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IN THIS ISSUE

RNA Analysis
UCLA's David Wong and UPenn's Rebekah O'Donnell discuss biomarkers and disease mechanism for oral squamous cell carcinoma

Dr. William Evans and Dr. Torsten Haferlach talk about the emerging role of microarray expression analysis in cancer classification and diagnosis

Wife and husband team, Drs. Amy and Keith Skubitz, discuss the challenges of expression analysis in ovarian cancer, renal cancer, and sarcomas

DNA Analysis
John Hopkins' Dr. Anirban Maitra and Duke University's Amanda Baumann weigh in on emerging methodologies and technologies for mitochondrial studies of cancer

Dana-Farber's Dr. Bill Sellers and UPenn's Dr. John Maris discuss the challenges of high-density SNP genotyping in copy number analysis

Special Section: Translational Medicine
Erasmus Medical Center scientists Prof. Peter van der Spek and Prof. John Foekens weigh in on their institution's evolving infrastructure to support translational medicine

RNA ANALYSIS

High-density Microarrays Help Oral Cancer Scientists Develop New Ways to Predict and Treat Disease

UCLA's Dr. David Wong and UPenn's Rebekah O'Donnell discuss biomarkers and disease mechanism for oral squamous cell carcinoma


Dr. David Wong

Scientists from the University of California Los Angeles (UCLA) and University of Pennsylvania (UPenn) are now using microarrays to perform the most detailed scan of the oral cancer genome ever undertaken. But they're approaching their studies with two different endpoints—finding new ways to predict disease and finding new ways to treat it.

Dr. David Wong, associate dean of research at the UCLA School of Dentistry, is leading a research group that's using new genomic and proteomic technologies to discover salivary markers that distinguish oral cancer patients from healthy controls; their goal is to develop a salivary diagnostic for oral cancer.

"The Holy Grail is for early cancer prediction," said Wong. "The problem with saliva has been *(cont'd on page 2)*

DNA ANALYSIS

Microarray Sequencing of Mitochondrial Genome Uncovers New Clues for Cancer Detection

Johns Hopkins' Dr. Anirban Maitra and Duke University's Amanda Baumann weigh in on emerging methodologies and technologies for mitochondrial studies of cancer

Scientists led by Dr. Anirban Maitra, assistant professor of pathology, genetic medicine and oncology at Johns Hopkins Medical Center, are using a new microarray-based method to rapidly sequence the genome of human mitochondria to find mutations linked to cancers, including prostate and pancreatic cancer. The microarray, called the MitoChip, sequences more than 12,000 bases of the mitochondrial genome, and takes less than a third the time of conventional sequencing methodologies.

"Right now we're using the MitoChip to look at a larger number of pancreatic tumor and juice samples to see if we are able to detect tumor-specific mutations *(cont'd on page 13)*


Dr. Anirban Maitra

Rebekah O'Donnell is a doctoral candidate in immunology at the University of Pennsylvania. Her research focuses on predicting lymph node metastasis by studying the genetic characteristics of primary tumors as well as the associated dendritic cells. She currently works in the laboratory of Dr. Ruth Muschel at the Children's Hospital of Pennsylvania.



that the analytes—the potential markers of disease—are present at very low magnitudes and are often below the limits of detection of an [enzyme-linked immunosorbent assay] ELISA, for example. Technology has changed that now."

While finding predictive markers is critical for early-detection efforts, discovering the underlying pathways that cause disease and then developing ways to block those pathways often requires a different study focused on a different endpoint.

Rebekah O'Donnell, a pre-doctoral fellow in Dr. Ruth Muschel's laboratory in the Immunology Graduate Group at the UPenn, is using the same microarray technology as Wong, but her studies investigate the molecular pathways that govern oral squamous cell carcinoma (OSCC), potentially leading to new targets for drug design.

AMB recently spoke with Wong and O'Donnell about the common challenges they face:

- statistical analysis methodologies
- data validation techniques
- the role of new methods and emerging technologies in improving signatures

Analysis methods

As the selection of statistical analysis packages for microarray data continues to grow—self-organizing maps, Fisher discriminant analysis, and logistic regression models comprise just a sample—choosing the right algorithm is critical.

Wong: We've used a number of tools, but by and large we really don't know which one of these will identify markers that are most discriminatory and validatable. That really is the six million dollar question! We started with S-plus, MAS 5.0 and dChip on the U133A data that was in the paper. Now we use the U133 Plus 2.0 set, and of course we're really looking forward to the new all-exon array which we'll hopefully start with in a month or so, and pass these data through all three of these classifying programs. And from that data, based on a number of statistical criteria—p-value fold change and percent present in a number of individuals—we use the discovery sets and identify candidate RNAs in saliva that are most discriminatory in a statistical sense. That's the discovery phase. And the next thing is to take these candidate markers and validate them in an independent population.

What we have learned so far, in the use of oral fluid for transcriptome analysis, is that dChip is very, very useful. It seems to yield the most discriminatory and validatable data in our Phase II analysis and is the most useful in yielding validatable RNA biomarkers in saliva for diagnostic applications. Our biomarker validation approach is based on the Early Disease Research Network five-phase model from the *Journal of the National Cancer Institute (JNCI)* paper in 2001.

Different expression analysis approaches: biomarkers vs. mechanism of disease

Wong: There's a national initiative to turn salivary diagnostic into a reality. The National Institute of Dental & Craniofacial Research (NIDCR) has invested $57 million into the extramural research community to do two things. One part is to build biosensors that can detect basically anything that's detectable in saliva—seven groups are funded to do that, including UCLA. The second part is to decipher the entire salivary proteome—to know all the proteins in saliva. Three groups are funded to do that, including UCLA. Once we decode the salivary proteome, we can use it as a roadmap to identify people with diabetes, Alzheimer's or major cancers and develop technology for screening and diagnostic applications.

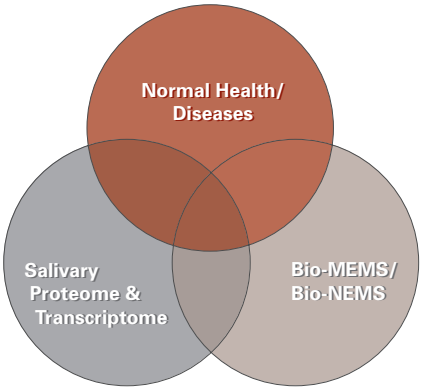
Saliva is not a segregated fluid in our body. Saliva is part of our circulation; physiologically, saliva is a filtrate of our blood, moving through the salivary gland into the oral cavity. Analytes that are known to be present in blood are present in saliva. That is a fact that we've known for decades. The problem is that these analytes are present in saliva at levels that are magnitudes lower than in blood. Therefore, ELISA cannot pick out informative salivary proteins. Technology has changed that, so we're looking at a new opportunity to use this non-invasive fluid, which basically has not been touched very much, unlike urine or blood.

So the question was: 'What RNA are in saliva?' What Charles Zhou—who works with me at UCLA—found, is that there are 3,000 species of RNA in the saliva of normal individuals like us. Interestingly, 185 of those were common in all normal individuals. That work was done two years ago using U95A microarrays.

For a proof-of-concept experimental study, we thought we needed to find a disease that happened close to the spit, or close to the mouth, and oral cancer is a logical choice. Additionally, we have clinical access to material here. That pilot study was published on December 15 last year in *Clinical Cancer Research*. Using a small population—32 cancer subjects and 32 matched-control subjects—we found that there are a handful of salivary RNA markers that could distinguish whether a saliva is from an oral cancer individual or from a control subject with 91 percent sensitivity and specificity. Obviously, this is a pilot study, but it gave us the early, encouraging result that there's RNA in saliva, and that these RNA signatures are disease-associated.

O'Donnell: We're actually using OSCC more as a model than an endpoint. And OSCC is a fantastic model because it doesn't have the conflicting distant metastases that exist in many other types of cancer, like breast cancer and lung cancer. If you find a signature for metastasis in those cancers, it's hard to be completely sure if you're looking at lymphatic metastasis or distant metastasis. But now that a few groups, including ours, are looking at cancers that primarily metastasize to the lymph nodes, the specific lymphatic signatures are starting to come out.

We're also doing some work modeling lymphatic metastasis in the mouse, and our goal is to eventually understand why lymphatic metastasis happens and what factors in the host may encourage or discourage it happening in a particular patient. And if we can identify it quickly, then we can change treatment. In OSCC, there are some non-metastatic patients who receive unnecessary neck dissections; they could just be treated with chemotherapy, which is much less invasive. There are also patients whose lymph node metastases are missed, and so we hope that by finding this signature, we'd be able to eventually change the clinical treatment to make cancer treatment more effective for these patients.



UCLA Human Salivary Proteome Project
UCLA Collaborative Oral Fluid Diagnostic Research Center
This ven diagram depicts the overlay and subset of human diseases that are detectable in saliva as reflected by transcriptome or proteome analytes. The third sphere denotes nanotechnology based biosensors that are currently being developed for saliva diagnostics.

O'Donnell: I agree with David that we just don't know the best method, and that's what makes all this difficult. I spent six months working with various tools and my general perception now is that I'm not a big fan of unsupervised clustering, which I think is starting to go out of style. Most of the very early papers use hierarchical clustering and various other kinds of clustering and the later analyses on those methods have shown that they're not very robust or stable, so I don't approve of them at all anymore.

But which supervised method to use, and where to get your initial gene list, is a very difficult question, and that's something that we're going to have to learn as we go along. I see the bar being raised in the microarray papers, but the statistics have to be stronger and the data sets have to be bigger.

I think we're waiting for the mathematicians to really do their thing—to work on neural networks and meta-analysis. Knowing what the best method is—that's probably several years away. The most important thing is validation. If you can't validate it, then you know it's not good. But if you can validate it, it's hard to argue that it's bad.

Data validation

Many scientists use leave-one-out cross validation (LOOCV) for data validation. However, the gold standard remains validating on a truly independent population.

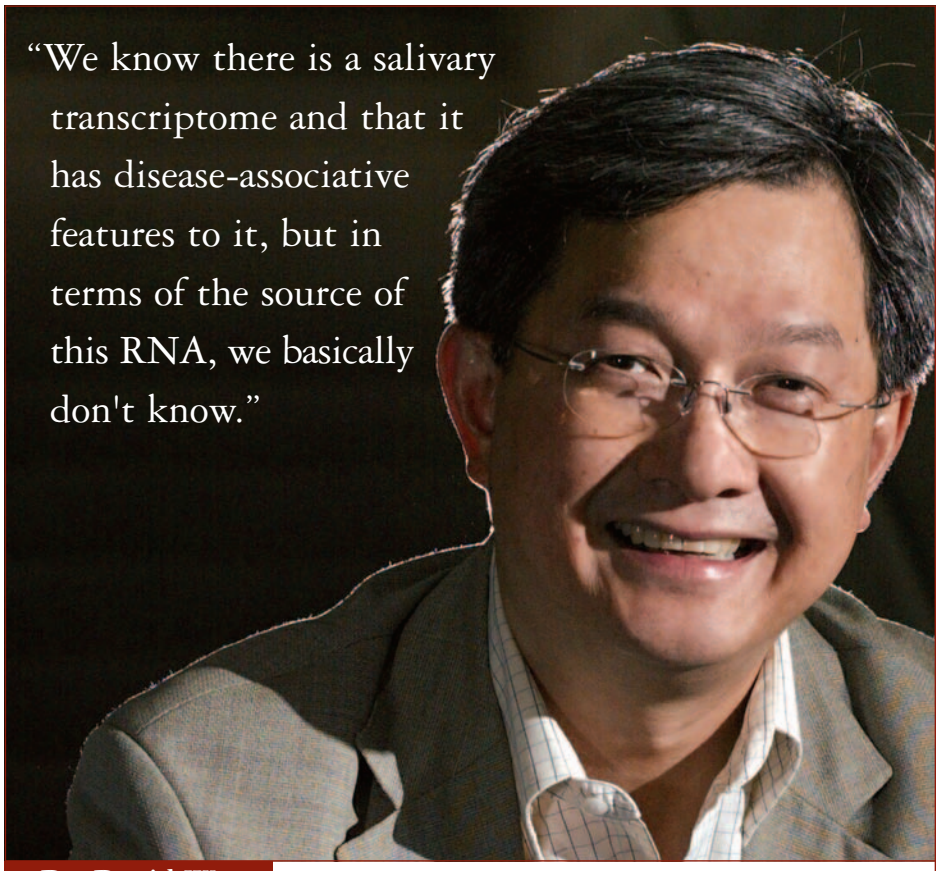
Wong: We absolutely need to do independent validation and that's the beauty of this five-step validation process. You know, we went only to a Phase II in that paper. In terms of the validation tool here, we have a Phase III detection trial that is currently ongoing. We are accruing 100 individuals and it is going to be a blinded study. But for an initial validation of our model, David Elashoff, a statistician that we work with, helped us with LOOCV.

O'Donnell: We use LOOCV as well. We consider it a kind of trip wire. If your initial testing set passes leave-one-out validation then it's worth going on to validate it, but if it doesn't survive, then you have a problem and you need to go back and look at your work. Unfortunately, a lot of studies have used LOOCV just on their original testing set and consider that a sufficient validation, but I think we're all pretty clear at this moment that we don't consider that to be sufficient anymore.

There really should be two kinds of validation though. First, you need a completely independent data set on your microarray that shows that your findings are reproducible. And then you need a molecular technique, which could be quantitative RT-PCR or which could be immunohistochemistry, and that shows that your findings are real. And you really need both of those to have a really high quality finding.

Wong: For what we do, the process here is fairly similar. But, I just want to add one idea here. In terms of our biomarker work, we approach it with an orientation towards diagnostic development. One can certainly embark on it on a direction of the mechanism of biology. And when biology is involved, there is no question one needs to have an additional level of validation, and that is at the proteomic level—at the protein level.

What I want to point out is that in terms of diagnostic or potential utility of these markers for disease association, the presence or absence of a panel of markers with disease phenotype—given a strong enough statistical association—could serve as an important endpoint. So, there are some subtle and perhaps obvious differences here, and the endpoint often has a bearing



Dr. David Wong is associate dean of research and a professor in the Division of Oral Biology and Medicine at the UCLA School of Dentistry. Dr. Wong is a dentist-scientist researching the molecular determinants of head and neck cancer. One of Dr. Wong's recent efforts includes the creation of the UCLA Collaborative Oral Fluid Diagnostic Research Center and the Human Salivary Proteome Project *http://www.hspp.ucla.edu* to identify the entire catalogue of proteins in human saliva.

"Unfortunately, a lot of studies have used LOOCV just on their original testing set and consider that a sufficient validation, but I think we're all pretty clear at this moment that we don't consider that to be sufficient anymore."

in terms of how far one needs to go in terms of validation.

Origins of salivary RNA

The cellular source of RNA used for microarray analysis can be one of the most important factors in experimental analysis; however, there are some major differences in diagnostic versus disease mechanism studies.

Wong: We have many, many questions that we need to answer, and we're beginning to work on them. We know there is a salivary transcriptome and that it has disease-associative features to it, but in terms of the source of this RNA, we basically don't know. But let me talk about the kind of experiments we're currently doing to find out.

We have stratified saliva into the five sources: from the three major salivary glands (parotid, submandibular and sublingual), and also from a space between the tooth and the gum, called the gingival crevicular fluid (GCF), and also from desquamated epithelial cells. And we profiled each one of these sources and found RNA to be in each one. Now, beyond that, we're currently tracking it.

We're also using rodent models, where we implant human cells—non-metastatic tumor cells—into distal parts of the rodent. So we use human cells in a mouse background and then try to track tumor growth. Once a tumor is formed—it takes about three to four weeks—we compare the RNA profile of the implanted tumor and also look at the presence of human RNA in the circulation of these mice, as well as in their oral fluids. Presently, our research is merely 18 months along and I think the data will come out fairly soon and hopefully these questions will be addressable.

For diagnostic purposes, I think it doesn't really matter where the RNA comes from, because the key factor you're looking for is just the presence of a disease-associative RNA signature, one that is always there associated with the disease, e.g. oral cancer. That's good. That's what a clinician needs to know and what a diagnostic kit needs to be able to determine. That's what a diagnostic company needs to know, if you have the sensitivity and specificity and can control all the confounding factors.

O'Donnell: But if you want to design a treatment based on the ribosomal RNA and what it's doing, you have to go back and figure out what's going on.

Wong: Absolutely.

O'Donnell: Right now we're doing a lot of immunohistochemistry, looking at the presence or absence of certain molecules in certain cells in the tumor and then going back to try and correlate that with the presence or absence of metastasis.

And there are issues with those experiments as well because it's a static system. We can see it, but we can't change anything. So then we have to go back to the mouse models. So I agree with David—the goal is to get a picture of how this process works, because once we thoroughly understand how it works, we should be able to do something about it. We're all coming at that from slightly different directions and different strengths, but hopefully we're all going to end up in the same place.

Improving Signatures

Current studies scan for transcriptional activity in nearly all known coding genes; however, by looking at the genome at greater detail, for instance by analyzing splice variation, scientists may be able to develop more specific signatures and more robust classifiers.

Wong: So far we are capturing only a fraction of the information from saliva. We know for certain that about 50 percent of the transcripts in saliva are devoid of a poly-A tail. So, using a T7-oligo dT primer in linear amplifications, we miss half of that information based just on that fact alone. And if you consider all the other degradative mechanisms of RNA-either decapping or clipping a piece of RNA in the middle—we'll miss more than 50 percent of those.

We are very excited and looking forward to the upcoming all-exon array—we are on the early-access program—because every coding sequence will have multiple 25mers tiled to it, so that, theoretically, every fragment of RNA in a biological fluid or tissue will be registered or captured. We're coupling this with an alternative amplification procedure, because using an oligo-dT procedure still requires RNA with a poly-A tail. The degraded RNA in saliva often don't have this tail, so we're currently experimenting with a German company, Artus, using an alternative amplification procedure for quote-unquote 'less than optimal RNA.'

O'Donnell: This array may potentially be the end of the arms race. A lot of the difficulty with comparing various papers is that they're all on different incarnations of microarrays. If there could be a consensus of what we're actually looking at—if we could really get everything—then I think that would go a long way to achieving standardization, which is one of the chief issues that we're facing.

FOR MORE INFORMATION

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■ **Artus Inc.** - http://www.artus-biotech.com/

Organizations

■ **University of California Los Angeles** - http://www.ucla.edu/
■ **University of Pennsylvania** - http://www.upenn.edu/
■ **Children's Hospital of Philadelphia** - http://www.chop.edu/consumer/index.jsp
■ **National Institute of Dental and Craniofacial Research** - http://www.nidcr.nih.gov/

Further Reading

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Background

■ **UCLA Collaborative Oral Fluid Diagnostics Research Center** - http://www.saliva.bme.ucla.edu
■ **UCLA Human Salivary Proteome Project** - http://www.hspp.ucla.edu/

Tools for download

■ **S-Plus** - http://www.insightful.com/
■ **MAS 5.0** - http://www.affymetrix.com/products/software/specific/mas.affx
■ **dChip** - http://www.dchip.org/

Expression Profiling Provides Most Detailed Classifications of Pediatric and Adult Acute Lymphoblastic Leukemia

Dr. William Evans and Dr. Torsten Haferlach discuss the emerging role of microarray expression analysis in cancer classification and diagnosis

Leukemia was one of the first cancers that researchers studied using microarray gene expression profiling. But recent advances in whole-genome analysis have enabled scientists to subtype the disease in totally new ways, including more detailed sub-classifications, prognoses, and drug-sensitivity status.

Dr. William Evans from St. Jude Children's Research Hospital in Memphis, Tennesse has studied pediatric acute lymphoblastic leukemia (ALL) for over 30 years, and his group was among the first to use high-density microarrays for ALL profiling.

"The expression patterns we've found are largely concordant with known ALL subtypes, but within any of those subtypes of ALL, even those with the best prognosis, we always had patients with a bad outcome," said Evans. "Our recent focus has been on trying to learn something new about why some kids respond and some don't to the same treatment."

Dr. Torsten Haferlach's research team at the Ludwig Maximilians University in Munich has taken a similar approach studying adult ALL. His group has developed expression signatures across a broad range of leukemias and leukemia-related disorders, including ALL, acute myeloid leukemia (AML) and acute promyelocytic leukemia.

Evans and Haferlach recently sat down with AMB Managing Editor Tommy Broudy to discuss the key challenges they face implementing microarray expression analysis in their leukemia research. Topics covered include:

- developing methodologies to assess tumor load and purity
- adequately powering studies for class discrimination, integrating different types of molecular data
- the role of technology in study validation

Tumor load and purity

Broudy: Tissue heterogeneity can greatly influence cancer classification. Traditionally, people have accounted for this through pathological and histological methods. What are your thoughts about putting heterogeneity classifier probes on the microarray itself to do this?

Evans: We're always struggling with this in terms of how much noise is being introduced by cells that aren't really what we are interested in. And the idea of putting



Dr. Torsten Haferlach is a Professor at the Medizinische Klinik III and head of the Laboratory for Leukemia Diagnostics at the Ludwig-Maximilians-University in Munich. Dr. Haferlach's research focuses on the accurate diagnosis and classification of leukemias to improve patient management. His lab uses high-density microarrays to analyze gene expression profiles in bone marrow and peripheral blood samples from patients with all clinically relevant leukemia subtypes and non-leukemic controls.

some classifier probes on the array is a good one. You would, of course, perhaps want some signatures that might tell you what other kinds of cells you might have contaminating the sample.

But, one reason that we work with leukemia is because it's easier to deal with that problem—we can just do purification and enrichment of bone marrow samples. In a lot of our work we have had minimum thresholds for percent of leukemia cells in a bone marrow before we'll include it in the analysis. In some of our earliest papers looking at classifications, we had data that said if we go from saying 70 percent cut-off of leukemia cells to 90 percent, then the data looks even cleaner, as you would expect.

Haferlach: Well, we have had the same experience and the question always is, 'What is a tumor cell?'

While we are happy having blood and bone marrow samples—even if their blast count is below 70 or 80 percent—we know from our interphase fluorescence in-situ hybridization (FISH) analysis that many other cells also belong to the tumor population.

So in our experience it does not matter if the tumor load is 90 percent or 100 percent, especially if what we are addressing is the diagnostic approach. The pattern of genes that can be recognized for a specific diagnostic pattern is so stable that you can do it with even lower numbers of tumor cells.

That does not mean that they are looking like blasts, so in our opinion it is much easier, and this is what is seen in the literature, that leukemia can be taken as an example in the paradigm for using tumors for diagnostics. For example, using microarrays, the tumor purity in our hands is normally above 70 to 75 percent, and that works as far as we use it. We don't purify in any case, we just do a Ficoll separation and then perform the microarray analysis.

Evans: We generally don't purify them either if they're in that range.

Broudy: What are the typical tumor cells counts when a patient is in blast crisis?

Evans: Well, we're dealing with acute leukemias, and these patients typically present with very high circulating

"In situations of uncertainty, you must go back and find additional cases that would allow you to develop a more robust signature and then test it in an independent cohort to see if it really is a unique subgroup of leukemia…"



Dr. William Evans is director and chief executive officer of St. Jude Children's Research Hospital and First Tennessee Bank Professor of Pediatrics, Clinical Pharmacy, and Pharmaceutics at the University of Tennessee Colleges of Medicine and Pharmacy. For the past 30 years, Dr. Evans' research has focused on the pharmacodynamics and pharmacogenomics of anti-cancer agents, exploring genetic and biochemical mechanisms for inter-individual differences in drug disposition and response. He has received three consecutive MERIT Awards from NCI, and was elected to the Institute of Medicine of the US National Academy of Sciences in 2002.

leukemia cell burdens. It's often 10,000 or so circulating blast count; but it can be 100,000 or more. And when we look at marrows at diagnosis, typically it's 70 to 90 percent or more leukemia cells in the bone marrow. That's at initial diagnosis.

Now, in a patient who has relapsed, it's a bit different, it may only be 25 percent leukemia cells in a marrow. That's one of the challenges we've seen with doing expression profiling of relapse samples—you have fewer cells in the marrow and it's a smaller percentage.

Haferlach: That's why our group is looking for diagnostic profiles. We only use samples from the primary diagnosis and mostly stick to bone marrow, not to peripheral blood. But it works the same. For example, if you look for chronic myeloid leukemia, you will see blast counts below 10 percent. However, if you use the same smears and apply interphase FISH, then you will see that the number of malignant cells are 85 or 95 percent. So it's different looking at this smear in the microscope and doing a specific analysis like FISH on the samples. But normally we've found the same thing as Dr. Evans—the number of tumor cells is, at least at the diagnosis of the primary disease, more than 70 or 75 percent in leukemia.

Power and Class Discrimination

Broudy: There seems to be an explosion of cancer subclassifications. What types of assumptions do you need to make when you're starting a study to achieve enough power to discriminate the smallest class?

Haferlach: That's a very difficult question because it depends on what you want to do. If you want to make a diagnosis, I think you need at least 10 to 15 or 20 samples from the same biological kind. Then you can start making an analysis using the biostatistic approaches and then find some genes. But, if, for example, you want to approach a group of AML with a normal karyotype that show several molecular mutations, you will need 100 or 200 or 300 or more samples to come up with a new classification. And so, it really depends on what you are looking for and where you go to. Then you can define the data set and the number of samples.

Evans: And sometimes you have unexpected subclusters that you didn't anticipate and it could be by chance or due to small numbers, or it could be of biological importance. In situations of uncertainty, you must go back and find additional cases that would allow you to develop a more robust signature and then test it in an independent cohort to see if it really is a unique subgroup of leukemia and let that take you to the biology of that subset. So there's an aspect to this that's unbiased in terms of the possibility of making some findings that you wouldn't have anticipated a priori, and that may need to be a preliminary finding the first time through, that you then go back and replicate or expand with adequate numbers. It may turn out to be noise, but you have to sort that out in the next experiments.

Haferlach: This is absolutely true. If you come up with some genes that do something or suggest to do something, then you need to study a cohort of patients that have never been in the analysis. Many, many experiments are hampered because the number of patients is too low and what was seen there was not tested in an unrelated cohort, and this makes it really weak from my perspective.

Broudy: Discriminating between the many different subtypes of ALL is not just a binary A or B type decision. What statistical methodologies, such as Support Vector Machines (SVM) or One-Verses-All (OVA) perhaps, are you using to take this complex set of conditions into account?

Evans: In some situations, you have multiple classes of phenotypes, and in other situations the phenotype can be a continuous variable (e.g. drug sensitivity). So, in the latter case, instead of using SVM, we have taken a linear-weighted average approach to initially look at linear correlations between gene expression and a phenotype. We published some of those data in the Lugthart et al. paper in the April 2005 issue of *Cancer Cell.*

In this case, we had an LC50 value, a sensitivity to drug value for 400 different patients. And there was a correlation struc-

ture that we thought we should take advantage of, in terms of identifying what the principal components were that we should be fitting to the model. And so we ended up identifying genes, and then essentially weighting each one of those 50 significant genes somewhat differently to create the best model for discriminating sensitivity to drugs among different leukemias. So that's quite a different approach than we had taken previously when we were asking other questions in a similar context. And it was because we had the informaticists, who are at every lab meeting and every research workshop and every discussion about how these experiments are going to be done and how to best analyze them.

I think you're just going to continue to see additional approaches; one vs. all is another option that you alluded to. In terms of a classifier, SVM's still our work horse.

Integrating expression, genotype and proteomic data

Broudy: Are you integrating multiple molecular sets, such as genotype data, proteomic data, or expression data to present a singular decision at the point of treatment?

Evans: There's power in all of those approaches, and sometimes they overlap. We're often using germ-line polymorphisms to identify the appropriate dose of a given drug or to select a drug for individual patients, and that's information that may or may not be imbedded in a gene-expression profile. Obviously, it's typically a germ-line polymorphism, and so in some settings we find that helpful in making treatment decisions.

Some of our work now is looking at germ-line polymorphisms in the promoter regions of genes that we see expressed at different levels in leukemias that are sensitive vs. resistant to a given drug. The question there becomes: 'Is there a polymorphism that is determining whether a child's leukemia cell is over- or under-expressing a given gene that is significant in our analysis?' Of course, in the simplest world, it would be. But it may be that there is a polymorphism in a transcription factor, not a transcription factor binding site, that is driving the level of expression. A lot of what's of interest now is the pathway, and not just a single gene or pattern recognition.

We've gotten lucky recently—this is not published—but on a couple of genes we do find single nucleotide polymorphisms (SNPs) in promoter regions that are predictive of expression levels in leukemia cells. But that won't be the case for all these genes.

Haferlach: We're just starting with these DNA arrays and p53, especially in AML with complex aberrant karyotypes, because we know that many of these patients have a p53 deletion and we are looking for mutations. If there are no deletions there, then we ask, 'Is there at least some mutation in the gene?' All these techniques are not in competition, but are used in combination. If we want to know more, not only about diagnosis, but also about drug response, biological backgrounds and pathways, we shouldn't just spend our time on one technique, but rather combine all these different approaches in one data set. We're doing this in our lab as well as combining all this with morphology, molecular genetic data and immunophenotyping. This is very, very interesting and we will come up with many more answers this way than relying on just one technique.

Technologies for validating results

Broudy: How do you see a technology like multiplex polymerase chain reaction (PCR) working with microarrays? Are you using them together now?

Haferlach: We are just using it for testing the genes that come up as most discriminative between several leukemia sub-classes just to see them on another platform. We use the ABI cards to do this and we found a very high correlation with these specific genes—we did it just to see if they work on the other platform as well. I'm quite sure that if you have 364 genes on this plate, you can also use it for several approaches, because normally you do not need 40,000 genes. But on the other hand, a platform like Affymetrix' in our hands is much more robust than the ABI platform using these gene cards. In the next three to five years, we will see which platform comes out on top, but from my experience right now, I think the gene expression profiling using the Affymetrix technology is ahead. Although you don't need all the genes that are on the array now for diagnostic questions and answers.

Evans: Yeah, I don't have much of a different point of view on that—we'll have to see. If we can do 30,000 probe sets for the same price as the 300-probe-set approach by multiplex PCR, then people are going to do 30,000.

Haferlach: It's absolutely true what Dr. Evans said—100 percent. We're happy to have the opportunity to have three or five different probe sets for the same gene on an array, rather than just having one on the ABI cards.

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Organizations

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University of Minnesota Scientists Find New Diagnostic Signatures and Therapeutic Targets in Gene Expression Data

Wife and husband team, Drs. Amy and Keith Skubitz, discuss the challenges of expression analysis in ovarian cancer, renal cancer, and sarcomas

Amy and Keith Skubitz are engaged in two major collaborations that vie for literally every minute of their time. The first project involves a small group of cohorts—their four young children—set against the variables of school, soccer practice, playtime and homework.

The second collaboration, finding new ways to diagnose and treat a wide range of cancers, gets most of their remaining attention, once the school bus leaves in the morning and after the kids fall asleep at night.

Dr. Keith Skubitz is a professor of medicine in the hematology, oncology, and transplantation division at the University of Minnesota and has studied gene expression of soft tissue cancers and renal carcinoma for the past half decade.

"We really have two problems when it comes to diagnosing cancers like soft-tissue tumors," said Keith Skubitz. "One, within a diagnostic category there's really more than one entity. And two, even the reproducibility of how we currently put samples into these categories is not great."

In a recent *Journal of Clinical Laboratory Medicine* publication, Keith Skubitz led the research that used microarray-based gene expression profiling to classify high grade pleomorphic sarcomas and malignant fibrous histiocytomas with known entities—results that were ultimately confirmed in several instances through more detailed pathological and biochemical exams.

Dr. Amy Skubitz, an associate professor in the department of laboratory medicine and pathology at the same institution focuses her research on developing new methods to detect ovarian cancer, a disease that is 90 percent curable when detected early, but is currently the fifth leading cause of death in women. Amy Skubitz has analyzed gene expression data from hundreds of tissue samples to discover 66 genes specifically expressed in ovarian cancer; the results may eventually lead to improved screening tests for the disease.

The Skubitz team recently spoke with AMB about the challenges they face in their combined research efforts.

Topics include:

- developing standardized methodologies for tissue collection
- choosing appropriate controls and analysis methods
- maintaining experimental reproducibility through generations of tissue samples and technologies

Tissue collection

The Skubitz research team has analyzed hundreds of tumor samples in their studies. Along the way, they've developed standardized methodologies for tissue collection and sample preparation.

Amy Skubitz: All of the tissues that we use undergo a very strict standardized protocol during procurement. We've tried to keep everything identical, and that includes the time from when the tissue is resected to the time that it is snap-frozen. This process must be completed within 30 minutes to ensure that RNA and protein degradation is kept to a minimum. The snap freezing technique is standardized and the tissue is then stored in the vapor phase of liquid nitrogen. Since everything is standardized, this helps us, as the end users of the tissue, feel very satisfied that there's no variability in sample preparation.

Other researchers with whom I've spoken get their tissue from sources, and they're not informed as to the procurement

Dr. Keith Skubitz is a professor of medicine in the division of hematology, oncology, and transplantation at the University of Minnesota. He received his M.D. and clinical pharmacology training from the Johns Hopkins University School of Medicine and his internal medicine and oncology training at the University of Minnesota. Dr. Skubitz cares for patients with a variety of malignancies and related diseases. His research primarily involves drug development and clinical trials, including the use of genomics to identify new drug targets and predict disease outcome.

Dr. Amy Skubitz is an associate professor in the Department of Laboratory Medicine and Pathology at the University of Minnesota. She received her Ph.D. at the Johns Hopkins University School of Medicine and performed her post-doctoral training at the University of Minnesota. Dr. Skubitz' primary research focus is the role of cell adhesion molecules in ovarian carcinoma. She is exploring the role of integrins and CD44 in the interaction between ovarian carcinoma and mesothelial cells, and is also investigating how ovarian carcinoma cells that are shed into the ascitic fluid are kept in a non-adherent and/or non-invasive state.



parameters. This leads to a potential for bias in the experiment of which the scientists themselves aren't even aware. It's really important that researchers know where the tissues come from and how they were procured—the whole process.

All of the tissues we use in our experiments were procured from the University of Minnesota Hospital's operating room suites by the Cancer Center's Tissue Procurement Facility. There's no question whether or not the tissues have been handled properly or shipped to us appropriately.

Keith Skubitz: Amy's being a little modest there. She founded that facility and set up all those parameters.

Amy Skubitz: I belong to the International Society of Biological and Experimental Repositories (ISBER). The society is comprised of repository staff and vendors involved in storing collections of samples ranging from human tissues to blood to environmental samples, such as soil, animals, and seeds.

Members of ISBER recognize issues such as consenting the patient, being in the operating room as soon as the tissue is resected, rushing the tissue to the pathology department for a diagnosis, and then processing and freezing the waste tissue immediately.

Every step along the way is very well documented at most of the ISBER sites since they are aware of the procedures. In fact, ISBER has just published our best-practices guidelines in *Cell Preservation Technology,* which outlines some of the procedures recommended for optimal human tissue processing and storage. The Society is really trying to make sure that scientists, who are the end users, are aware of the issues surrounding human tissue use in their experiments.

Keith Skubitz: It's also important to have a histologic confirmation of the tissues that you're examining because a piece of tumor might have a necrotic area or some extra non-tumor tissues in the sample.

We make sure that nearly our entire sample is tumor and that there is not much necrosis. In some tumors that may be difficult to achieve. The pathologist needs to look at a slice before cutting up the sample and doing the frozen section. And then, the researchers who get a sample for RNA or DNA extraction need to know what they're working with, usually by examining a section from that piece.

But, it can get kind of complicated with some tumors that actually have different histological patterns within them. For instance, there might be a little patch of one type of thing here, and another type of thing there.

Amy Skubitz: For quality control, a pathologist looks at a frozen section at the time of surgery and then excess waste tissue is given to the staff of the Tissue Procurement Facility. The procurement facility staff makes their own quality control slide from tissue that is adjacent to what is later given to researchers. A pathologist will then verify the diagnosis, percent tumor, and percent necrosis on the H&E [hematoxylin and eosin] slide. We then select for those tissues that are greater than 90 percent tumor and less than 10 percent necrotic.

Keith Skubitz: The microarray data that we've been publishing was generated by Gene Logic Inc. based on a contract with the University of Minnesota.

Amy Skubitz: Each of the tissues that was provided to Gene Logic Inc. was excess; in

"It may sound simple, but when selecting controls, the key thing is figuring out what question you want to answer. Without formulating the question, you can't really address anything else."

other words, additional samples of the tissue were maintained at the University of Minnesota. For example, after pathology keeps the tissue that they need in order to make a diagnosis, the Tissue Procurement Facility may be provided with 10 grams of tissue, which they cut into multiple pieces. We use a piece of that tissue for immunohistochemistry, while Gene Logic Inc. generates gene expression data with an adjacent piece of the tissue. So we have matching tissue that we can use to validate the gene expression data by immunohistochemistry or other types of experiments.

Appropriate control selection

Obtaining healthy control tissue for many internal organ diseases is often a difficult task. The Skubitz' weigh in on the challenges and solutions they've reached for collecting control tissue in ovarian and kidney cancer studies.

Keith Skubitz: It may sound simple, but when selecting controls, the key thing is figuring out what question you want to answer. Without formulating the question, you can't really address anything else.

For kidney cancer, we wanted to know if we could find therapeutic targets that would be expressed in the tumor, but would not be expressed in normal tissues. So in that case, the normal control is really the rest of the body.

You could modify those conditions if you're willing to accept certain toxicity. Let's say you want the drug target expressed in kidney cancer, but not expressed in any essential tissue like heart or lung. In that case, you may not care if it's expressed in non-essential tissues—you don't need a thyroid, you don't need a prostate, you don't need an ovary for example.

Amy Skubitz: We obtain the normal ovaries from women who come to the University of Minnesota for prophylactic oophorectomies. For example, women may require a hysterectomy or they may have a family history of some gynecological disease. When their ovaries are removed and the pathologist determines that the ovaries are normal, we can use them for our research projects.

> "The problem is that a kidney is not just a kidney. There's a cortex and a medulla; so depending on how much of your 'normal kidney' sample came from one part of the kidney versus another, the gene expression signal could be quite different."

What complicates our studies is that the most common type of ovarian cancer arises from epithelial cells that are on the surface of the ovary. Therefore, it is difficult to do a direct correlation with surface epithelial cells from normal ovaries since the ovary may be handled by surgeons, residents, nurses, and pathologists. By the time the Tissue Procurement Facility staff gets the normal ovary, the surface epithelial cells may be rubbed off. When we eventually look at the gene expression data, it's with the understanding that we're comparing a sample of ovarian carcinoma, which has lots of cancerous epithelial cells in it, to a normal ovary, which may have a very small percentage of the normal counterpart of normal surface epithelial cells. That's why it is important for us to then do the immunohistochemistry and to see if the surface epithelial cells on normal ovaries stain for the proteins corresponding to the upregulated genes in the ovarian carcinoma samples.

Furthermore, we've looked at over 300 other tissues, comprising tumors and normal tissues, to see whether the ovarian cancer expression signature would be differentially-regulated in them.

Statistics and software

Eisen Clustering and principle components analysis (PCA) are two major microarray analysis solutions frequently used by scientists. Each has strengths, but limitations as well.

Keith Skubitz: Eisen Clustering does have a nice visual effect, so it's very easy to readily understand what you are looking at. But it also has the obvious limitation that it's restricting the distribution to two-dimensional space. So PCA is definitely preferable, and that's what we've been using primarily, but we still do use Eisen Clustering as well.

Amy Skubitz: One concern with PCA might be that you can rotate it any way you want in order to get clusters to appear. But, it's very satisfying when the clusters just leap right out at you.

Keith Skubitz: PCA is usually preferable, but it is true that with either approach, depending on how you analyze the data, you can get different results.

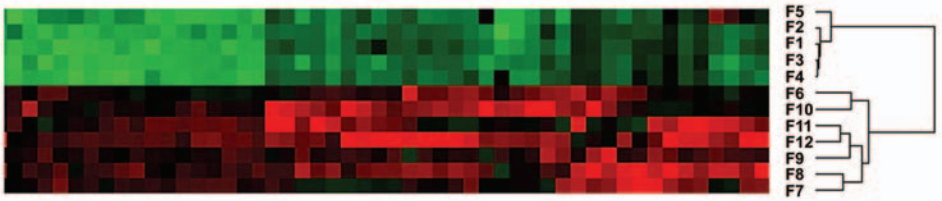
But these things we're manipulating are numbers, they're measurements, they're not magic. So we have to convince ourselves that whatever data or conclusion we're generating is actually true. One way to do that is by analyzing the data in several different ways and getting the same answer several different ways.

One place where things can get confusing is in fold change analysis. A gene may be reported to be 50-fold up in group A versus group B. But before we would believe these results, we would like to actually look at a graphical representation of the data in each sample group, so that we can say, 'Yes, we believe this.' For example, it may be that 20 percent of the samples are contributing 90 percent of the signal, or something like that, but one would not be aware of that unless one looked at the individual data itself.

Amy Skubitz: It's also important to look at the standard errors and p values for each of the data sets too. You can look at the actual data plotted out and see if there's a big spread or if they're all clustered at one end or the other. It's very time-consuming to do it that way, but if you don't, you can fall into this trap of believing that 50-fold up value. And then you end up choosing that gene without ever actually seeing the data. That's a concern.

Common cancer signatures

Information from one type of cancer can help scientists learn more about another. By comparing data, key genes and pathways can emerge that might otherwise be missed.



Heterogeneity in fibromatosis. *The heatmap portrays clustering of gene expression in aggressive fibromatosis samples analyzed with HG U133 microarrays and Eisen clustering software Cluster.*

Keith Skubitz: Right now we're focusing on specific tumors. One interesting point related to that question was a study we did looking for genes upregulated in leiomyosarcoma compared with normal tissues. We looked first at leiomyomas, which are benign smooth muscle tumors of the uterus, and we found some genes that were uniquely expressed compared with all the normal tissues. Later, we did an analysis of leiomyosarcomas, which are malignant tumors of smooth muscle.

We looked for genes that were uniquely expressed in that sarcoma compared with a number of normal tissues, and we found some. But we then decided to go back and ask specifically, 'Were any of the genes that we found uniquely expressed in leiomyomas also selectively over-expressed in leiomyosarcomas compared with normal tissues?' In fact, this approach yielded new genes selectively expressed in leiomyosarcomas, but only a fraction of them. In other words, this approach identified heterogeneity in the leiomyosarcoma population, and allowed the identification of potential targets that were not found in our general screen.

For example, we found that doublecortin was expressed in about half of the leiomyosarcomas. That's interesting, because we identified a subset of leiomyosarcomas and a potential drug target that we wouldn't have found without this clue from the earlier studies of related tumors.

Experimental reproducibility

As the duration of their cancer research has grown, the Skubitz' have run into the challenge of comparing data from different generations of sample collections and different generations of microarrays. The data are validating toward each other, but extra caution needs to be taken in these instances.

Amy Skubitz: The experimental data that we initially analyzed was generated using the Human Genome U95 microarrays. It's just recently that we've used the Human Genome U133 chips and we've gone back to see if we could get some of the same data. Right now we're doing a different series of experiments with our ovarian cancer samples, and we're coming up with the same expression trends.

Keith Skubitz: In kidney cancer, we're also finding a lot of the same genes with the U133 set that we found with the U95 set. So that's reassuring.

Amy Skubitz: The HG U133 experiments are using the exact same tissue samples that were used in the HG U95 experiments. So it's not like taking a totally different tissue and seeing if we see the same trend. I would say that it's working well.

Keith Skubitz: In many of the studies that we're doing we're looking at genes that are unique to the tumor versus a lot of different tissue types. I'd say it's been pretty reproducible. But let's say, for example, that you wanted to compare kidney cancer with normal kidney. The problem is that a kidney is not just a kidney. There's a cortex and a medulla; so depending on how much of your 'normal kidney' sample came from one part of the kidney versus another, the gene expression signal could be quite different. Also, in our first kidney cancer paper, we looked at gene expression in not only normal kidney and kidney cancer, but also in so-called diseased kidney. Not surprisingly, diseased kidney is not the same as normal kidney.

So if one study looked at kidney cancer versus perfectly normal kidney, the results could vary depending on how much of the cortex or medulla were in the samples. Also, if there were inflammation in one of the kidneys, or it were somewhat atrophic, or they had significant hypertension or diabetes, or who knows what, the results could also differ. So, things that might at first appear to be the same comparison in different experiments, might not be.

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Organizations

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http://www1.umn.edu/twincities/index.php

■ **International Society of Biological and Experimental Repositories** -
http://www.isber.org

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Tools for download

■ **Eisen Clustering Software** -
http://rana.lbl.gov/EisenSoftware.htm

Microarray Sequencing of Mitochondrial Genome Uncovers New Clues for Cancer Detection *(cont'd from page1)*

in pancreatic juice samples," said Maitra. "While we developed the MitoChip to study cancer biomarkers, it certainly has uses in non-cancer applications as well, everything from evolutionary studies to metabolic diseases to aging."

Maitra recently spoke with Amanda Baumann, a pre-doctoral fellow in Duke University's department of Pharmacology and Cancer Biology regarding the new challenges facing mitochondrial research now that the genome sequence can be readily accessed. The two discussed:

- the various types of mitochondrial mutations linked to cancer and models available for validation
- the "mass advantage" of mitochondrial genetics and the associated difficulties of distinguishing heteroplasmies
- the sensitivity, sample preparation, and genome coverage of microarray-based resequencing technology

Formerly at Emory University, Baumann worked with Dr. John Petros to prove that mitochondrial DNA mutations increase tumorigenicity in prostate cancer. The group published their work in the January 2005 *Proceedings of the National Academy of Sciences, USA.*

Mutation discovery and in vivo models

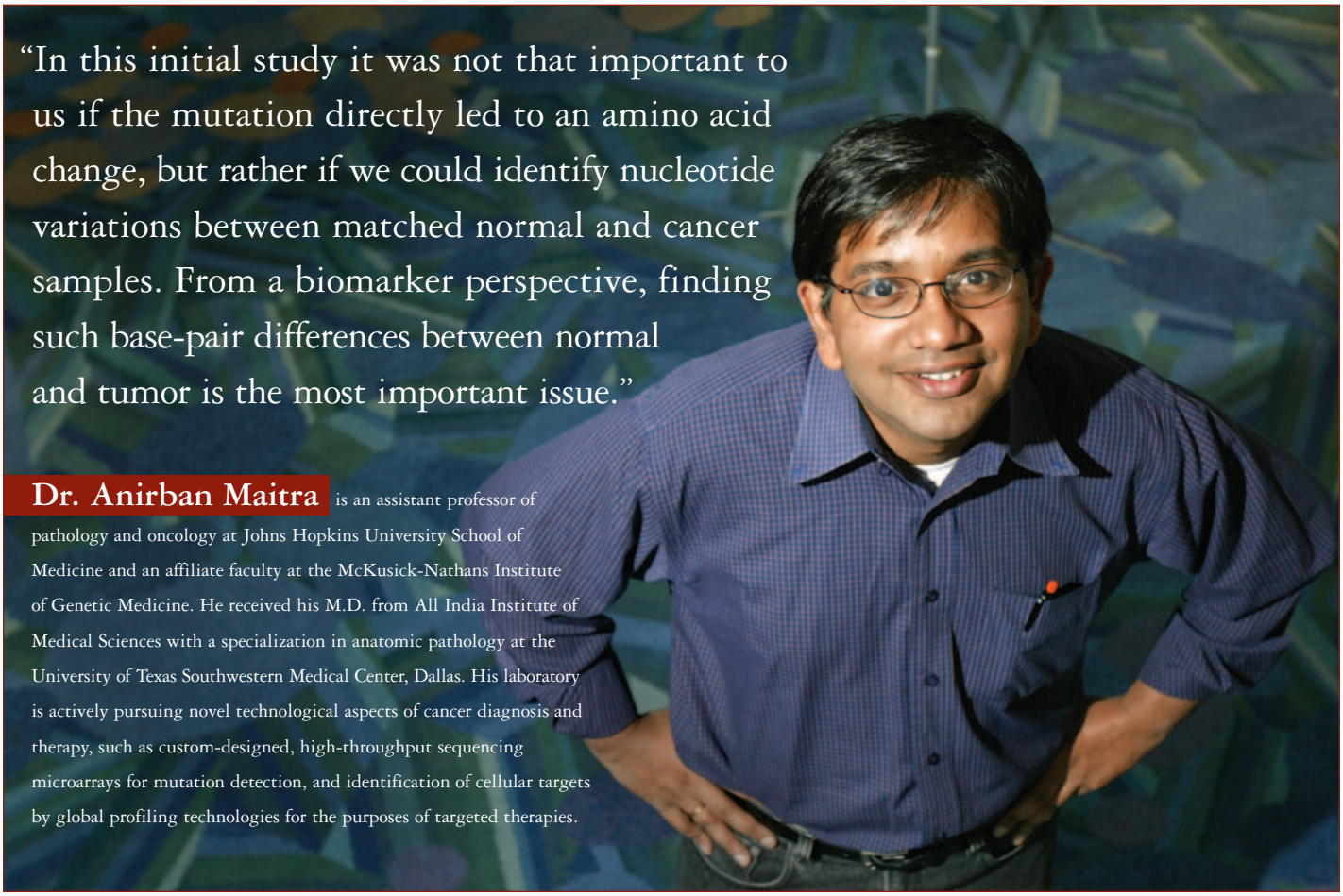
Baumann: Did you use the MitoMap website and the Cambridge Reference Sequence to identify the mutations that you published in your 2004 *Genome Research* publication? Were they all amino-acid altering or did you just distinguish them as polymorphic versus non-polymorphic base changes?

Maitra: Actually, it was a mixture of synonymous and non-synonymous changes. We were primarily interested in finding differences between normal and cancer samples. In this initial study it was not that important to us if the mutation directly led to an amino acid change, but rather if we could identify nucleotide variations between matched normal and cancer samples. From a biomarker perspective, finding such base-pair differences between normal and tumor is the most important issue.

Having said that, if you are actually studying, for example, genotype-phenotype correlations

MITOCHONDRIAL GENOME FACT BOX

Size:	16,500 bases
Coding sequence:	Over 12,000 bases
Mitochondrial mutations:	Present in 70 to 90 percent of cancers
Cancer histologies:	Include breast, lung, prostate, pancreatic, head, neck, and bladder
Nuclear mutations:	p53 is mutated in only about 50 percent of human cancers



Dr. Anirban Maitra is an assistant professor of pathology and oncology at Johns Hopkins University School of Medicine and an affiliate faculty at the McKusick-Nathans Institute of Genetic Medicine. He received his M.D. from All India Institute of Medical Sciences with a specialization in anatomic pathology at the University of Texas Southwestern Medical Center, Dallas. His laboratory is actively pursuing novel technological aspects of cancer diagnosis and therapy, such as custom-designed, high-throughput sequencing microarrays for mutation detection, and identification of cellular targets by global profiling technologies for the purposes of targeted therapies.

or doing a bit more of a functional type of analysis, then that information becomes very important. And we did find many of those examples that had been referenced in MitoMap, or in previous studies in the literature. But at the same time, we found a bunch of other changes as well. I'm not sure what the significance of them is, except that they were not present in the matched normal tissue.

Baumann: We were absolutely amazed at the number of amino-acid altering mutations we found when we started sequencing whole mitochondrial genomes. How can we find more of an in-vivo model for some of these mutations? Obviously, it's not currently possible to really make a transgenic mouse where you can alter specific bases in the mitochondrial genome.

Maitra: One of the biggest challenges has always been attributing exactly what these mutations mean in cancer. The implications of mitochondrial mutations are far better known in many metabolic diseases, but the implications for cancer pathogenesis and progression have always been somewhat in doubt.

The first really conclusive evidence actually came from John Petros' work, that you obviously were involved in, which showed that when you over-express a mutant mitochondria in a cancer, you were able to make it grow faster in vivo, while if you express the wild type, you actually make it grow slower than it normally does.

I suppose the final sort of proof will be when we are able to overcome the technical challenge of making a transgenic mouse model of a mutant mitochondrial gene and show that these mice are more predisposed to cancer, aging and so forth. But just the demonstration that you can take a single point mutation in the mitochondrial gene and can actually increase in vivo tumorigenicity is, by itself, a very nice demonstration of functionality.

The "mass advantage" and heteroplasmy

Maitra: The 'mass advantage' of mitochondria means that you only have two copies of every gene in the nucleus, but you have many more copies of mitochondrial DNA. So if you're dealing with limited clinical samples, for example body fluids that have limited amounts of DNA, you have a better chance of detecting mutations with mitochondria than nuclear DNA. This has been shown by several groups at Johns Hopkins in a variety of human cancer types.

Baumann: But the quantity of DNA you're able to obtain with mitochondria may also create a disadvantage in distinguishing between different types of heteroplasmy. Within a mitochondrion you might have both wild type and mutant base pairs or maybe within a cell, you have different mitochondria that are wild type and mutant. Even within a tissue, you may have cells that are wildtype or mutant for certain mitochondrial DNA mutations. How are you able to distinguish between these different types of heteroplasmy?

Maitra: The problem at this point is technically challenging to answer: `Is there a difference if you have an intracellular heteroplasmy versus an intercellular heterocellular heteroplasmy?'

You'd have to fall back on in vitro studies where you basically make cells with designer mitochondria (so-called "cybrids") within which you can actually titrate the dose of heteroplasmy. Then you can say, 'Okay, if I have this many mitochondria in this cell that are mutant versus this many that are wild type, is there a difference compared to mixed populations of mutant and wild-type cells?' These are questions that have to be answered in vitro using hybrid techniques that we have access to and gradually titrating the doses. But in terms of what happens in vivo, I don't think we have the expertise at this point to answer that question.

Baumann: I agree. The hybrid-hybrid cells are really the best in vitro system that we have, especially if you can get matched wild-type and mutant DNA, so you know the only nucleotide at which the genome is different.

In terms of the heteroplasmic patients we looked at, something we talked a lot about was whether it might actually be better—instead of obtaining a pure wild-type and mutant cybrid—to have a 90 percent and 10 percent wild type across the spectrum to find the threshold at which a mutation becomes dominant.

Maitra: The cybrid technology allows you to actually plan some very elegant experiments looking at mutational thresholds that put a cell over the edge. You know, with the nuclear DNA it's either heterozygote or homozygote, so you're dealing with basically a binary system. But with mitochondria it's sort of a continuous bell curve, if you will, and so the experiments you have to conduct are quite different than what you would do with a nuclear gene.

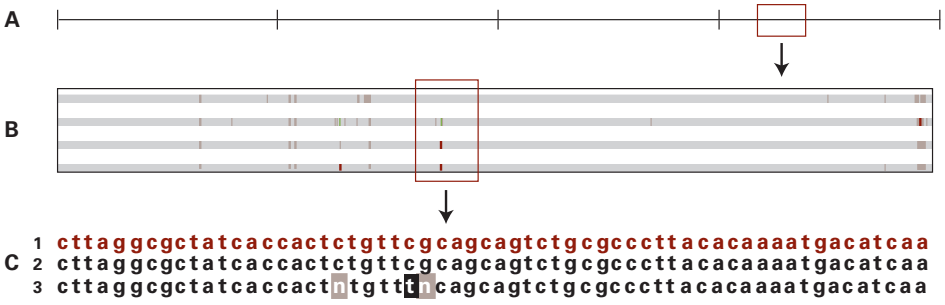
Array-based mitochondrial genome sequencing

Baumann: What does the MitoChip offer to researchers who are currently doing manual and automated DNA sequencing?

Maitra: We discussed several of the advantages of the MitoChip in our *Genome Research* paper, but there are three key ones worth recapping:

First, we cut down the number of polymerase chain reactions (PCR) required to sequence the entire mitochondrial DNA to three. As you know, most routine mitochondrial protocols will use anywhere from 20 to 40 individual reactions.

Second, we reduced the amount of starting template that you need. We cited 100 nanograms in the paper, but we were able to amplify mitochondria with as low as 10



The MitoChip microarray sequences the 16,500 base pair mitochondrial genome (represented in panel A). Upon detailed examination of a small section of the genome sequence (enlarged in Panel B), scientists discover specific nucleotide sequence variations (Panel C). This sequence alignment shows a mutation (black highlight) in the tumor (black second row) compared to the normal (black first row), which is wild type as shown in the reference sequence (red row on top).

Amanda Baumann is a pre-doctoral fellow in Dr. Sally Kornbluth's lab within the department of Pharmacology and Cancer Biology at Duke University. In her earlier research at Emory University, Baumann sequenced patient samples to discover mitochondrial DNA mutations linked to prostate and renal cancers. She has also developed several cell-fusion protocols to explore the role of mitochondrial DNA mutations in prostate cancer.



nanograms of starting template. I think that's because on a per-cell basis you're dealing with a lot more DNA than you would with nuclear DNA.

Third, of course, now you're able to sequence the entire mitochondria on a single chip without having to pour over 16,000 bases of chromatograms. The first generation MitoChip we described had the entire coding sequence short of the D-loop. Now, Affymetrix has developed a second-generation chip that has the entire mitochondria on it, including the D-loop, as well as redundant tiling for deciphering mitochondrial haplotypes.

We've been collaborating with an investigator here at Hopkins, David Cutler, who has a lot of experience in designing high-throughput genotyping analysis software. Dr. Cutler has developed an algorithm for automated sequence analysis of MitoChips; his software is available as freeware online. Affymetrix also has its own software analysis platform that will basically spew out the mutations for you in a beautiful Excel format, with the exact position, the wild-type sequence and the base change.

Then the other thing is the time factor. It takes about one and a half days for a full-time technician to get from starting template to a scanned chip with 16,000 bases of associated information. And you can multiplex eight chips at a time on most Affymetrix wash stations, so obviously that significantly cuts down on the time required as well.

Sequencing method sensitivities

Maitra: The sensitivity of the assay is particularly important when you're looking at this as a potential biomarker-detection tool. We showed that this assay is more sensitive than some of the more conventional fluorescent dye-sequencing technologies that are out there. We've basically done sequential dilution studies and shown that in a mixed population of normal and mutant cells you can detect mutations—80 percent of the mutations in a sample—even at a dilution of 1:50 (i.e., when only two percent of the cellular population is comprised of tumor cells and 98 percent are normal, wild-type cells). I think the limit of detection for fluorescent dye sequencing technologies is about 10 percent. We've managed to achieve about five-fold better rates.

It's well-known that allele-specific hybridization techniques are always going to be more sensitive than fluorescent dye-sequencing technologies. While it used to be the gold standard to prove mixed populations of cells existed within a sample, it's really time consuming and tedious. Affymetrix technology basically is allele-specific hybridization on a chip, so it's not surprising that it's more sensitive than regular fluorescent sequencing techniques.

What's interesting is that we have been collaborating with the National Institute of Standards and Technologies (NIST), located in Gaithersburg, Maryland, and they actually used a two-stage PCR base-sequencing protocol that they developed to sequence many of the same tumor samples that we described in the original MitoChip paper. So they had these chromatograms, and when we gave them the MitoChip data, there were several mutations they had actually not found using their sequencing software. Anyone that has worked with chromatograms knows—especially with 16,000 bases worth of chromatograms—that you're bound to miss small heteroplasmies. When the NIST group went back, they found that every one of the heteroplasmies was present; they were just little blips that were not picked up by manual scanning or even by automated sequencing software. That paper just came out in the May issue of Journal of *Molecular Diagnostics.*

Baumann: I definitely agree. Those chromatograms can be very time consuming to read and it's often difficult to know what is background and what might be considered heteroplasmy. From just a data collection point of view, being able to verify and obtain more accurate results has to be one of the biggest advantages.

Sample preparation

Baumann: What types of samples are you able to sequence off the microarray?

Maitra: Well, we've used either snap-frozen samples or body fluids like urine or pancreatic juice. Certainly, body fluids work very well; there's enough mitochondria floating around to amplify. We've actually never tried paraffin-embedded or laser-capture micro-dissected samples.

We've designed the assay for long-PCRs and there are three fragments about 5 to 6 kB long. So, as long as the DNA quality is adequate enough to get those long products, the assay should work. If you can do laser capture of cryostat-embedded sections that should do pretty well. But unless the existing long-PCRs are modified, I don't know at this time if paraffin-embedded, archival material will work, so that's one drawback.

Baumann: We used about 18 sets of PCR fragments to span the whole genome. How did you choose the three sets of PCR fragments?

Maitra: I designed the primers, made sure they worked, and then we went to the MitoChip. I designed these mitochondrial DNA long PCR primers using a web-based protocol called 'Amplify' that I found is the best long-PCR program on the web. It's free on the web and I highly recommend it; it really works very well.

I arbitrarily divided the mitochondrial DNA into three fragments and designed two alternative primers for each, such that we would have amplicons of 5 to 6 kB each. So we had six sets of primers, each of which was sort of an alternative pair covering three of the fragments. And of each pair, one of them worked beautifully.

We chose such long-PCR protocols for two reasons: number one was, of course, you wanted to cover the mitochondria in as few PCRs as possible, without having to do too many amplification reactions. And the way the chip works, you need to pool all of them in equi-molar quantities. So if

you have 20 PCR reactions, you'd have to clean them each up, you'd have to measure the DNA quantity, and you'd have to aliquot equi-molar quantities from each fragment. The fewer reactions you have, the better it is for you to actually be working with the MitoChip, or for that matter, any DNA sequencing array.

The other more technical issue we had is that there are significantly long stretches of mitochondrial DNA insertions in the nuclear DNA—sort of like pseudo genes if you will. If you have too short an amplicon, you could potentially be amplifying a nuclear insert, as well as a true mitochondrial DNA. And if they are different in sequence, you'd think that you are getting a mutation or a heteroplasmy, but it is really nuclear DNA. To overcome that, we actually designed these long-PCR primers; we tested them on cells depleted of mitochondria; and we found that there was no product. So we know that these are mitochondria-specific primers.

We've also tested them on mouse DNA, and they do not amplify. So fortunately for us, they are also human-specific. So, if you're looking at xenografted tumors, for example, you can safely use these primer sets to amplify, and you know that the only thing you are amplifying is human mitochondrial tumor DNA and not the mouse stroma.

Genome coverage

Baumann: You said that initially you had left out the D-loop in the MitoChip?

Maitra: Yes. However, the new second-generation MitoChip has several differences.

For one, it has a smaller feature size; so it has about 50 kB of sequence as compared to the 32 kB that we had in the first generation chip. That 50 kB comprises the entire mitochondrial coding sequence—top to bottom, forward and reverse strand—as well as redundant tiling for mitochondrial haplotypes and known mutations from common websites such as what is referenced in MitoMap, and other online databases out there.

That gets me to one of the problems of chip-based hybridization chemistries, chips are very good at detecting single-base changes, such as missense or nonsense mutations or single-base polymorphisms; however, they're not as good when it comes to detecting insertion-deletions. Affymetrix has included redundant tiling features in the new MitoChip that will enable researchers to detect potential insertion-deletion mutations especially in the non-coding D-loop region where these are not uncommon. The advantage of an iterative tiling strategy for regions that demonstrate frequent insertion-deletion mutations is that you can now actually look for the sequence that gives you the best hybridization and use that as a guide for detecting such mutations with greater confidence than you could in the older generation of arrays. In other words, having 50 kb worth of sequence to 'play with' has given Affymetrix the freedom to introduce additional features that I believe strongly enhance the utility of resequencing arrays.

We've now worked with this new MitoChip for about six to eight months using human cancer samples, and although we haven't published the data yet, it looks very good; for example, we are able to detect additional mutations that we missed using the older generation microarrays. A lot of the artifacts associated with, for example, dye bleeding through and so forth in the older generation chips have now gone as well.

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■ **Johns Hopkins University School of Medicine** - http://www.jhu.edu/

■ **McKusick-Nathans Institute of Genetic Medicine** - http://www.hopkinsmedicine.org/geneticmedicine/

■ **The Sol Goldman Pancreatic Cancer Research Center** - http://pathology2.jhu.edu/pancreas

■ **Duke University** - http://www.duke.edu/

■ **National Institute of Standards and Technology** - http://www.nist.gov/

■ **MitoMap Database** - http://www.mitomap.org/

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People

■ **John Petros** - http://www.emoryhealthcare.org/departments/wci/sub_menu/Prostate_Cancer.html

Tools for download

■ **Amplify** (web-based primer design software) - http://www.bioinf-club.com/pages/download.htm#soft1

100,000 SNP Copy Number Studies Find New Genes Linked to Cancer

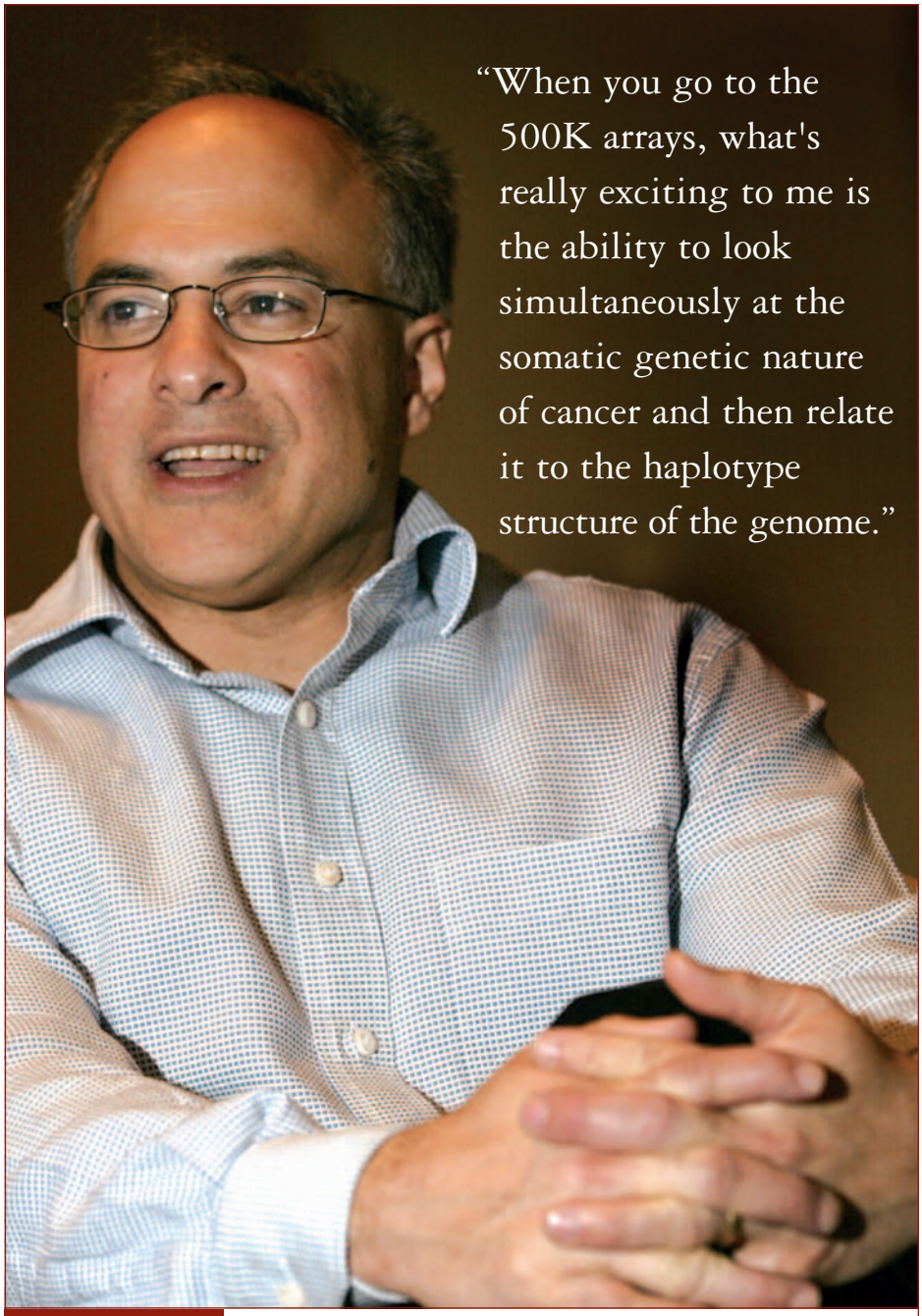
Dana-Farber's Dr. Bill Sellers and UPenn's Dr. John Maris discuss the challenges of high-density SNP genotyping in copy number analysis

Chromosomal copy number and loss of heterozygosity (LOH) studies analyzing more than 100,000 single nucleotide polymorphisms (SNPs) are generating promising new results in the search for cancer-causing genes that are mutated, missing or duplicated. While both methods have been around for awhile, by using high-density SNP microarrays to conduct these experiments, scientists can now search the genome faster and in greater detail than ever before.

Dr. Bill Sellers together with his colleague Matthew Meyerson, both of the Dana-Farber Cancer Research Institute, were among the first scientists in the world to use genotyping microarrays for cancer copy number research. A few years later, Seller's has upgraded from studying a few thousand SNPs with a microarray to studying more than 100,000. And in the July issue of Nature, Seller's reported a critical gene, MITF, that was amplified in 20 percent of metastatic melanoma patients studied and was also functionally required to transform human melanocytes.

Dr. John Maris, an associate professor at the University of Pennsylvania's (UPenn) School of Medicine studies pediatric cancer and in particular the correlation between DNA copy number aberrations and tumor genotype and clinical phenotype.

"We're very interested in understanding the pattern of genomic alterations and how they associate with tumor aggressiveness," said Maris. "The potential of SNP arrays is in having a very high-resolution survey of the entire genome—and that resolution continues to increase. That's why the SNP chips are a very attractive platform for cancer genomics."



Dr. Bill Sellers is an associate professor of medicine at the Dana-Farber Cancer Institute and Harvard Medical School. He is also an Associate Member of the Broad Institute. His research focuses on generating a genetic and molecular understanding of various cancer types, including prostate and lung; his lab was among the first to apply SNP-array based LOH detection to prostate cancer. Sellers' research has two primary aspects: understanding the role of the PTEN tumor suppressor gene in the progression of prostate cancer, and discovering expression and genetic profiles for poor outcome prediction in prostate cancer.

While technological advances in genotyping density have helped both researchers find new genes linked to disease, the advances have also generated a new set of challenges. Sellers and Maris recently discussed some of these emerging issues with AMB; topics included:

- methods for dealing with cellular contamination during sample preparation
- the merits of differing copy number analysis technologies
- ways to improve copy number algorithms and assays for cancer-specific research
- the value of higher-density SNP analysis in resolving haplotype structure within the cancer genome and the contribution of germ-line polymorphisms to disease

Potential of copy number and LOH analysis

Maris: We look at LOH and copy number for two reasons: to identify prognostic biomarkers and to identify therapeutic targets.

There is a long history in pediatric cancer literature, in particular in the disease neuroblastoma, and there is a lot of information showing specific regional gains or losses to be very powerful biomarkers. This is currently being applied to the clinic and used to help stratify therapy; examples include MYCN gene copy number status in neuroblastoma and measurement of 1p or 16q LOH in Wilms' tumor.

The second more recent, and perhaps more important goal, is to use these data to help us identify the genes that are driving the phenotype. Certainly my bias is that it really comes back to DNA and DNA mutations. The most important therapeutic targets are going to be genes that are targeted by DNA aberrations—either gross DNA aberrations, gains and losses, and/or DNA mutations—and we hope to use SNP chips to help us streamline identification of these.

Sellers: I agree; those are the two major applications. To expand a little bit on distinguishing LOH and copy number, which is one of the things that the SNP array will give you, I think many people end up equating LOH with hemizygous deletion. This is only one way in which you get LOH. But you can also get LOH through gene duplication of a mutant allele. For example, instead of having a wild-type and a mutant allele, you now have two copies of a mutant allele. This is not uncommon in the p53 locus. So LOH analysis gives you a different view of the genome than you can get by straight copy number alteration.

Maris: Yes, that's a very important point and I can give you an anecdote where this is very important in our disease: LOH in chromosome 11q is a very important biomarker, but if one were to use something like [fluorescence in-situ hybridization] FISH to just count the number of alleles, you very frequently see two alleles, despite the fact that there is LOH. Mitotic recombination gene duplication is very, very common. If you just use FISH to survey for this aberration you miss it most of the time.

Sample preparation and sample purity

Sellers: The challenges of array-based genotyping for copy number analysis are mainly due to sample preparation. To get the maximal amount of information across the array currently requires a highly purified tumor cell population, especially if you want to get accurate calls and accurate determination of LOH.

That's actually the rate-limiting step right now for common solid epithelial cancers where the cancer cells are intermixed with stroma. The primary challenge has been to optimize conditions of laser-capture microdissection and amplification of the DNA. Initially, the informatics was the biggest challenge, but we've made a lot of progress there and it no longer poses a great challenge for us.

Maris: Currently, each of our labs spends quite a bit of time carefully preparing high-quality DNA out of carefully acquired and prepared samples. But to take this to the next step, to really get at the 99 percent of human cancer samples that aren't archived in a way that's amenable to developing high-quality DNA probes, that's really going to be a challenge. The big question is: 'Can you do this in paraffin?' And right now, the answer is, 'no.' But the lab efforts are going to be directed towards either getting high-quality DNA probes out of sub-optimal tumor preparations and/or finding an assay that allows lower-quality DNA probes to work.

Sellers: I agree with all of those points; paraffin is a big obstacle.

Pluses and minuses of copy number analysis technologies: SNP arrays and CGH

Maris: I have two specific examples I can give you from our own work. First, using a BAC array platform we identified a homozygous deletion that also happens to be on 3p, but not near Bill's locus. On the BAC array it was quite a large homozygous deletion, but just by putting it on the 10K SNP chip we were able to cut the size of the homozygous deletion in half, just in one experiment! Now, as we speak, we're putting it on 100K. So, just in terms of mapping, it dramatically helps hone in on the region where the genes are located.

The other example involves low level gain of 17q—a frequent occurrence in many human cancers. In neuroblastoma, it has been assumed that the gain is even along the chromosomal arm—i.e. there is one or two additional copies of each nucleotide. However, our recent SNP experiments strongly suggest that there are regional amplifications many fold higher within the background of low level copy number gain. These data may help us identify the true oncogenes located at 17q.

Sellers: We've clearly seen that the 100K arrays pick up deletions that you can't see on the 10K array. The best example might be a gene like EGFR, which in malignant glioma has a deletion of exons 2 through 7 or 8 known as vIII. You can't see that on a cDNA array, or a BAC array, or the 10K array. But on the 100K array, we start to see the vIII deletion in occasional samples because there are probes for four SNPs within that deleted region, but that's not really enough to be highly confident about the deletion every time. You can imagine that if there are 20 SNPs in that region, like on the 500K array, you'll see that deletion almost every time. There are a number of genetic events that are very small that you'd really like to see. And as you get better and better resolution you'll see more and more of them.

Maris: BAC arrays are also cumbersome to create and it's difficult to keep the clone sets and verify the clone sets. Additionally, they can't approach the resolution of the 100K chips, and certainly not the 500K chips. There are full tiling path arrays, but the spot is still a [polymerase chain reaction] PCR product from an on-average

Dr. John Maris is an associate professor at the University of Pennsylvania School of Medicine and an oncologist at the Children's Hospital of Pennsylvania. Dr. Maris received his MD at the University of Pennsylvania and did his post-doctoral research and clinical fellowship in pediatric hematology and oncology there as well. His research interests include profiling for LOH and copy number with genotyping arrays and using this information to develop biomarkers and or therapeutic targets in cancers such as neuroblastoma.



"The big question is: 'Can you do this in paraffin?'"

200 kB piece of DNA, so there are some limits in the resolution of the BACs and they are inconvenient.

That said, BAC arrays do not rely on high-level PCR amplification of your sample. Data from our lab, and from many others, have shown that this issue of normal stromal DNA contamination—while it remains an issue—is not critical in BAC arrays as compared to any sort of SNP oligo array platform. And although these numbers are a little bit soft right now, my guess is that when you have more than 10 percent contamination with normal DNA, you lose your calls pretty precipitously with the SNPs. But on the BAC array, you can get away with up to 50 percent normal contamination. Bill, I don't know if you would agree with those numbers or have looked at that in any detail.

Sellers: With SNP arrays, we've found that at 10 percent contamination, accuracy for LOH calls—allele calls—is pretty much gone. You can go down to about 30 percent stromal contamination and still get pretty good copy number and homozygous deletion measurements if you don't mind losing the LOH; of course, we'd rather not lose it.

Improving algorithms and assays

Sellers: But, there's some hope that new informatics methods with improved call algorithms might correct aspects of stromal contamination. I think Affymetrix has optimized the algorithm for germ-line DNA analysis, so with even the slightest deviation from a homozygous call—you might happen to have a little bit more B allele than A allele—the algorithm starts to make 'no call' designations because it really doesn't want uncertainty. But often, if you visually inspect the probes for each allele in a contaminated sample where a 'no-call' determination was made, you can pretty much see what the genotype call should have been; of course for 100,000 SNPs, it's impossible to manually score all of the genotypes.

It would be really nice to have some help from Affymetrix looking at how to improve the call accuracy from mixed samples. We would imagine taking a tumor sample and contaminating it with normal genomic DNA in order to create an artificial genotype set that usually would result in 'no call' designations, and then try to develop algorithms that still allow calls to be made. Maybe you could decide what algorithm to use for your sample; it might say, 'when working with the cancer sample, please don't use the perfect-call algorithm, use the cancer-call algorithm.'

Maris: I like the idea of there being a germ-line DNA algorithm and there being a cancer DNA algorithm.

In terms of assay optimization, I guess one of the other overriding features in getting this assay to work is the highly multiplexed nature. I don't know if there would be any opportunity to cut back on the number of cycles of PCR which is sort of the key step without losing the call rate pretty significantly.

Sellers: Yes, I think that's a good point. We have spent almost no time optimizing the lab process part for copy number alteration detection. We thought about using quantitative PCR to look for genome copies that are produced during the PCR cycling, to see if you hit a certain number of genome copies; that might give you an optimal copy number detection point as well as still having robust calls.

Moving to the shorter fragments will potentially give more uniform amplification during PCR and there may be less variation around mean copy number across the genome. Hopefully on the 500K array we'll move away from the longer fragments that happened with 100K—there was a transition between 10K and 100K where a lot longer fragments got put on the 100K. If you look at the way long fragments behave with respect to copy number, it's much worse than the shorter fragments. It's also worse with respect to call rates. So their behavior in general is not as good.

The value of higher resolution analysis

Sellers: When you go to the 500K arrays, what's really exciting to me is the ability to look simultaneously at the somatic genetic nature of cancer and then relate it to the haplotype structure of the genome. So for the 500K, I believe you'll start to see things like haplotype-specific deletions and you'll see associations of haplotypes of amplifications. In other words, we will begin to see how germ-line polymorphic alleles might be contributing to the cancer that develops. I think that's one very exciting area which can really only be looked at using the SNP array platform.

Maris: Yes, I agree with that completely. The 500K especially is going to open up a whole new world in terms of doing classic genetic association studies as well as pretty novel cancer-specific genomics. So we're excited about that as well.

For mainly technological reasons, we have had a very, uni-focal, looking-under-the-lamppost type of approach, and prognostic biomarkers have been assessed one at a time, a deletion here, or an amplification there by itself and correlated with corollary measures, clinical factors. And it is very clear that it's the pattern of DNA aberrations across the genome that really gives you a much more refined picture of the phenotype. There are cooperating loci and associations that if you can consider those together, may be more powerful than the uni-focal approach that we have now. So that's one of the big areas of interest in our lab, what is the pattern of aberrations and can you learn more from the entire profile than you can from the region-by-region approach, considering things one at a time.

The only downside I see is as we've gotten higher and higher resolution, we've really run into this problem of how complex the human genome is in terms of copy number polymorphisms, and separating out the real signal from the noise is still a bit of a challenge.

Sellers: Some of that will be offset by the scale of some of the germ-line studies that are planned by the medical population geneticists using the 500K. With researchers running 10,000 normal germ-line DNAs on the 500K, you're going to have a pretty good map of polymorphisms pretty soon. It'll never find the ones that are less than one in a thousand, but I think we can count on our medical population genetics colleagues to identify many of these, and that will help us just simply subtract these by database searches.

Maris: I agree. In the meantime, we are going to be scratching our heads until there is a big database of these copy number polymorphisms.

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Tools for Download

■ **Affymetrix Chromosome Copy Number Analysis Tool v2.0** - http://www.affymetrix.com/support/developer/tools/affytools.affx

■ **dChip** - http://biosun1.harvard.edu/complab/dchip/snp/

SPECIAL SECTION: TRANSLATIONAL MEDICINE

Dutch Hospital Offers Heavy Dose of Translational Medicine

Erasmus Medical Center scientists Prof. Peter van der Spek and Prof. John Foekens weigh in on their institution's evolving infrastructure to support translational medicine

From Pasteur to Salk, the purpose of biomedical research has always been to take research and turn it into a new diagnostic or treatment that improves health. Translational medicine aims to speed that process up by taking advantage of modern technologies and performing the primary research in such a way that it can be more quickly converted into an applied tool.

While translational medicine is a phrase that baffles a lot of people, they would clearly support its benefits of cheaper and more effective healthcare.

Erasmus Medical Center (MC) scientists John Foekens and Peter van der Spek have been working in the field for more than 20 years and to them translational medicine means an institutional commitment to a cross-functional infrastructure, a heavy dose of multidisciplinary teamwork, an unbiased approach to genome exploration, and developing alliances early with business partners that can help develop a diagnostic or therapeutic for the marketplace.

"The bioinformatics team really ranges from IT [information technology] folks to molecular biologists to clinicians to statisticians. It's a variety of people," said van der Spek. "But the multi-disciplinary angle of how the team works makes it possible to cross borders and perform research that we wouldn't otherwise be able to do."

Dr. van der Spek is a professor of bioinformatics, who uses modern genomic and proteomic approaches to study cancer predisposition genes and neuroscience relevant pathways at the molecular level. Dr. John Foekens is a biochemist who has published more than 200 articles focusing on breast cancer prognosis and therapeutics. Two recent studies he's been involved in have discovered expression signatures predictive of tamoxifen-resistant breast cancer and metastatic breast cancer; the studies were reported in the *Journal of Clinical Oncology* and *The Lancet,* respectively.

The Erasmus MC scientists spoke with AMB Managing Editor Tommy Broudy about some of the challenges of setting up a translational medicine institute and some of the successes that have resulted.

Setting up a translational medicine hospital

Broudy: Erasmus MC is now positioned as an incredible site for clinical trials using technologies like microarrays. How did you transition a traditional hospital to a clinical center for translational medicine like the one you have now?

van der Spek: We have set up the bioinformatics department in such a way that it can also be used as a certified environment for supporting clinical trials and multi-center efforts. The bioinformatics department requires substantial hardware to look at complex volumes of data, as do the medical imaging and molecular imaging departments—two new areas that are expanding very rapidly. We have managed to combine the hardware for the molecular array data, proteomics data, and imaging data. We are seeing more and more biomarkers being discovered in projects, and of course, we can monitor these biomarkers in 3-D scans. In fact, we have built a 3-D cinema for the purpose of moving molecular biology towards medical imaging, a field now called molecular imaging.

Foekens: We are already working with Peter's bioinformatics group on a couple of new projects. For the breast cancer study that was just published in our *Lancet* paper, all the data-mining and bioinformatics were done by the group of Dr. Yixin Wang and Dr. David Atkins, Veridex LLC, a Johnson & Johnson Company, San Diego/New Jersey. We started the collaboration at the end of 2002 to help bring this signature to the clinic. By that time, Peter was at Johnson & Johnson and linked David onto the well documented patient archive in Rotterdam.



Dr. Peter van der Spek

Dr. Peter van der Spek is head of the department of bioinformatics at Erasmus MC in Rotterdam. His bioinformatics group focuses on the support of data mining and analysis of fundamental and translational research using cutting-edge bioinformatics tools. The Erasmus MC is a test and development site for technology and software tools such as Affymetrix, Inxight, OmniViz and Spotfire. This expertise is being applied in the fields of molecular diagnostics, molecular imaging, forensics, and molecular biology and in clinical trials in one of the largest medical centers of the Netherlands.

Broudy: Translational research accelerates the process of bringing research from bench to bedside, but the work that goes on in each area typically has very different regulatory and quality control criteria. How can one institution really accommodate both?

van der Spek: In terms of working with patient material for doing diagnostic classifications, there are actually three labs equipped with Affymetrix equipment in order to fulfill the regulatory restrictions. One lab is focusing on DNA SNP [single nucleotide polymorphism] profiling for linkage and cytogenetics purpose. Then we have a lab which focuses on the human RNA profiling for pathway analysis, and we have a third lab for animal models—the biomics core facility.

Certification of the Affymetrix system last year was a major step forward in order to tie these things more immediately into our efforts in the clinic. That's because when you work with a certified machine and certified software, there won't be any more changes in the system. When you change the software halfway through the procedure, you cannot correlate things back to the samples that you had run previously. So that's what we also keep very strongly in mind. But in case changes are made somewhere in the analysis pipeline, we basically store the CEL, as well as the DAT files, so that we can go back to the raw data and push everything through the pipeline again completely once new ways of normalization or data mining get established.

Foekens: We have separate labs to isolate RNA and DNA in the facility that our group uses. It's just isolation in these rooms because you need to, of course, control for contamination of aerosols. And then we take the RNA or DNA back to our lab for qPCRs [quantitative polymerase chain reactions] or we take it to the central facility lab for microarray hybridization.

Multi-disciplinary approach

Broudy: It wasn't too long ago that you really couldn't find two people with less in common than a statistician and a physician. How are you and the Erasmus research teams integrating informatics and statistics with the clinical team?

van der Spek: We have set up a multi-disciplinary team that includes experts on IT, software, and on how to use these pieces of software, experts on genomics technologies and proteomics technologies, and statistics. And to tie all this together, we defined a five-pillar approach:

1 The first pillar is based on IT. So we focused two people on the computers that everything runs on.

2 Part two is the software which runs on the hardware. These are generally client-server applications. But the software needs adjustments, so we have a pillar called, 'Software Development.' There, we optimize certain ways of viewing the data.

For instance, with the leukemia study of Valk et al. published in the *New England Journal of Medicine* in 2004, we made a picture where we combined clinical data with the molecular data coming off the arrays. The first picture was born by analyzing one correlation view with large volumes of patients. So if we've already compared 300 patients, and number 301 walks into the clinic, we can immediately position that individual into the history of all the patients that we have previously seen on the arrays. For the diagnostic decision-making process, that's part one, a clinician wants to see where the patient falls out.

3 Now, of course, we have a pillar which focuses on education. This is because we are a university. Moreover we need to train people in using visualization and data-mining solutions. We realized that we needed to create certain views that clinicians and biologists could see via a web environment, because almost all clinicians are nowadays used to working with Internet Explorer or with their email. When we did that, the learning curve went down enormously, because then we didn't have to train them as much and could actually go for end results.

4 Another pillar covers our genomics group. All the array profiling expertise is in there.

5 And finally, we have a pillar which focuses on mass spectrometry and other proteomic approaches. And, as mentioned, the five pillars are loaded with a variety of multi-disciplinary people. We have a nuclear physicist in our proteomics pillar that is not afraid to deal with the vast, vast volumes of data that are generated in proteomic experiments.

Foekens: In my own group, together with Professor Jan Klijn (he's the medical oncologist we work with), we also recognized the importance of this multi-disciplinary approach. Our group is about 20 people, and we have a biostatistician, together with a medical doctor, molecular



Dr. John Foekens is head of the laboratory of tumor endocrinology in the Department of Medical Oncology at the Josephine Nefkens Institute at Erasmus MC in Rotterdam. His recent research focuses on high-throughput gene-expression and DNA-methylation array analyses for prognosis and therapy prediction in patients with primary and metastatic breast cancer. He was honored for being one of the 100 investigators published most frequently in the 20th century for studies advancing breast cancer treatment (San Antonio Breast Cancer Symposium 2000).

biologists, and technicians specializing in pathology, biochemistry, and molecular biology. We also have two people who specialize in animal studies.

Just look at our prognosis or response to therapy studies. We have information coming from DNA methylation, from RT-PCR [real-time reverse-transcriptase polymerase chain reaction], from the microarrays, and now it's coming from proteomics. It's all different, and we need a team that can combine it.

Whole genome vs. focused study

Broudy: The benefits of whole-genome exploration in the discovery phase of translational research seem clear. What are your thoughts about whole genome vs. focused analysis when the data coming off these high-density technologies make their way to the actual patient?

Foekens: For the whole genome, you need frozen tissue. But it's always preferable to do the whole-genome analysis, because you can always come back later to ask new questions and to find new profiles. The breast cancer study which we have done with Veridex is a good example. We found the profile and validated it in our own patient set—that's the *Lancet* paper. But now we have to move on to bring it to the clinic, so what we need is an independent validation set, and preferably a prospective trial.

But before you can enter a prospective trial, you need to validate the data. So we have performed a multi-center study with several institutes in Europe. For this validation study, we are working with Veridex and they have a custom Affymetrix microarray with the 76 genes on it. The whole-genome approach may be preferable, but in practice it seems that a dedicated chip is more pragmatic. The whole-genome chip allows you to do discovery studies, but you also get your problem of intellectual property-related issues where everybody wants a piece of the cake, so it gets very complicated.

We may bring our 76-gene breast cancer signature down to a multiple-qPCR. In the end, it may be a diagnostic chip. I'm just not sure yet.

van der Spek: In my eyes, whole-genome analysis is easier also with respect to the statistics of doing the analysis. The genes which don't change also tell you a lot in terms of the stability of the experiments.

And a full-genome chip allows you to measure and study pathways which you initially would not study in the context of the disease. With the hematological type of questions, you could be studying leukemia, but the analysis may give you added information about everything from blood group to high cholesterol levels. You can measure so many more things, which you can't do when you have a theme. And in order to gain insight into the underlying molecular pathways involved in cancer we use the Ingenuity pathway database.

Developing better signatures

Broudy: John, you have a lot of experience with multi-centered studies. What are your thoughts about maintaining data quality with so many groups involved?

Foekens: I don't think location of the participating institutions is a major concern. If you set pre-defined criteria for the inclusion of patients in your studies before analyzing any RNA on the microarray, you should be okay.

We have now performed multi-center studies where the centers sent tissues to our institute and we isolated the RNA. The quality is perfect. There's hardly any drop-out because of poor quality. It would be different, of course, if you asked the separate small institutions to isolate the RNA themselves. We know from the early days in the quality control rounds organized by Professor Theo Benraad, and nowadays by Professor Fred Sweep in Nijmegen, that the variability for something like a protein assay can be over 20 to 30 percent. That does not mean that the specific laboratory is performing badly; it's

> "We have information coming from DNA methylation, from RT-PCR, from the microarrays, and now it's coming from proteomics. It's all different, and we need a team that can combine it."

just that everybody uses his own standard to calculate something like the protein content or the estrogen-receptor or progesterone-receptor level.

There can be a difference of a factor of 10 between two laboratories, while their intra-laboratory variation is extremely low. So it's just a matter of standardization.

Developing diagnostics and impacting healthcare

Broudy: Your recent work in the *Lancet* has identified a very strong signature predictive of breast cancer metastases. What strategies are you using to move this into an actual diagnostic?

Foekens: In the *Lancet* paper we validated our profile in an independent set of 171 patients. But to bring it to the clinic, you need more. First of all, you need a company, because as a research group you cannot bring something to the clinic.

We are now validating the profile in two multi-center patient cohorts. One is an untreated set of 180 patients. That data has already been analyzed and the data look excellent. We are now analyzing a cohort of lymph-node negative breast cancer patients treated with tamoxifen, so that will be a second independent validation. We're still working up samples, isolating RNA and sending it to Veridex for microarray analysis. We'll need at least 200 samples in this second set because there are fewer failures, fewer relapses, in tamoxifen-treated patients compared with the untreated.

Then, we would like to go, of course, into prospective clinical trials, but that's for the business people at Veridex (Johnson & Johnson) to decide. We generated the profile together with Veridex and they wish to bring it to the clinic if the validation studies hold like they do now. In the first multicenter validation study, it looks excellent.

Broudy: Integrating expression data and clinical data along with other molecular data will be key. How do the software packages that you're developing now work towards this very large goal of integration?

van der Spek: That's a very important point. If you have programmers that build particular solutions, these solutions have to be supported over time. The aim of a medical center is not to support software, so you need to build software in collaboration with professional players in this field. We can build very fancy new ways of looking at and analyzing data, but we do it in collaboration with preferred software partners like Spotfire and OmniViz . They can take over the routines, support them over time and migrate them into new versions. We could never do this from a medical center.

Also, pharmas are now interested in using web based tools, such as Inxight technology to couple microarray data onto the latest version of the human genome, which has the most current annotation, and link all their historical experiments onto the newer annotation files. Of course, you can do this via the NetAFFX web site, but that's still quite labor intensive, and you don't want your scientists to have to worry whether they are working with the latest annotation. You just want to have it there. We are working on these kinds of solutions with EBI, the European Bioinformatics Institute in Cambridge, UK.

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Companies

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Organizations

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■ **Dutch Cancer Society** - http://www.kwfkankerbestrijding.nl/content/pages/English_information.html

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