Welcome everyone to the Science/AAAS live webinar on the “Discovery of Autoantibody Biomarkers for Cancer and Autoimmune Disease”. My name is Sean Sanders and I’m the commercial editor at Science Magazine.

The focus of today’s online seminar will be to explore currently available detection and discovery systems for autoantibodies, in particular protein array technology. Autoantibodies hold significant promise as tools for early diagnosis and better prognosis of a number of diseases including cancers and autoimmune diseases.

Since the immune system is a ready-made and highly sensitive surveillance system, there is the expectation that it will sense aberrations caused by diseases and generate autoantibodies as a result well before our current medical technologies can detect them. In order to take advantage of this, we require sufficiently sensitive ways to detect specific autoantibody biomarkers or signatures, a not insignificant challenge. The price, however, is a powerful means to not only allow for early detection of disease including many cancers, but also a mechanism to track patient’s response to treatment and to improve prognosis.

We have three exceptional speakers here today; all of whom have had many years of experience in this field. First, we will hear from Dr. Eng Tan who will talk about the importance of autoantibodies as biomarkers for disease as well their reliability, specificity and sensitivity. Next, Dr. Paul Predki from Invitrogen will discuss current approaches and technologies for autoantibody biomarker discovery. And finally, Dr. Michael Snyder from Yale University will talk about the application of autoantibody biomarker discovery technology to ovarian cancer including some recent data.

Don’t forget that you can submit questions to the panelists at any time by typing them into the box below the video and clicking submit. We have a large online audience today so please keep your queries short and to the point. This will give you a better chance of the questions being asked.

On to our first speaker, Dr. Eng Tan is Professor Emeritus in the Department of Molecular & Experimental Medicine at The Scripps Research Institute. He obtained his M.D. from Johns Hopkins University School of Medicine. And besides a five-year period as a professor in the
Department of Medicine at University of Colorado Health Sciences Center, Dr. Tan has been at the Scripps Institute since his first assistant professorship there in 1965. Dr. Tan sits on the editorial boards of a number of prestigious journals including the “Journal of Molecular Medicine”, the “Journal of Clinical Immunology” and “Clinical and Experimental Medicine”. And he has been recognized by the broader scientific community through numerous honors and awards as a top researcher in his field. Most recently, Dr. Tan received the European League Against Rheumatism Meritorious Service Award in 2005. Currently, the focus of Dr. Tan’s research is the molecular and cell biology of autoantibodies and autoimmunity. His work seeks to identify autoantibodies and their requisite antigens to enable the development of better diagnosis and future treatments for autoimmune diseases and cancer.

Dr. Tan?

Dr. Eng Tan: Thank you, Sean.

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For the past 30 or 40 years, we have been very interested and have worked on characterizing autoantibodies in a prototype autoimmune disease called Systemic Lupus Erythematosus. And the reason why it’s called a prototype autoimmune disease is that in lupus there are many, many different types of autoantibodies that can be detected.

Now back in 1948, the LE cell, a diagnostic marker for lupus, was discovered by Malcolm Hargraves and his colleagues at the Mayo Clinic. And until the 1960s, it was the only diagnostic marker available for lupus. And it was detectable in about 30% to 40% of patients, but these were often patients in the severe or advanced states of the disease.

Slide 3

Now until the 1960s and 1970s, the 10-year survival for a lupus patient after initial diagnosis of the disease was a very dismal 50% or less. And the reason for this was the diagnosis was made late, using the LE cell test quite often, but after multisystem organ damage to the kidney, central nervous system, heart, lung and other organs had already occurred.

[0:04:47]

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In the late 1950s and early 1960s, autoantibodies in lupus were discovered, and these were primarily autoantibodies that were directed against nuclear components of the cell. Among these were antibodies, the double-strand DNA, for example, antibodies to a molecule or a group of proteins called the Sm antigen.
Now, these tests for ANAs became increasingly available in the 1970s and were widely used by clinicians in the diagnosis of lupus. And they were extremely useful because they could be used for diagnosis in the early stages of the disease, in other words, before severe organ damage had occurred in the patient.

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The methods that were in use at that time were several. I think the most powerful and most the informative method was the immunofluorescence imaging of autoantibodies that were directed against antigens that are present in tissue culture cells, which were used as the substrate.

Another technique, which was used, was immunodiffusion analysis in agar looking for antibody-antigen precipitation lines. And other essays that were used quite widely were Western blotting and enzyme immunoassays.

And in the next two slides, I’ll be showing a couple of examples of the types of immunofluorescence imaging that one can see with autoantibodies in patients with lupus.

**Slide 6**

This one for example shows an almost homogenous immunofluorescence, and that’s the green that you see throughout the nucleoplasm of the cell. The cytoplasm has been counterstained with methylene blue. And you can see there’s kind of an even staining throughout the nucleoplasm with the nucleolus slightly less strongly positive. This is the image that one gets from autoantibodies that were responsible for the LE cell phenomenon and this is an antibody that’s directly against chromatin. The antibodies can be either antibodies, the double-strand DNA or antibodies to histones.

**Slide 7**

The next one I’m showing you is a completely different pattern. You can see here that the nucleoplasm is stained but in a speckled fashion. And these speckles represent autoantibodies in the patient’s serum, which are reacting with spliceosomes -- the particles within the nucleoplasm that are responsible of doing the job of splicing of precursor messenger RNA. And you can see these speckles are of different sizes indicating perhaps that splicing activity is more highly present or highly active in larger speckles than in smaller speckles. And you can see that the nucleolus are relatively absent for staining. So, this has antibody to the Sm antigen, which was named after a patient with lupus who provided the antibody to these spliceosome particles so we called it the antibody to Sm antigen.

**Slide 8**
Now, one of the important things that emerged from these early studies was that in many of these autoimmune diseases, there were profiles of autoantibodies. And in the case of lupus for example, one saw that there were antibodies, the double-strand DNA, that’s the first line there on the table, which was present in about 40% to 60% of patients with lupus, but the specificity was extremely high. In other words, if a patient was found or a person was found to have antibodies to double-strand DNA, one could be fairly sure that this person had lupus.

Now third line down on the table is antibody to Sm, the antigen; the kind of antibody that gives the speckled nucleus staining. And here the antibody is present in only from 20% to 40% of patients with lupus, but again the specificity is very high.

So, this table here illustrates that in disease conditions like lupus and many other autoimmune diseases, there are profiles of autoantibodies that are characteristic of a particular disease.

Now, what is the clinical outcome of these biomarker discoveries?

From 1960 until now, treatment for lupus has not changed significantly: The main modalities have continued to be corticosteroids and immunosuppressive agents. However, since biomarkers for lupus became available, the 10-year survival for lupus after initial diagnosis is now over 90%; a huge improvement over the 50% described above. Most of this advance is widely attributed to early diagnosis with the use of these antibody biomarkers I have just described, that is, before irreversible organ damage has occurred in these patients.

Now, let’s turn now to cancer. And as far as autoantibodies as biomarkers in cancer are concerned, I would like to point out a special case for hepatocellular carcinoma.

In HCC, one can identify a cohort of patients, one-third of whom will eventually develop HCC. And these are patients with liver cirrhosis or chronic hepatitis due to viral hepatitis or other conditions such as alcoholic liver cirrhosis.

The autoantibody response that some of these patients make occurs during the transition from chronic liver disease like cirrhosis or chronic hepatitis to malignancy. And we think it appears to be reporting abnormal cellular mechanisms, which are associated with malignant
transformation. In other words, the malignant transformation is instigating the antibody response.

Slide 11

Now here are four patients for example; three of them with liver cirrhosis and one with chronic hepatitis. If you will look at the first patient NK, for example, you’ll see that the diagnosis of liver cirrhosis was made at time point 0 and the diagnosis of hepatocellular carcinoma was not made about 6 years later. But you can see that in this patient, there was a rise in the titer of his antinuclear antibody about a year and a half before the diagnosis of liver cancer was made. And you can see this in all of these four patients that are shown here.

It’s important to point out that three of these patients did not show a rise in alpha-fetoprotein, which is one of the markers for liver cancer. Only one patient, the patient number four in the right lower panel, shows a rise in the AFP, alpha-fetoprotein. But the important point is that antibodies seem to appear -- a new antibody seemed to appear before the clinical diagnosis of cancer was made.

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And here is the immunological characterization of that first patient; what happened when the antinuclear antibody titer rose. And you can see that in the lower series of -- sorry -- the lower series of panels A and B, A was taken at the time point with the stars and you can see there’s a change of almost invisible antinuclear antibody staining to a very strong antibody -- to the appearance of new autoantibodies. And in the analysis up on the upper right-hand corner you can see that time points 3 and 4 show the appearance of new antibodies that occurred during this transition from chronic liver disease to hepatocellular carcinoma.

So, it points out that with the transformation to malignancy, new immune responses are made in the form of new antibodies.

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[0:14:57]

On this is slide, which I want to show very quickly, and that is the -- I’m sorry. I thought these were pointers, but these are not pointers. In this slide, I want to show the frequency of antibodies to two tumor associated antigens called TAAs. These are p62 and Koc. These two are actually proteins that are -- that bind to messenger RNA, which encode or which code for (IGF2) insulin-like growth factor 2. Insulin-like growth factor 2 or IGF2 is known to be a growth factor and has been implicated in malignant transformation.
And the important feature in this table is that for example looking at colorectal cancer, you have antibodies, the p62 in about 9% of patients and antibodies to Koc in about -- I can’t see for sure.

Male Voice: 13.8%.

Dr. Eng Tan: In about 13.8%. But in the last column, if you look to see what is the percentage of antibody present in these patients when they are reacting with either p62 or Koc, the percentage increases to 20%. You see in this study, we looked at 777 cancer patients, quite a number of normal human sera and many autoimmune disease sera, 139 autoimmune disease sera. You can see that the cumulative positive percentage of antibodies to these two tumor associated antigens is quite different between cancer patients and normal sera or autoimmune disease sera.

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This is the study that led us to look at a panel; this is a mini-panel of tumor associated antigens. And the important feature is that the more -- sorry again -- the more antigens you add to the panel, the frequency of antibodies, the tumor associated antigens increase. So with the successive addition of more selected tumor associated antigens, one then can increase the percentage of positives as far as antibodies are concerned in cancer patients. In breast cancer with these seven TAAs, it’s about 43%. In lung cancer, it’s about 67% or 70%. So that the figure emerges or the information emerges here that if one can get a correct mini array of tumor associated antigens, one can really increase the sensitivity and retain the specificity as far as diagnostic markers for cancer are concerned.

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And I just want to quickly end by saying that autoantibodies -- autoantibody profiles in cancer are probably expected to occur. We, for example, looked at 7 TAAs and looked at a number of different tumor associated antigens including c-Myc, cyclin B1 and the insulin like growth factor 2 mRNA binding proteins. We looked at cancer patients as well as normal individuals and our statistician did a recursive partitioning.

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And you can see here that autoantibody to cyclin B1 was the initial discriminator in these different cancers, for gastric, lung and hepatocellular carcinoma. When you look at c-Myc, c-Myc was the initial discriminator in breast cancer. Because in breast cancer there were more patient with anti-c-Myc than with other types of antibodies. P62, one of the IGF2 messenger RNA binding proteins, was the initial discriminator in prostate cancer. IMP1, another one of the family of IGF2 messenger RNA binding proteins, was the initial discriminator in colon cancer.
So, I think, that with further work into getting the correct type of arrays of tumor associated antigens, one can arrive at the point where these arrays can be highly sensitive and very specific and will be extremely useful markers for cancer.

[0:20:02]

Sean Sanders: Excellent. Thank you very much Dr. Tan. It was a great overview and a great historical perspective as well.

We’re going to ask a couple of questions that came in from our online audience. The first question I have for Dr. Tan is, in your opinion what impact will biomarker research have on the advancement of personalized medicine?

Dr. Eng Tan: As far as personalized medicine is concerned, I think that one has to remember that autoantibody titers change, and the change is largely reflected on the tumor load. In other words, if you have the tumor and it’s a large tumor, you generally get a good immune response, high antibody titers. And if a patient with tumor is being treated and the antigen load or its tumor load is much decreased, one can see a fall in the antibody titer. So, as far as personalized medicine is concerned and these autoantibodies, how can one use these autoantibodies to personalize treatment, it might be a very useful method of following the response to treatment.

Sean Sanders: Excellent. And we have one more question, very quickly. Some researchers doubt the role of autoantibodies as early markers of disease. How would you reconcile that point of view with some of the more recent clinical data?

Dr. Eng Tan: Well, the autoantibody response to cancers, for example, is not seen in every patient, but I think that it may be related almost to the sensitivity of the detection method. We have, for example, now studies that look at antinuclear antibodies as the detection method. If you have more sensitive detection methods, your chance of probably finding an antibody response in cancer may be much higher. That’s number one. Secondly, I have showed in one of the previous slides that the immune response can sometimes predate all the current methods we have for detecting cancer. And this is simply because we were able to say that here is a cohort of patients with chronic liver disease whom we know will ultimately develop cancer down the line. We do not have any knowledge of identifying cohorts like these for other types of cancer yet.

Sean Sanders: Excellent.
So, we’re going to move on to our next speaker. Dr. Paul Predki serves as Vice President for Proteomics Research and Development at Invitrogen Corporation. Dr. Predki joined Invitrogen as part of the acquisition of Protometrix in 2004. This is a functional protein microarray company he helped start in 2001 as a spinoff from Yale University. Prior to that, he served as Associate Director at the Department of Energy Joint Genome Institute where he was responsible for the development and implementation of the institute’s high-throughput production sequencing and genomics programs. Prior to joining the JGI, Dr. Predki spent four years at the genomics biotechnology company, CuraGen Corporation. Dr. Predki has over 10 years of experience in industrial-scale biology plus postdoctoral experience at Yale University, where he investigated protein engineering and protein biochemistry. He obtained his Ph.D. in Biochemistry from the University of Toronto. Dr. Predki has published in a variety of high-profile journals, including “Science, Nature, and Cell”, and most recently served as editor of the book, “Functional Protein Microarrays in Drug Discovery”.

Dr. Predki.

Dr. Paul Predki: Thank you, Sean.

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So, my first slide outlines the recent evolution of technologies for the discovery of autoantibody biomarkers. You can see that some of the early approaches like expression library screening and phage-display selection very heavily employed techniques of molecular biology. More recent additions to this toolset, if you will, relied on innovations in the fields of proteomics and genomics.

The mass spectrometry based approaches for instance, which use mass spectrometry for the identification of antigens were inspired heavily by advancements in the field of proteomics.

The microarray approaches on the other hand while strictly speaking are proteomics technologies, because we’re talking about protein microarrays, were in fact inspired by technologies developed for genomics most specifically, DNA microarrays.

Now, I think it’s these microarray approaches that in recent years have generated maybe some of the most excitement in terms of autoantibody biomarker discovery. And I think that one of the big reasons for that is that it makes it possible for the first time to screen large numbers of
autoantigens and potential autoantigens against large numbers of sera or human samples in a statistically rigorous fashion.

What we’re going to do over the next couple of slides is delve into some of these approaches. And I’m going to focus most of my time on some of the recent developments in high content discovery arrays.

So, as I mentioned, really the initial large scale approach to discovery was that of cDNA expression libraries, good old lambda libraries with plaque lifts screening those with sera. Those have evolved into a technique called SEREX, which is really just a variant of this, which employs cDNA libraries from patient’s tumors themselves and screening those with autologous sera.

A little later on, phage display selection became a little bit more common. We saw essentially the same type of libraries that were used for SEREX now used in phage display. Instead of screening, we’re now selecting for autoantibody binders. Now, these techniques have the advantages of being very well-established, relatively simple techniques, the disadvantage of being quite labor-intensive certainly not friendly to a high throughput environment. And they have the drawback that most library-based techniques do and that is that they tend to be favored towards the higher abundance transcripts.

The next approaches I’d like to discuss are the mass spectrometry based approaches, and there are really two main approaches that have been taken here. One is that of two-dimensional gels where protein lysates from again tumorous or cancer cell lines are separated on two-dimensional gels. Those proteins transferred via Western methodologies to a membrane and that is screened with sera. And I’ll show you as an example on the image on the right from some of the early work from Sam Hanash’s Lab. And this of course requires mass spec follow-up to identify spots of interest putative antigens.

Another approach is the reverse phase arrays. In this case, protein lysates are fractionated via chromatography, and these fractions are in fact spotted out on arrays and those are used for screening sera. And once again, mass spec is required for antigen identification.

Now the mass spectrometry based approaches have the major advantages that the proteins that are being used are actually extracted from the source of interest. And so, they can detect, at least in principle, post translational modifications to which autoimmune events are directed. I think the biggest disadvantage of these is that one has to use
mass spectrometry based approaches to identify the antigens. These approaches tend to be biased towards denatured epitopes. For the 2D approach, in particular, reproducibility and sensitivity are quite low and the labor required is relatively high.

And this is brings to the microarray approaches. These were initially pioneered in terms of antigen arrays by Bill Robinson and PJ Utz as well as Thomas Joos. And these are essentially arrays of known autoimmune antigens, which are profiled against sera.

Later on, discovery-based arrays using the same basic principles developed. The same sort of expression libraries and phage display libraries that I described earlier are now being screened in a microarray sort of platform. And more recently, the development of high content functional protein microarrays, which I’ll focus more on momentarily, have been added to this arsenal, if you will.

The main advantages of these microarray approaches is that they’re relatively simple and fast to do, at least once you’ve got some arrays in your hands. And it’s possible to do relatively high-throughput experimentation with them. Depending on the exact type of array proteins maybe folded, so in the high content functional protein microarrays and antigen arrays that’s generally the case. It’s generally not the case with expression libraries and phage display type of arrays. And at least some post translational modifications maybe present.

The biggest disadvantage of the arrays is that actually making them robustly, which is required for the success of these approaches can be quite challenging.

So, over the next couple of slides I’m going to focus on some of the work that we’ve done at Invitrogen.

This slide here describes our most recently launched commercial microarray, which contains over 8000 purified human proteins. These proteins are expressed through a Baculoviral System and Sf9. They’re all GST tagged and purified based on that tag to at least 90% purity and purified under non-denaturing conditions.

The library of proteins is a relatively unbiased collection. You can see at the bottom some example counts of numbers of proteins from different classes. The main bias would be that we tend to have an overrepresentation of kinases, a family of proteins that Invitrogen has
been doing a lot of work in, and an underrepresentation of integral membrane proteins.

In addition to these 8000 proteins, which are double spotted on the arrays, there are a large number of control spots, both manufacturing and quality control, and application specific controls in each array.

**Slide 23**

Now, we’ve developed a highly automated and controlled manufacturing process to generate these arrays. They start with our Ultimate ORF Clone Collection, which is a fully sequenced, full-length human open reading frame collection, which are transitioned into a Baculoviral Expression System for high-throughput expression of proteins, and then moving into a cool germ environment with automated equipment once they gain high-throughput purification of these proteins under native conditions, followed by protein arraying. This whole process of course tracked by a LIMS System that’s been customized for this purpose. And much of the information around the various QCs in the identities of proteins is in fact available through and encoded by the bar code on each of these slides.

**Slide 24**

So, this slide illustrates a generic sort of protocol or workflow for biomarker discovery. The first stage is sample acquisition. I won’t say too much about sample acquisition except that one needs to have basically at least two groups, and the selection of these groups is absolutely critical. This is definitely a process where garbage in leads to garbage out -- certainly, another topic for a webinar in of itself. In terms of sample processing, a relatively simple process, the arrays are blocked and then probed with biological sample, often sera. Any autoantibodies in that sample will bind to their cognate antigens on the array. And then we do a wash in the secondary detection with a fluorescent antibody and imaged on a standard slide scanner. And the next step after that is the data analysis, comparing the disease or treated with the controls.

**Slide 25**

This slide delves into that in to a little bit more detail. Roughly, there are three general steps that are required. The first one, normalization, is required because we’re actually measuring relative signals of proteins, not absolute signals. So, normalization includes things such as data correction, intra-slide and inter-slide normalization. And we make available free of charge a computer program called ProtoArray Prospector, which facilitates those types of algorithms. The second step is training, which is basically development of an algorithm, which is able to differentiate control from test samples and the various methods by which
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this can be done. And then finally, testing, an independent validation of the training algorithm.

In order for these to be successful, these technologies require high sensitivity and reproducibility. The graph on the left shows you a series of dose-response curves of sera, which test positive for NY-ESO1, which is a known autoantigen. And each of this is being performed with protein spots at different concentrations. And from this you can see that except for the lowest concentration of protein, we are easily able to detect dilutions down to 1/50,000, which is more than sufficient for high level antigen or antibody responses.

And similarly, reproducibility on the right we’re, just in the image below, comparing two samples of serum diluted 1/500. The CVs on intra-assay, inter-lot, and inter-operator experimentation are, I think, getting towards the limits of the reproducibility of the manufacture of these arrays themselves.

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We’ve investigated a large number and have been involved in a large number of different types of studies. Clinical condition study include obviously autoimmune in cancer. But we’ve looked at inflammatory, therapeutic response and transplantation as well. In terms of samples, serum is certainly our workhorse, but we have tested urine, tears, aqueous humor, CSF, saliva and a variety other tissues -- or sorry -- of other samples. And in terms of antibodies, well we tend to rely on IgG. We’ve also tested IgA and IgM. And if you’ll look over to the bottom right, you see a comparison of IgA and IgG responses. You can see that they actually can be quite distinct from another.

So, over the next couple of slides, I’m going to give you kind of a snapshot of two studies, one autoimmune and one cancer. Both of them tested based on sera and IgG.

[0:35:00] Slide 28

The first one here is cancer and if you take a look at the left, you see 9 plots. Any one of those plots represents one protein on the array. So, you can imagine that, you know, on an 8000 protein array, you would generate 8000 of these plots. On the Y axis is a normalized amount of signal coming from that spot and on the X axis on the left-hand side normal sera, and circled in red cancer sera.

And for each of these 9, you can see there’s definitely an enrichment in the response from the cancer sera versus normal. But the magic really starts to happen when you combine all 9 of these together into a
classifier. And this here is at the sort of training set stage and with this classifier; we’re able to reach about 85%, sensitivity and specificity. And of course the real proof for these experiments is in the test and that’s something that’s in progress.

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This other example here is in fact with lupus and I’ll just show you sort of a different way to look at the data. This is with hierarchical clustering on the left. In this case, we’ve got a 64-marker panel that’s been culled out of these array experiments. Each column in that graph represents a different protein. The colored response from green up to red indicates the amount of reactivity we’re seeing. And you can see clearly that we’re able to just differentiate controls from the lupus condition itself.

On the right, I gave you an example of two of those proteins. The one on the top is one that’s already an established diagnostic biomarker for lupus, and on the bottom, an example of one that is a candidate for this.

So, these two examples are really snapshots. In the next presentation from Mike Snyder, he will dig into some more detail on how these studies are really done.

Sean Sanders: Excellent. Thank you very much, Dr. Predki. That was a great primer [0:36:54] [Phonetic] on particularly these protein array technologies. And I think we got a nice perspective and a nice link-in with Dr. Tan’s talk as far as lupus is concerned.

So, we have some questions that have arrived in our inbox. The first one I have for Dr. Predki is, why are protein arrays particularly suited to autoantibody discovery and detection?

Dr. Paul Predki: As I’ve mentioned towards the beginning, I think that sort of the biggest reason why this technology is suited for this purpose is that it enables one to do two things. One is to screen large numbers of proteins. We’re working with 8000 here and that number will only increase. And it enables one to do this in a relatively simple high-throughput manner such that one can generate statistics necessary for real biomarker discovery. So, it’s the one technology that brings those two facets together.

Sean Sanders: Right. And another question that we have is what is the minimum amount of antibody that is detectable using these arrays? Do you have a number for us?

Dr. Paul Predki: Well, it depends how it’s specified. I guess, maybe the most relevant example would be in the slide that I showed earlier where sera that we
took, which was NY-ESO1 positive, and I believe that was from a lupus patient. We could easily detect down to 1/50,000 dilution of that sera. The sensitivity seems to be comparable to ELISA sensitivity.

Sean Sanders: Excellent.

Slide 30

Okay. We’re going to move on quickly to our next speaker. We have here Dr. Michael Snyder. He is the Lewis B. Cullman Professor of Molecular and Cellular Biology and Professor of Molecular Biophysics and Biochemistry at Yale University. He is also the Director of the Yale Center of Genomics and Proteomics. Dr. Snyder received his Ph.D. training in the laboratory of Dr. Norman Davidson at the California Institute of Technology and carried out postdoctoral training in Dr. Ronald Davis’s lab at Stanford University. He is a leader in the field of functional genomics and proteomics. His laboratory currently carries out a variety of projects in the areas of genomics and proteomics both in yeast and in humans. These include the large-scale analysis of proteins using protein microarrays and the global mapping of the binding sites for chromosomal proteins. His laboratory built the first proteome chip and the first high resolution tiling array for the entire human genome. Dr. Snyder has published over 200 manuscripts and he is the editor of a number of journals. He also sits on many international advisory boards and was cofounder of Protometrix, which was purchased by Invitrogen in 2004.

Dr. Snyder?

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Dr. Michael Snyder: Okay. Thanks Sean. It’s a great pleasure to be here.

So, I’m sure many of you know that ovarian cancer is an important problem in the United Stated and worldwide. In fact, in the US, it’s the fourth most common cause of cancer. There are about 15,000 deaths attributed to this disease each year. Like most cancers, if you diagnose it early, you can actually do things about it, and that will ultimately lead to survival. But unfortunately, also like most cancers, if diagnosed late, say at stage 3 or stage 4, the prognosis is quite poor with estimates on the order of 15% to 30% survival over five years.

Currently, there’s only one biomarker test out there for ovarian cancer and it has a lot to be desired. It’s elevated in women with advanced ovarian cancer, so 80% of women have this marker. But it’s not usually found in the early stages of the disease and, moreover, has a very high
false-positive rate. So, it’s clear we need new biomarkers to be able to detect this disease at early stages.

**Slide 32**

Furthermore, what we’d like to do is ultimately come up with markers for cancer, and certainly one goal of our research is to try and use protein chips to be able to identify ovarian cancer markers. Ultimately, what we’d like to do is be able to find markers that would enable us to accurately prognose the outcome of the disease and track its progression. And also one important aspect of ovarian cancer is that we need effective measures to determine, which treatment individual patients should get.

So, for example there are some patients who actually have adverse effects to certain treatments, that is the cancer becomes much worse and other patients in which the disease is improved upon treatment. So, you’d like to be able to understand which patients are which and if we had appropriate markers that would be a terrific thing to have.

As both Dr. Tan and Dr. Predki indicated and certainly lots of evidence in the literature that diseased patients will produce antibodies that are reflective of the disease state, and there’s a number of lines of evidence now in the literature that cancer patients may have such antibodies, such autoantibodies.

**Slide 33**

And so what we wanted to do was use the technology that Paul just described to see if we could actually find markers that would say something about the disease state of ovarian cancer. And to do this, what we did is use the approach outlined on this slide. We took 30 ovarian cancer patients at different stages of the disease. So some were stage 1, some were stage 2, some were stage 3 and some were stage 4. We also took sera from 30 age-matched healthy women, so who did not have any signs of the disease. And for each of these sera, we probed the Invitrogen ProtoArray chip, which at the time had 5005 human proteins. So we took each of these sera, probed the chip and looked for reactive antigens.

**Slide 34**

Some examples of the types of reactivity we saw are shown on this slide. So if you start out looking at the middle panel there, this was probing with a sera from an ovarian cancer patient. And in red boxes are actually antigens that are reacting with the sera specifically from this particular patient and not showing up in healthy individuals. And occasionally, it’s quite rare, but we also will find some antigens that will be reacting with sera from the healthy individuals but not from the cancer. Those are indicated in the yellow boxes on the slide and you can see that if you look hard in the top panel relative to the middle panel. And then there are
additional antigens that will react with secondary antibodies as well so they’ll show up on all the slides.

So, when we completed our study of probing with 30 cancer sera and 30 normal sera, we found a total of 94 different proteins that showed very high reactivity in the cancer patient relative to the healthy individuals. And these antigens actually come from a very diverse set of types of proteins. So for example, some antigens were involved in nuclear envelop formation, others are involved in transcription, others are involved in splicing and cell cycle control or signal transduction and ion transporter. So, it’s really a very diverse array of antigens that were found by this.

Now what we are hoping for is that when we actually looked at the reactive markers, we could come up with a predictive test or diagnostic test of sera that would say that this was from a cancer person relative to a person who does not have cancer. And by screening these different antigens and looking at them in different stages of disease, we actually didn’t come up with prognostic signature like we had hoped or a diagnostic or a prognostic signature. So, we did not find a nice signature that would say these patients have cancer and these are healthy.

So, what we decided to do was look at these cancer markers a little further. It remained possible that actually the cancer markers were still quite useful for this -- or these reactive markers were still quite useful for this, but perhaps the autoantibody response was not as indicative as we had hoped.

So, what we did for eight of these antigens, some of the more reactive antigens, shall we say, we actually took antibodies directly against those proteins and screened those. We those screened those in two types of assays. We screened those on immunoblot analysis and also dot blot analysis and then we also screened them in tissue stainings. And I’ll show you examples of both of these in these next slides.

So, here’s staining with one of the antigens, one of the antigen was actually a very common protein called lamin A and lamin C. It’s actually two proteins that are derived from the same gene. And on the left shows probing of tissue lysates from healthy tissue and from cancer tissue. And what you’ll notice is that we can actually get strong reactivity with samples from the cancer tissue, but not the healthy tissue for this particular antigen, again, when the antibody directly against the protein is used. So these are not autoantibodies, these are antibodies directly against the protein.
Likewise, for Western blots, we can find that these antigens are overproduced in tumor tissue relative to healthy tissue. And actually, we’ve seen this with two different sera for lamin. So we’re quite confident it’s the antigen we think it is.

We’ve also used these antibodies to look at tissue sections and there we can look at large numbers of patients and try and get an idea of what kind of staining pattern we might see.

Slide 37

And so on this slide shows staining of 40 different ovarian tumor tissues, so tissues from ovarian cancer patients. And there are 40 different tissues here. And there’s actually very, very strong reactivity with the anti-lamin antibody as you’ll see on the slide.

Slide 38

And if we probed the tissues from 40 healthy individuals that’s shown on this slide, you still get reactivity, but I think you can appreciate that it’s much less than that of the cancer patients. So again here’s the healthy tissue, I’ll just flip back and show you this once again. Here is cancer material, here is healthy and what you’ll notice, there’s much stronger reactivity in the cancer patients relative to healthy.

Slide 39

We can also look at matched samples from the same patients. That is, we can look at cancer material, which is shown on the top of this part relative to healthy tissue taken from the same patients, but 1 cm away from the cancerous material. Now, once again, for most patients, you can actually see stronger reactivity in the cancer tissue relative to the healthy. And so again, these seem to be much, much -- this protein seems to be highly elevated in cancer material relative to healthy.

Slide 40

If we actually start looking at the one marker that’s commonly used, the CA-125 antigen that I mentioned at the outset, this shows much higher staining in cancer tissue than healthy, which is shown on the next slide. But it’s actually not quite as pronounced as the lamin marker that I just showed you.

Slide 41
Slide 40

So again here is healthy, here is cancer. Here is healthy. It’s elevated, but not to the same degree as the lamin.

Slide 42
Slide 43

And I’ll just show you one more example. Here’s another marker we found that shows much higher staining in the cancer tissue sections once again. So look at this compared to healthy shown up in the next slide, you
can see quite dramatic changes. The protein is present. These are normal cellular proteins, but they are at much higher levels in the cancer material.

**Slide 44**

And likewise for marker B, we’ll see increased staining even from tissues from the same healthy -- from the same patient when we compared a cancerous and healthy material.

**Slide 45**

Now we can quantify these data. As Dr. Tan mentioned, if you actually can -- we can get nice differential staining with individual markers, but if we combine the results of several markers, we can actually get even improved diagnostics for this. So, the left 40 samples are samples from patients; and 38 of 40 show higher reactivity and only one in the other sample, and in fact that one we think is actually cancerous material.

**Slide 46**

We’ve started looking at different stages of disease. So, this is looking at stage 2, seven samples, a number of samples from stage 3 and a number of samples from stage 4. And the control is not on here, but healthy samples from healthy individuals are similar to the top three left panels of stage 2. You tend to see low levels of staining, but it’s pretty clear even in stage 2 patients we can see very strong reactivity with this lamin A and the same is true actually for the marker B I mentioned, and it’s also true we think for three other markers as well. So, five of the eight markers are showing elevated staining in these tissue sections relative to healthy individuals. And again, for stage 3 and stage 4 actually, virtually all of those tissue samples show very, very high staining.

**Slide 47**

Lastly, we looked at whether this was specific for just ovarian cancer for these different markers and we’ve analyzed three of these so far. And for all three, they’re not actually specific for ovarian cancer. They’re staining several different types of cancer, but they don’t stain in all types. And so for example lamin A, and this is true for two other markers as well, they show higher staining in the ovarian and the uterine and the lung samples for cancer patients and less, we think, for healthy. For breast cancer, there may be elevated staining in both. And kidney interestingly enough is showing higher staining in the healthy relative to cancer, and for liver it’s the same.

**[0:50:23]**

So, certain kinds of tissues are staining in cancer relative to healthy and so we think it’s not specific for ovarian cancer. A number of markers or a number of different types of tissues are elevated for these. We think that at least some of these markers are probably elevated in specific kinds of epithelial cells associated with cancer, but not other types. It’s not all
epithelial cells that stain based on these and some other experiments. And actually, for some of the markers, we noticed staining of stromal cells as well.

**Slide 48**

So in conclusion then, we could actually use the protein microarrays that Paul mentioned and by using autoantibody screening, we could find differentially expressed proteins, and we came up with five differentially expressed proteins.

In tissue microarrays, at least some of these appear to be detecting early stage cancers, which is quite nice. And at least two and we now think three of them are probably better in these tissue sections than CA-125. We certainly need to actually investigate whether we can get them showing this differential staining in sera. So whether they’ll be useful sera markers that remains to be seen, but that’s something we’re exploring now.

And lastly, I want to emphasize, as the speakers have said, actually by combining the results of the several markers, you get more accurate tests using these.

**Slide 49**

And lastly, I’d just like to acknowledge the people who did the work. This work was primarily done by Mike Hudson. Li Kung also helped towards the end of the research, and all of this is a collaboration with Dr. Gil Mor at Yale University.

**Slide 50**

**Sean Sanders:** Excellent. Thank you very much, Dr. Snyder.

So, we’re going to some questions for Dr. Snyder and then we’re going to have our question and answer session. So firstly, Dr. Snyder, one of the questions that came in is, wanting to know about any other ProtoArray based sera profiling projects that you’ve been conducting in your lab if it’s anything you can talk about.

**Dr. Michael Snyder:** Right. Well, we carried out a very large study looking for coronavirus. We are active in coronavirus diagnostics. And as many of you know the SARS virus is in fact a member of this family. And what we were able to do in a very large study was show that in fact we could detect which individuals had SARS reactivity and which individuals had reactivity to other human coronaviruses that weren’t as pathogenic; although, they certainly caused sickness in humans, they tended not to be as lethal.
And the nice thing was from this coronavirus study we could again figure out which patients seemed to have which coronaviruses based on their autoantibody reactivity, or in that case antibody reactivity profile.

Sean Sanders: Excellent. Another question that came in that I think we can probably open up to the panel is -- or possibly Dr. Predki would best be able to answer this. The false-positive rate with this technology; what is the false-positive rate and how do you address this?

Dr. Paul Predki: Yeah. It’s actually kind of a difficult a question to answer. I mean, it starts really with what your definition of a false-positive is. So, there are a couple of areas one could focus in on. If you focus in on the various sort of terminal stages of this and the diagnostic itself, what is a false-positive on the diagnostic, you know, I think we’ve seen that these studies are capable of false-positives as low as say 10%, 15% and maybe more so. But that’s highly contingent on a couple of things and it starts out with a selection of your samples, right, and how you define the study. And that sort of -- that aspect of it is outside of the realm of the technology itself but terribly important to the final sort of result.

If we think of false-positives just in terms of a hit on the array, if you will, where the array is telling there’s an antibody binding to something and you ask, you know, in what fraction of the case is that actually true and which is that not true. That answer can actually be tweaked depending on how one sets the parameters and kind of the thresholds for calling something a positive or not calling it a positive. And again, there’s an interplay between specificity and sensitivity when you do this, for false-positives or false-negatives. But certainly, it’s capable of pretty low false-positive rates in the order of 5% to 10%.

Sean Sanders: It seems like it’s similar to a lot of other technologies of this type, DNA microarrays, you have the same issues.

Dr. Paul Predki: Yeah. These are kind of general challenges.

Sean Sanders: Right. Great. Okay. So, I want to move on to another question for Dr. Snyder. This is talking about appropriate study size. How many disease and normal samples are required in order to get statistically meaningful results?

Dr. Michael Snyder: Okay. That’s a really good question and also a very difficult question to answer. It really depends on the statistical significance of the results that comes from your sample population. So that is to say that if robust
results come from a small number of samples that separate the two populations, you’re okay. But if the reactivity is lower for example maybe fewer proteins on the chip are reacting or they’re at a lower signal, you may need many more. So, in a good study, you might get by with as few as 20 healthy and 20 diseased individuals. But if there aren’t -- if the frequency with which these markers appear in the disease population relative to the healthy individual is not so penetrant, you will need to use many more, possibly as many as a hundred.

Sean Sanders: Uh-hum.

Dr. Michael Snyder: And under those circumstance, then it probably becomes more practical to do a more limited study like we did, find candidate antigens and then analyzed those further in a larger population. So that’s probably a more cost-effective way to go if the penetrate is low. But the one lesson I can say is at the end, you need to have a robust statistician to give you confidence in the results you get out for following all of your markers and sets of markers.

Sean Sanders: What will we do without biostatisticians?

Dr. Michael Snyder: Right. We’d be in trouble.

Sean Sanders: [Laughs] So, this is a question, I think, for Dr. Tan asking about whether the antibodies that were mentioned are free in the blood and what major subtypes of these antibodies do you detect?

Dr. Eng Tan: As Dr. Predki has mentioned we have actually looked or it has been actually studied for many years to see whether IgG antibody versus IgM versus IgA or IgG, IgE and so forth; what kinds of antibodies are important to look for when we are looking at these autoantibody markers. And except for the allergic diseases where IgE is the important antibody, most of the clinically relevant autoantibodies are of the IgG isotype. There may be some IgA, especially I guess when one is looking at immune responses from the gastrointestinal tract where a lot of the IgA cells are present. But, by and large, IgG is the type of antibody that most of us are looking at.

Sean Sanders: Okay. The next question probably for Dr. Predki asks whether the array has been run with samples from other cancers -- actually, maybe Dr. Snyder could answer that. And the second part of the question is how do you tell ovarian cancer specific versus proteins upregulated in some cancers generally. I know this is a problem with this type of analysis. You
know, that there are a number of proteins that are upregulated in a variety of cancers that may not be cancer specific.

Dr. Paul Predki: It’s a challenge with any biomarker sort of approach.

Sean Sanders: Uh-hum.

Dr. Paul Predki: Let me address from my perspective the first half of that question. I think that the second is really targeted more towards Mike.

Sean Sanders: Uh-hum.

Dr. Paul Predki: With cancer studies, we ourselves just strictly in an R&D mode and in collaborations have been involved in just two or three studies for cancer. But we do offer this as a collaborative service and the numbers start to jump up from there. But in those cases, obviously those really aren’t in the end our results. So we’ve got -- you know, we’ve got the most experience with about three different types of cancers.

Dr. Michael Snyder: Okay, yeah. And as far as the issue about specificity of the makers, that’s certainly one of the first things that has to be checked. Obviously, once you get candidate markers you need to look at a variety of different kinds of cancers if you’re looking at cancer markers, and also other tissues from inflammatory diseases, things like that. So, one does need to look at specificity for these things.

I think the markers will still be valuable in many cases even if they’re not specific for just one kind of cancer. It’d still be nice to know for example in an individual, if you could find something early that they have cancer and you can pin it down to several types, that’s probably more valuable than not knowing at all. But certainly obviously at the end, it would be very, very nice to have specific markers, but even general markers will be very valuable.

[1:00:15]

Sean Sanders: Uh-hum.

Dr. Michael Snyder: I think one thing biology has taught us about though is that there is rarely one marker that’s perfect for anything.

Sean Sanders: Uh-hum.

Dr. Michael Snyder: So, I think at the end of the day, we’re going to need multiple markers for any type of disease we want to study, and by the collective ensemble of
these markers, we hope to get signatures for various diseases. In that way, if you’re not so reactive for one or another, you’ll still be able to get diagnosed early if you have one of these, you know, problematic diseases so...

Sean Sanders: Uh-huh.

Dr. Eng Tan: Sean, I’d like to --

Sean Sanders: Sure, Dr. Tan.

Dr. Eng Tan: -- really support what Dr. Snyder said. And that is, the Holy Grail almost is that people think that there will be one autoantibody that would suffice to mark a certain disease, but that’s not a case.

Sean Sanders: Uh-huh.

Dr. Eng Tan: Wherever people have looked in these autoimmune responses, there are multiple antibody responses. It’s not limited to just one antibody response.

Sean Sanders: So, a question related to that to Dr. Tan is how do you validate your biomarkers?

Dr. Eng Tan: Well, in our case, as we showed in one of our slides; we try to look at a large number of, say, cancer patients but in addition to a large number of normal patients as well as patients known to have antibody responses but to other markers, to see whether this antibody response in cancer is really more specific for cancer. And as I have said in one of the slides previously, even some of these antibody markers are not 100% specific as, I guess, Dr. Snyder even showed in his anti-lamins A and C are not really present in all kinds of cancers. You see overexpression or underexpression actually of lamins A and C in different types of cancers. So, that is you can’t depend on one autoantibody marker and think that that will -- see, it would make life much simpler if it were the case, but it’s not the case.

Sean Sanders: Sure. So, we’re running short on time, but I have one more question for Dr. Predki that came in. What are the most important performance characteristics for protein microarray technology with respect to autoantibody profiling specifically?

Dr. Paul Predki: Okay. Well, in terms of sort of direct specifications, if you will, I think that the sensitivity of the assay is important and the reproducibility of the
assay. So, the assay obviously needs to be sensitive enough to be able to
detect these autoantibodies at the types of levels that they exist at in
disease cases. And our evidence is that generally that is the case. The
second one is reproducibility. At end of the day, this all requires a
rigorous statistical analysis and the more reproducible this data can be
from array to array, from study to study, the more rigorous the
conclusions will be.

And as I said before, I think that the big sort of attraction and excitement
around arrays over the last couple of years has been that it is a unique
technology in that it brings together the capabilities to do this in ways
that haven’t been really possible before. So, large numbers of proteins
versus large numbers of samples with suitable sensitivity and
reproducibility that one can generate statistically rigorous results.

Sean Sanders: Right.

Dr. Eng Tan: Sean, could I --

Sean Sanders: Sure.

Dr. Eng Tan: -- add something? Dr. Predki has spent some time kind of looking at
antibody cross-reactivity. I think this is extremely important in these
array techniques where you have so many different antigens that are
present. And there’s a likelihood that the antibody you’re looking for
maybe very highly reactive with its copy of the antigen but partially cross-
reactive with others.

Sean Sanders: Uh-hum.

Dr. Eng Tan: And I wonder whether he would expand on that since he has thought
about it a lot.

Dr. Paul Predki: Yeah. In fact, we’ve done a lot of independent experiments with just
purified antibodies. And, you know, generally there’s -- we can see this in
terms of the specificity of antibodies, but when you bind them against a
high content array like this. Generally, if you take a therapeutic antibody
they tend to be exquisitely specific and just hit their target and only in
very high concentrations do you see binding to other proteins. However,
when you take a look at research, antibodies polyclonals are worse than
monoclonals, but monoclonals as well you’ll see a lot of nonspecific
cross-reactivity.

[1:05:05]
So, I think it’s true with any of these technologies, if you see a hit on a protein, you definitely can’t say that that is the protein to which that antibody was initially targeted. You do tend to see higher reactivity towards the targets. So, such signals can serve as markers, but they may not necessarily be indicative of the disease process and that’s an important thing to realize in interpreting this sort of data.

Dr. Michael Snyder: But if I could add one thing, if you want to see whether it’s the marker, you go and get an antibody directly to that protein and test it, which was exactly what we do.

Dr. Paul Predki: Exactly as you did.

Dr. Michael Snyder: So you can in fact -- you can sort that out.

Dr. Paul Predki: You can follow it up, yes.

Dr. Michael Snyder: Yes.

Dr. Paul Predki: That’s great.

Sean Sanders: Actually, a question that came in related or it’s something that you touched on there is somebody was asking whether autoantibodies can be used for therapy. So, Dr. Tan, I guess, you would like to comment on that?

Dr. Eng Tan: Well, certainly, that’s been the objective of many people in the field of immunology; that immunotherapy can be tailor-made for a particular patient, for a particular antigen-antibody system. But so far, it has been fairly elusive and not been very successful. And maybe there is something in there, I don’t know what the other experts here may think. But I think when people come up with certain antibodies or certain cells that target a specific antigen, one has to probably take into consideration the fact that the epitope that’s targeted by either a T-cell or a killer cell and the epitope that’s targeted by an antibody, there may be a very, very kind of limited sequence, and maybe a confirmation sequence, confirmational sequence too. So that maybe that’s what’s important. And we have at least talked about the fact that the immune system, the patient’s or a person’s immune system, some of these patients that are making immune responses are really telling us what they can see in vivo.

Sean Sanders: Uh-hum.
Dr. Eng Tan: In the patient himself, you can only see a specific sequence. And if you make, if you make immunotherapy directed against other sequences it may not be very effective.

Dr. Paul Predki: Right. Okay.

Sean Sanders: Well, unfortunately, we’re out of time so I’d like to thank our speakers who are kind enough to give off their time and expertise to be here today and help us understand more about this very interesting and rapidly evolving subject. Dr. Eng Tan from Scripps, Dr. Paul Predki from Invitrogen and Dr. Michael Snyder from Yale.

Many thanks for the excellent questions submitted by our online audience. I apologize that we didn’t manage to get to all of them.

Please use the URL that is up in the slide viewer right now if you’d like to learn more about the technology that we discussed today. We also encourage everyone to provide feedback on this webinar by going to the link displayed at the bottom of that same slide.

Again, thank you to all our participants and to Invitrogen for their sponsorship of this educational seminar.

Goodbye.

[1:08:20] End of Audio