercialization, however. Felsher’s group reported in *Nature Medicine* in 2009 that NIA could detect and quantify expression of a variety of proteins in human samples from several tumor types. The technique worked for solid tumor specimens, and monitored cell signaling network changes over time, by using serial biopsies from patients.

Nano-Capillary Method



Proteomics in a Pipe: For the nanofluidic proteomic immunoassay (NIA) diagnostic test, the tumor sample is loaded into a tiny capillary tube. Phosphorylated proteins, separated by isoelectric focusing, stick to the inner surface of the capillary. Washed with antibodies and a luminescent material, they’re identified and quantified. This information, researchers hope, will predict individual patient response to drugs. (Image courtesy of Cell Biosciences.)

NIA traces its lineage to Western blotting, a routine method for detecting protein expression that uses gel electrophoresis to separate proteins by size, using antibodies to identify them on a nitrocellulose sheet. NIA instead takes advantage of the fact that every protein has its own isoelectric point, a pH at which it carries no net electrical charge. Placed along a pH gradient, proteins naturally separate, a process called isoelectric focusing. NIA “really is like a mini-Western blot, except . . . it’s isoelectric focusing to separate the proteins, rather than size based,” said Alice Fan, M.D., a Stanford lymphoma specialist who led NIA’s development in Felsher’s lab. Protein separation occurs in tiny fused-silica capillaries inside a benchtop machine, so that multiple phosphorylated proteins can be identified by using tiny quantities of tumor material (see illustration). Proteins stick to the inner surface of the capillaries, where antibodies wash over them, followed by a chemiluminescent material. Tiny spots of light mark proteins, which the instrument detects, quantifies, and interprets.

Fine-needle aspiration—the least invasive form of biopsy—yields more than enough tumor cells, and these don’t need to be in suspension. The disadvantage of NIA is that cells must be lysed and analyzed in bulk, as opposed to flow cytometry’s ability to monitor individual cells. Felsher’s group is now working on applying NIA and flow cytometry together in some cases.

Stanford is testing NIA in a prospective phase II trial of atorvastatin (Lipitor) to treat indolent low-grade lymphomas. So far, NIA has revealed that during treatment, phosphorylation of the transcription factors STAT3 and STAT5 decreases in tumor cells from patients, evidence that the drug is altering this pathway. Researchers hope to link these changes to clinical outcome and eventually create a test that will predict which patients will respond to atorvastatin treatment. “At this point, this is exploratory work, and we would need to prove this again prospectively” to validate such a test, said Fan.

One biotech company, Onconova, is already using both SCNP and NIA experimentally. Onconova is completing a phase II trial of its kinase inhibitor, ON 1919.Na, in myelodysplastic syndrome. Researchers take serial bone marrow biopsy samples from patients and run tumor tissue through both tests. “This is very much exploratory, but what we’re trying to do is to find some determinants that could predict further response to the drug,” said Onconova Chief Medical Officer Francois Wilhelm, M.D., Ph.D. At the American Society of Hematology meeting in December,

Onconova and Stanford researchers reported that patients who responded to therapy had a 36% decrease in phosphorylation of AKT2, on the basis of NIA analysis. Onconova continues to use NIA and SCNP to identify other changes that could eventually help guide treatment decisions.

Molecular Diagnostics on Trial

Commercializing SCNP and NIA will require rigorous validation, especially in the wake of a scandal at Duke University, which launched three clinical trials using pathway-based gene expression signatures, based on DNA microarrays, to direct cancer patients to different chemotherapy drugs. Researchers at M. D. Anderson, working independently, could not reproduce some of Duke's results linking the signatures to clinical outcomes and found serious methodological errors. The trials were eventually halted, and Duke has retracted some of the findings.

Nodality, Parkinson said, is taking steps to avoid Duke's mistakes. "The Duke example is a real lesson to everyone working in the field about how important it is to do it right," he said. "You have to be extremely careful, because when you're making so many measurements, the possibilities of finding a [spurious] positive result are always there." Internally, the company has been careful to ensure data accuracy and reproducibility, using methods and software that meet federal regulatory standards, Parkinson said. Externally, experts from the National Cancer Institute's clinical trial cooperative groups that conducted the original AML trials review the validation study protocols for the diagnostic tests. The company, said Parkinson, is also consulting with the FDA on validation procedures.

Tests such as SCNP and NIA, if they succeed commercially as well as scientifically, will give network profiling a foothold in the clinical diagnostics world. Newer technologies are already emerging. Nodality is

exploring the application of mass spectrometry to a future generation of tests. Other companies are developing network profiling tests based on microfluidic "lab on a chip" devices, and various other novel approaches are under development in academic labs worldwide. "Point of care" tests, as opposed to analyzing tumor samples in a central lab, are probably the future. "You can envision [that] you take a sample, load it into the instrument right at the bedside," said Fan. "You can have an answer in 2 hours."

Yet development of all types of molecular tests has been slow. Molecular diagnostics now account for only \$3.5 billion of a \$40 billion total annual worldwide, in vitro diagnostics market. Success of pathway and network profiling, in the clinic and in the market, would show that the field is finally beginning to realize its potential.

Dr. Fan is coinventor on a Stanford University patent application for NIA.

© Oxford University Press 2011. DOI: 10.1093/jnci/djr042